The impact of stroma and ionising radiation on the plasticity of Dendritic Cells and Macrophages.

Anna Malecka

Thesis submitted to the University of Nottingham

for the degree of

Doctor of Philosophy

July 2017

University of Nottingham

Faculty of Medicine and Health Sciences

School of Medicine

Academic Unit of Clinical Oncology

Publications and abstracts arising from this study and related projects

Published papers

Jeyaraman J, **Malecka A**, Billimoria P, Shukla A, Marandi B, Patel PM, Jackson AM, Sri S. Immuno-silent polymer capsules encapsulating nanoparticles for bioimaging applications. *Journal of Materials Chemistry* B. 2017. *In Press* doi: 10.1039/C7TB01044C

Crittenden MR, Baird J, Friedman D, Savage T, Uhde L, Alice A, Cottam B, Young K, Newell P, Nguyen C, Bambina S, Kramer G, Akporiaye E, **Malecka A**, Jackson A, Gough MJ. Mertk on tumor macrophages is a therapeutic target to prevent tumor recurrence following radiation therapy. *Oncotarget.* 2016 Nov 29;7(48):78653-78666.

Malecka A, Wang Q, Shah S, Sutavani RV, Spendlove I, Ramage JM, Greensmith J, Franks HA, Gough MJ, Saalbach A, Patel PM, Jackson AM. Stromal fibroblasts support dendritic cells to maintain IL-23/Th17 responses after exposure to ionizing radiation. *J Leukoc Biol.* 2016 Aug;100(2):381-9.

Langosch S, Wehner R, **Malecka A**, Franks HA, Schäkel K, Bachmann M, Jackson AM, Schmitz M. Impact of p38 mitogen-activated protein kinase inhibition on immunostimulatory properties of human 6-sulfo LacNAc dendritic cells. *Immunobiology.* 2016 Feb;221(2):166-74.

Wang Q, Franks HA, Lax SJ, El Refaee M, **Malecka A**, Shah S, Spendlove I, Gough MJ, Seedhouse C, Madhusudan S, Patel PM, Jackson AM. The ataxia telangiectasia mutated kinase pathway regulates IL-23 expression by human dendritic cells. *J Immunol*. 2013 Apr 1;190(7):3246-55.

Conference abstracts:

OPERRA Workshop "Radiation effects on the immune system: an update on state of the art and future research needs" REIS-2017 Budapest, Hungary: The response of Dendritic Cells and Macrophages to ionizing radiation. A.M.Jackson, **A.Malecka**, M.J.Gough, P.M.Patel

ECI 2015:, Vienna, Austria: Modulation of dendritic cells function by dermal fibroblasts. **A.Malecka**, Q.Wang, S.Shah, A.Saalbach, M.J.Gough, P.M.Patel, A.M.Jackson

Keystone 2013: Stromal fibroblasts support the radio-resistance of Dendritic cells to maintain IL-23/Th17 responses. **A.Malecka**, Q.Wang, H.A. Franks, P.M.Patel, M.Gough, A.M.Jackson

Abstract

The basic model of antigen-presenting cells (APC) activation describes a change of state from resting (immature) to activated (mature) upon encounter with a first signal. However, it is apparent that APC maturation is not binary but instead has a spectrum of outcomes. This likely reflects the complex and dynamic crosstalk resulting from their encounter with a wide variety of stromal cells. A deeper knowledge of these interactions, and how they are processed by APC to instruct immunity is required. This study explores the plasticity of the major APC, namely macrophage (Mφ) and dendritic cells (DC). We test the hypothesis that plasticity in APC function depends on the sequence of signals encountered during generation and activation. Human laboratory models of Mφ and DC were generated from monocytes as follows; monocyte-derived Mφ were cultured with M-CSF (M-Mφ) or GM-CSF (GM-Mφ) and DC with IL-4 and GM-CSF. These were characterised and the impact of cellular (e.g. fibroblasts (FB)) or external factors (e.g. ionising radiation (IR)) studied.

Changes in microenvironment considerably alter the functional-phenotype of Mφ however it remains to be shown if their initial polarisation restricts responses. Activation of GM-Mφ elicited IL-12 and -23 whilst M-Mφ expressed IL-10. However, the classic pro-inflammatory signal interferon γ (IFNγ) did not elicit M-Mφ that secreted cytokines to the level expected of GM- Mφ. The M-Mφ displayed mixed phenotype with both pro-inflammatory cytokines (albeit at lower levels, 75% less IL-23, 21% less IL-12) on a background of high IL-10. The IL-23 response of Mφ to re-stimulation was

reduced or abrogated upon pre-treatment with LPS but enhanced in Mφ conditioned by IFNγ. These findings demonstrate limitations to Mφ plasticity reflecting initial conditioning and suggest the development of innate memory with a degree of specificity in Mφ responses to external factors.

We applied ionising radiation (IR) to our models at doses commonly used for radiotherapy (1-20Gy). Irradiation markedly suppressed IL-23 secretion by both Mφ and DC ($p<0.01$) in a dose-dependent manner without affecting viability. This was selective for IL-23 as there was no change in IL-1β, -6, -10, -27 or TNFα, and only a modest decrease in IL-12 secretion. To investigate the mechanism for IL-23 regulation we showed that IR activated Ataxia telangiectasia mutated kinase (ATM) which in turn inhibited the function of cAMP response element-binding protein (CREB) through phosphorylation of an inhibitory residue (S121). ATM activation suppressed IL-23 as inhibition of ATM with KU55933 increased its expression. Furthermore, the small-molecule CREB inhibitor C-Ci reduced IL-23 levels. As a consequence of modulation of IL-23 in APC, IR indirectly affected the generation of T-cell responses, namely Th-17. Cross-talk between APC and stromal cells was modelled by co-culture of DC with FB. The presence of FB prevented IL-23 downregulation by IR. This was dependent on TNFα and IL-1β secretion by DC which were unaffected by IR. In response, FB secreted PGE_2 which resulted in a feed-back loop to enforce IL-23 secretion via activation of cAMP. Therefore, whilst IR could be seen to regulate IL-23 in a single cell culture system, this did not occur in co-cultures that simulated the interaction APC have with stroma.

In conclusion, we shown that the order in which APC receive conditioning signals ultimately affects how they respond to re-challenge. This is further influenced by a mixture of micro-environmental and external factors. Understanding the molecular pathways through which these cues manifest will result in *in vitro* models that better reflect the biology of APC and create opportunity for intervention.

Acknowledgements

I would like to thank my supervisors Andrew Jackson and Poulam Patel for their guidance and teaching throughout my PhD. Their continuous support and encouragement while giving me the opportunity to explore my own ideas made it a very enjoyable experience. In particular, I would like to thank Andrew Jackson for always being available to talk when I needed and his help with overcoming the many obstacles in my work and life. My thanks go to Michael Gough (Earle A. Chiles Research Institute) for his suggestions and comments regarding radiation work. I would also like to thank Anja Saalbach (Leipzig University) and Lloyd Hamilton for help with fibroblasts and Mark Schmitz (Technical University of Dresden) for his help with SlanDC. I am grateful to Derfogail Delcassian and Julie Greensmith for their comments regarding macrophage plasticity.

I am very grateful to the members of the "Host-Tumour Interactions Group", Sabaria Shah, Qunwei Wang, Aanchal Preet Kaur and Rob Silk for their advice and practical assistance. Their positive attitude and readiness to talk and help at any time of day or night helped me to overcome many difficulties in my studies. Special thanks to Ruhcha Sutavani for her help with Th17 cells and talks till 5am. I am grateful to Joanne Porte and Wim Clarke for their help with senescent fibroblasts. Finally, I would like to thank Rob Moss for his kindness and practical advice and Richard McIntosh for his help with reagents. I feel truly honoured to have had the opportunity to work with and

learn from amazing and kind group of people who made me feel a part of family.

My thanks also go to the University of Nottingham which awarded me the Vice Chancellor's Research Excellence Scholarship supporting me financially during PhD.

This work would not have been possible without the continuous support and encouragement from my amazing partner, Chris Kozarzewski. I would like to thank my son Andrew. His patience and everlasting optimism have been a constant and wonderful source of enjoyment. I am also very lucky to have my daughter Eliza with me during the last year of my PhD. Her incredible smile gave me the strength to continue my work. I would like to thank my grandmother for her encouragement through my life and studies and my father for teaching me to think. Finally, I would like to thank my mother who sacrificed so much to give me education and never stopped believing in me. She supported me at every step of my life and she is and will always remain my hero.

Contents

Publications and abstracts arising from this study and related projects 2

Published papers 2

Conference abstracts: 3

Abstract 4

Acknowledgements 7

Contents 9

Figures 15

Tables 21

Abbreviations 22

Chapter 1 Introduction 26

1.1 Antigen presenting cells - function and place in the immune system .. 26

1.1.1 Antigen processing and presentation 26

1.1.2 PRR 27

1.1.3 Modulation of T-cell responses 30

1.2 Macrophages 33

1.2.1 The ontogeny of tissue macrophages. 33

1.2.2 Environmental programing in homeostasis and disease 39

1.2.3 The diversity of macrophage activation 43

1.2.4 The variability of Mφ phenotypes 53

1.3 Dendritic cells in immune system 60

1.3.1 Dendritic cells subsets 60

1.3.2 Dendritic cell ontogeny 63

1.3.3 Dendritic Cell function 64

1.4 IL-23 68
1.4.1 IL-12 cytokine family 68
1.4.2 Signalling pathways involved in regulation of IL-23 secretion. 71
1.4.3 IL-23 function 75
1.5 Innate memory 78
1.6 The role of stroma in governing immune responses 82
1.7 Inflammation in the tumour environment 87
1.7.1 Immune-mediated mobilisation and activation of FB as crucial step in carcinogenesis. 89
1.7.2 TAF-dependent regulation of trafficking and function of monocytes and macrophages. 91
1.7.3 Role of COX2 and PGE_2 in cancer 93
1.8 Radiotherapy 95
1.8.1 Ionising radiation 96
1.8.2. Biology of cancer radiotherapy 99
1.8.3 ATM kinase 101
1.8.4 ATM kinase functions in immunity 105
1.9 Hypotheses and aims 108
1.9.1 Hypotheses 108
1.9.2 Aims and objectives 108
Chapter 2 Materials and methods 109
2.1 Regents 109
2.2 Primary cell isolation 109
2.2.1 Isolation of CD14+ve monocytes from blood 109
2.2.2 Isolation of monocytes 110

2.2.3 Isolation of slanDC 110
2.2.4 Naïve T-cells 111
2.3 Generation of MoMφ 111
2.4 Generation of MoDC 112
2.5 Culture of BJ6 and primary FB 112
2.6 Freezing FB 113
2.7 Stimulation of Mφ for phenotypic characterisation and secretory profile 113
2.8 Stimulation of MoDC and SlanDC for phenotypic characterisation and secretory profile 113
2.9 Co-culture of FB with APC 114
2.10 Irradiation of cells 114
2.11 Generation of Th17 responses 115
2.12 Determination cytokine secretion in supernatants 115
2.13 Flow Cytometry 116
2.13.1 Characterisation of APC phenotype by flow cytometry 116
2.13.2 Determination of Viability 117
2.13.3 Intracellular staining for CD68 118
2.13.4 Intracellular staining for phospho-ATM 118
2.14 Quantitative PCR 119
2.15 Western blotting 119
2.16 β-galactosidase assay 120
2.17 Statistical analysis 121
Chapter 3 Optimisation of culture conditions for macrophages 122
3.1 Introduction 122

3.2 Results 122
3.2.1 Isolation of CD14+ve cells 122
3.2.2 The effect of FBS type on cytokine secretion 124
3.2.3 Characterisation of macrophages differentiated on normal vs low adherence plates 125
3.2.4 Time-course of MoMφ generation 126
3.2.5 Requirement for GM-CSF and M-CSF during activation 129
3.2.6 MoMφ phenotype 137
3.3 Discussion 141
Chapter 4 Macrophage plasticity in response to environmental factors 145
4.1 Introduction 145
4.2 Results 151
4.2.1 Effect of LPS and IFNγ on GM-Mφ and M-Mφ 151
4.2.2 Effect of IFNγ vs IL4 on MoMφ 155
4.2.3 Effect of switching polarisation signals on MoMφ behaviour 161
4.2.4 Modulation of the effect of IFNγ and IL-4 on MoMφ by TLR activation 169
4.3 Discussion 172
Chapter 5. Ionising radiation down-regulates macrophage IL-23 preserving their pro-inflammatory phenotype 178
5.1 Introduction 178
5.2 Results 184
5.2.1 IR inhibits IL-23 secretion by MoMφ 184
5.2.2 Effect of IR is independent of MoMφ generation method 186

5.2.3 Effect of IR on cytokine secretion and expression of HLA-DR and CD86 by MoMφ..... 187
5.2.4 Regulation of ATM activation by IR..... 190
5.2.5 Effect of IR on cytokine secretion is propagated by ATM kinase 193
5.2.6 ATM-dependent IL-23 secretion by MoMφ is TLR4-dependent 197
5.2.7 Inhibition of ATM triggers IL-23 secretion from Mφ2-like MoMφ 198
5.2.8 Effect of IR-induced ATM is mediated by CREB/CBP interactions. 201
5.3 Discussion..... 203
Chapter 6. Effect of IR on cytokine secretion by MoDC..... 209
6.1 Introduction..... 209
6.2 Results 211
6.2.1 DC characterisation and phenotype..... 211
6.2.2 IR does not affect DC viability 216
6.2.3 IR modulates cytokine secretion by MoDC..... 219
6.2.4 IL-23 secretion by MoDC is regulated by ATM..... 222
6.2.5 The effect of ATM on IL-23 is mediated through CREB/CBP interactions..... 228
6.2.6 Effect of IR-mediated IL-23 down-regulation on Th-17..... 229
6.3 Discussion..... 232
Chapter 7. Effect of Fibroblasts on the response of antigen presenting cells to radiation..... 237
7.1 Introduction..... 237
7.2 Results 240

7.2.1 Effect of FB on cytokine secretion by MoDC, slanDC and MDMφ 240

7.2.2. FB rescue IL-23 from irradiated DC 245

7.2.3 FB are unaffected by IR..... 246

7.2.4 FB promote IL-23 secretion by irradiated DC through COX2-dependent PGE_2 release..... 250

7.2.5 FB promote irradiated DC IL-23 responses through the cAMP pathway 252

7.2.6 FB permit irradiated DC to promote Th17 responses 254

7.3 Discussion..... 257

Chapter 8 Final discussion 264

The role of research on environmental modulation of APC in designing modern immunotherapy 264

8.1 The importance of environmental factors for designing improved immunotherapy modulations. 264

8.2 The importance of environmental factors in understanding immune-mediated consequences of therapeutic applications 267

8.3 Employing discoveries from studies on environmental regulation of immune system to create novel targets for immunotherapy 269

8.4 Conclusions and future directions..... 271

Appendices: 274

References 282

Figures

Figure 1.1 Ontogeny of tissue Mφ..... 35

Figure 1.2 Schematic presentation of Mφ activation states 46

Figure1.3 Classification of DC subsets based on ontogeny and phenotype ... 64

Figure 1.4 Schematic of IL-12 family cytokines, their receptors and signalling partners..... 69

Fig.1.5 Direct vs Indirect IR..... 97

Figure1.6 Comparison of exposure ranges of IR..... 99

Figure1.7 Cell cycle and DNA repair mechanisms 100

Figure 3.1 Isolation of CD14+ve population from PBMC 123

Figure 3.2 Determination of cytokine secretion depending on the type of FBS in media..... 124

Figure 3.3 Morphology of MoMφ differentiated on normal vs low adherence plates 125

Figure 3.4 Comparison of IL-12p70 secretion by GM-Mφ generated in normal (NA) or low (LA) adherence plates..... 126

Figure 3.5 Time-course of Mφ generation..... 128

Figure 3.6 Effect of GM-CSF and M-CSF on the viability of Mφ 130

Figure 3.7 Effect of the presence of GM-CSF/M-CSF on cytokine secretion by MoMφ 132

Figure 3.8A. Effect of GM-CSF/M-CSF presence on HLA-DR and D86 expression by MoMφ. 134

Figure 3.8A. Effect of GM-CSF/M-CSF presence on HLA-DR and D86 expression by MoMφ. 135

Figure 3.8B, C Effect of GM-CSF/M-CSF presence on HLA-DR and D86 expression by MoMφ. 136

Figure 3.9 Secretion of cytokines by MoMφ 138

Figure 3.10A Phenotype of Mo-Mφ 139

Figure 3.10B Phenotype of Mo-Mφ 140

Figure 3.10 C Phenotype of Mo-Mφ 141

Figure 4.1 Effect of LPS and IFNγ dose on cytokine secretion by MoMφ .. 154

Figure 4.2 Effect of IFNγ and IL-4 on cytokine secretion by MoMφ 156

Figure 4.3A, B Effect of IFNγ and IL-4 on the expression of co-stimulatory molecules by MoMφ 159

Figure 4.3C Effect of IFNγ and IL-4 on the expression of co-stimulatory molecules by MoMφ 160

Figure 4.4 Repolarisation protocol 162

Figure 4.5 A,B. Effect of Mφ repolarisation on cytokine secretion 163

Figure 4.5 C,D. Effect of Mφ repolarisation on cytokine secretion 164

Figure 4.6A,B Effect of repolarisation on MoMφ phenotype..... 165

Figure 4.6C Effect of repolarisation on MoMφ phenotype 166

Figure 4.6.D,E Effect of repolarisation on MoMφ phenotype 167

Figure 4.6.F Effect of repolarisation on MoMφ phenotype 168

Figure 4.7 Titration of R848 on MoMφ..... 170

Figure 4.8 Comparison of the effect of LPS and R848 stimulation on MoMφ in the presence or absence of IFNγ and IL-4..... 172

Figure 5.1 Effect of IR on IL-23 secretion by GM-Mφ $_{(LPS/IFN\gamma)}$..... 185

Figure 5.2 Effect of IR on MoMφ viability 186

Figure 5.3 Comparison of the effect of IR on GM-Mφ and Mφ-M 187

Figure 5.4 Effect of IR on cytokine secretion by GM-Mφ(LPS/IFNγ) and M-M(LPS)..... 188

Figure 5.5 Expression of HLA-DR and CD86 by irradiated MoMφ..... 190

Figure 5.6 IR activates ATM kinase in MoMφ..... 192

Figure 5.7 Effect of IR on ATM kinase is dose dependent..... 193

Fig5.8 KU55933 effectively inhibits ATM kinase..... 194

Figure 5.9 Effect of ATM inhibition on IL-23 secretion by MoMφ 195

Figure 5.10 Effect of ATM inhibition on viability of irradiated MoMφ..... 196

Figure 5.11 Inhibitory effect of ATM on IL-23 is TLR-dependent 198

Figure 5.12 Inhibition of ATM triggers IL-23 secretion by Mφ2 MoMφ.... 200

Figure 5.13 Regulation of CREB activity by IR-induced ATM 202

Figure 5.14 Effect of the inhibition of CREB-CBP interaction on ATM-dependent IL-23 secretion..... 202

Figure 6.1 Phenotype of immature and mature MoDC..... 212

Figure 6.2 A,B. Effect of FBS in medium on DC generation..... 215

Figure 6.2C. Effect of FBS in medium on DC generation..... 216

Fig 6.3A Determination of DC viability following exposure to IR..... 218

Fig 6.3B Determination of DC viability following exposure to IR..... 219

Figure 6.4 Effect of IR on IL-23 secretion by MoDC 220

Figure 6.5. Effect of IR on cytokine secretion by mDC 221

Figure 6.6 Activation of ATM following IR..... 223

Figure 6.7 Phosphorylation of ATM following irradiation and treatment with KU55933 223

Figure 6.8 Effect of ATM inhibition on IL-23 secretion by MoDC..... 225

Figure 6.9 Effect of ATM inhibition on MoDC survival..... 227

Figure 6.10 ATM-dependent inhibition of IL-23 is mediated through CREB/CBP interactions..... 229

Figure 6.11A Effect of IR on DC ability to stimulate Th17 responses 231

Figure 6.11B Effect of IR on DC ability to stimulate Th17 responses..... 232

Figure 7.1 Effect of FB-DC co-culture on cytokine secretion by MoDC..... 241

Figure 7.2 Effect of FB on cytokine secretion by slan DC 243

Figure 7.3 Effect of FB on cytokine secretion by Mφ 244

Figure 7.4 FB up-regulate IL-23 secretion by irradiated DC..... 246

Figure 7.5 FB are functionally impaired by IR 248

Figure 7.6 Determination of FB viability following exposure to IR..... 249

Figure 7.7 β-galactosidase staining of primary FB 7 days after treatment with 0-15 Gy IR..... 250

Figure 7.8 FB dependent increase of IL-23 is mediated by soluble factors.. 251

Figure 7.9 FB up-regulate IL-23 secretion by DC through PGE2 in a COX2-dependent manner 252

Figure 7.10 Effect of Fb on ATM phosphorylation in irradiated DC..... 253

Figure 7.11 Effect of c-AMP activation on IL-23 secretion by IR DC. 254

Figure 7.12A FB promote Th17 responses 255

Figure 7.12B. FB promote Th17 responses 256

Figure 7.13. Proposed signalling transduction pathways involved in regulation of IL-23 secretion by irradiated DC..... 260

Figure 7.14. Model of DC-FB crosstalk governing IL-23 dependent Th17 responses after irradiation 262

Fig S.1A Effect of LPS and IFNγ dose on IL-12 secretion by MoMφ..... 274

Fig S.1B Effect of LPS and IFNγ dose on IL-23 secretion by MoMφ..... 275

Fig S.1C Effect of LPS and IFNγ dose on IL-10 secretion by MoMφ..... 276

Figure S.2A Comparison of the effect of LPS and R848 stimulation on cytokine secretion by MoMφ 277

Figure S.2B Comparison of the effect of LPS and R848 stimulation on cytokine secretion by MoMφ 278

Figure S.2C Comparison of the effect of LPS and R848 stimulation on cytokine secretion by MoMφ 279

Figure S.2D Comparison of the effect of LPS and R848 stimulation on cytokine secretion by MoMφ 280

Figure S.2E Comparison of the effect of LPS and R848 stimulation on cytokine secretion by MoMφ 281

Tables

Table 1.1 Human PRR, their location and ligands 28

Table 1.2 Types of human and murine Mo 39

Table 1.3A Characteristics of most prevalent Mφ activation types 51

Table 1.3B Characteristics of most prevalent Mφ activation types..... 52

Table1.4A Characterization of surface markers typically expressed on Mφ1 57

Table 1.4B Characterization of surface markers typically expressed on Mφ2 58

Table 1.5 TLR expression patterns in human DC subsets 67

Table 1.6 Types of ionizing radiation..... 96

Table2.1 Antibodies used for flow cytometry..... 117

Table 4.1 Effect of Mφ type on the cytokine secretion in response to LPS and IFNγ 154

Table 5.1 Effect of IR on cytokine secretion by MoMφ..... 189

Table 6.1 Effect of IR on cytokine secretion by mDC 222

Abbreviations

AD atopic dermatitis, **Ag** antigen, **AOM** azoxymethane, **AP-1** Activator protein 1, **APC** antigen presenting cells, **ArgI** arginase I, **A-T** Ataxia-telangiectasia, **ATF-2** Activating transcription factor 2, **ATP** adenosine triphosphate, **ATM**- ataxia teleangiectasia mutated, **ATR** - ataxia telangiectasia and Rad3-related, **BCG** bacillus Calmette Guerin, **Bcl3** B-cell leukaemia 3, **BCL-10** B cell lymphoma 1, **BM** bone marrow, **CARD** caspase recruitment domain family, **CBP** CREB binding protein, **C-Ci** 2-Naphthol-AS-E phosphate, **CCL** Chemokine (C-C motif) ligand, **CCR** Chemokine (C-C motif) receptor, **cDC** conventional DC, **CDK** Cyclin-dependent kinase, **CDP** common DC precursor, **Chk** Checkpoint kinase, **CLR** C-type lectin receptors, **CREB** - Ca^{2+}/cAMP response element-binding protein, **CTLA-4** cytotoxic T-lymphocyte-associated protein 4, **CXCR** C-X-C motif chemokine receptor, **CXCL** Chemokine (C-X-C motif) ligand, **CLP** common lymphoid progenitors, **cMoP** Mo-specific progenitor, **CMP** common myeloid progenitor, **COPD** chronic obstructive pulmonary disease, **COX2** Cyclooxygenase 2, **C/EBPβ** CCAAT-enhancer-binding protein, **DAMP** damage-associated molecular patterns, **DC** dendritic cells, **DCreg** regulatory DC, **DDR** DNA damage response, **Dex** dexamethasone, **DMSO** Dimethylsulfoxide, **DNA** deoxyribonucleic acid, **DNA-PKcs** DNA-dependent protein kinase catalytic subunit, **DMBA/TPA** 12-dimethylbenz-(a)anthracene/12-O-tetradec-anoylphorbol-13-acetate, **ds** - double stranded, **DSB** double strand break, **DSS** dextran sulfate sodium, **E** embryonic day, **EAE** experimental autoimmune encephalomyelitis, **EBI3** Epstein Bar virus–

induced protein 3, **EC** endothelial cells, **ECM** extra-cellular matrix, **EMP** erythro-myeloid progenitors, **EPAC** exchange factor directly activated by cAMP, **EpCAM** epithelial cell adhesion molecule, **FBS** foetal bovine serum, **FcγR** Fc receptors for immunoglobulin G, **FL** foetal liver, **GCS** glucocorticosteroids, **GM-CSF** Granulocyte-macrophage colony-stimulating factor, **GM-Mφ** MoMφ generated in the presence of GM-CSF, **HBEC** Immortalised human brain microvascular hCMEC/D3 endothelial cells, **HEAT** Huntington-elongation factor 3-protein phosphatase 2A-TOR1, **HIF1α** Hypoxia-inducible factor 1α, **HPA** hypothalamic-pituitary-adrenal, **HR** homologous recombination, **HSC** Haematopoietic Stem Cells, **HSPC** hematopoietic stem and progenitor cells, **IBD** inflammatory bowel disease, **IC** immune complexes, **ICAM** intracellular adhesion molecule, **ICOSL** inducible costimulator-ligand, **IDO** indoleamine 2,3 deoxygenase, **IFN** interferon, **Ig** Immunoglobulin, **IgSF** immunoglobulin superfamily, **IKK** IκB kinase, **IL-1R** Interleukin 1 receptor, **infDC** inflammatory DC, **IRF- 1** Interferon regulatory factor 1, **ITAM** immunoreceptor tyrosine-based inhibitory motif, **JNK** c-Jun-N-terminal kinases, **KC** keratinocytes, **KID** kinase-inducible domain, **LA** low adherence, **LC** Langerhans cells, **LIF** Leukemia Inhibitory Factor, **LFA-1** Lymphocyte function-associated antigen-1, **LMN** lymph nodes, **MALT1** mucosa-associated lymphoid tissue lymphoma translocation gene 1, **MAPK** Mitogen activated protein kinase, **MCA** methycholanthrene, **M-CSF** macrophage colony-stimulating factor, **MCP-1** monocyte chemotactic protein-1, **MDM2** Mouse double minute 2 homolog, **MDP** Mo-Mφ/DC progenitor, **Mφ** macrophages, **MHC** major histocompatibility complex, **M-Mφ** MoMφ

generated in the presence of M-CSF, **Mo** monocytes, **MoDC** monocyte-derived DC, **MoMφ** monocyte-derived macrophages, **MoP** Mo precursors **MPL** 1-8 only 3-O-desacyl-4'-monophosphoryl lipid, **MR** mannose receptor, **MS** multiple sclerosis, **mTOR/FRAP** mammalian target of rapamycin (mTOR)/FKBP-rapamycin-associated protein, **MyDC** myeloid DC, **NA** normal adherence, **NFκB** nuclear factor kappa-light-chain-enhancer of activated B cells, **NHEJ** non-homologous end-joining, **NSAID** nonsteroidal anti-inflammatory drugs, **NSCLC** Non-small-cell lung carcinoma, **NLR** NOD-like receptors, **nT-reg** natural T-reg, **OX40L** OX40 ligand, **PAMP** pathogen associated molecular patterns, **PBS** Phosphate-buffered saline, **PD** Programmed death, **pDC** plasmacytoid DC, **PDGF** Platelet-derived growth factor, **PDX** patient-derived xenograft, **PGE2** Prostaglandin E2, **PIKK** phosphoinositide 3-kinase-like protein kinases, **PKA** Protein kinase A, **PP2A** Protein phosphatase 2A, **PRR** pattern recognition receptors, **Rac1** Ras-related C3 botulinum toxin substrate 1, **RAE** retinoic acid early inducible, **RCC** renal cell carcinoma, **Rh** recombinant human, **RIG** Retinoic acid-inducible gene, **RLR** RIG-I-like receptors, **ROS** reactive oxygen species, **SCS** subcapsular sinus, **SEA** soluble egg Ag, **Ser** Serine, **slanDC** Sulpho LacNAc DC, **SLE** systemic lupus erythematodes, **SpA** spondyloarthritis, **SCC** Squamous cell carcinoma, **SSB** single strand break, **STAT** Signal transducer and activator of transcription, **Syk** spleen tyrosine kinase, **TAF** tumour associated FB, **TCR** T-cell receptor, **TGF-β** transforming growth factor-β, **TIDC** tumour-infiltrating DC, **TIL** tumour-infiltrating lymphocytes, **TLR** Toll-like receptors, **TLSP** thymic stromal lymphopoietin, **TNFα** tumour necrosis factor α, **TNFSRF** tumour necrosis factor receptor superfamily, **T-reg** regulatory T-cell, **VCAM-**

1 vascular cell adhesion molecule -1, **VEGF** Vascular endothelial growth factor, **YS** yolk sac

Chapter 1 Introduction

1.1 Antigen presenting cells - function and place in the immune system

Professional antigen presenting cells (APC) include dendritic cells (DC), macrophages (Mφ) and B-cells forming a highly heterogeneous group of immune cells which mediate adaptive immune responses by processing and presenting antigens (Ag) to T-cells and B-cells, thereby delivering activating signal [1]. Importantly, Ag presentation is not a unidirectional process. APC exhibit dynamic responses to their environment and other leucocytes as well as stromal cells can greatly influence the functions of APC through secreted mediators (e.g. cytokines, chemokines) and direct cell-cell contact (e.g. Lymphocyte function-associated antigen-1 (LFA-1)/CD54, CD40-CD40L) [2-5].

1.1.1 Antigen processing and presentation

The unifying feature of professional APC is their ability to present Ag in the required context resulting in triggering and directing adaptive immunity. The Ag are classically presented on major histocompatibility complex (MHC) class I and II molecules. The Ag presented by MHCI are derived from endogenous proteins which are degraded in the cytosol by the proteasome [6]. On the other hand, epitopes presented by MHCII are derived from exogenous proteins degraded in the endosomes [6]. In addition, DC and possibly some Mφ and Mo have the ability to cross-present Ag to T-cells by expressing exogenous proteins on MHCI which play a key role for immunity against viruses and intracellular bacteria [7, 8].

There are four major pathways used by DC and Mφ to internalise exogenous Ag which include: endocytosis, pinocytosis, phagocytosis and micropinocytosis [9]. The internalisation route is determined by the nature of the pathogen [9]. The uptake of small soluble Ag is implemented by pinocytosis and micropinocytosis [9]. Small particulate Ag are internalised by receptor-mediated endocytosis while large-particular Ag such as opsonised bacteria or apoptotic cells enter the cell by phagocytosis [9]. Receptors involved in phagocytosis and endocytosis include Fc receptors for immunoglobulin G (FcγR), complement receptors, scavenger receptors and CD36 [9].

1.1.2 PRR

Microorganisms are sensed by germline-encoded pattern recognition receptors (PRR) which recognise highly conserved structures among microbial species called pathogen associated molecular patterns (PAMP) [10, 11]. Currently there are four recognised types of PRR including transmembrane proteins such as the Toll-like receptors (TLR) and C-type lectin receptors (CLR), as well as proteins localised in the cytoplasm such as the Retinoic acid-inducible gene (RIG)-I-like receptors (RLR) and NOD-like receptors (NLR) [10, 12, 13]. Each PRR recognise different molecular patterns and therefore leads to a distinctive immune response. (Table 1.1).

PRRs	Location	Ligand	Origin of the ligand
TLR			
TLR1	Plasma membrane	Triacyl lipoprotein	Bacteria
TLR2	Plasma membrane	Lipoprotein	Bacteria, viruses, parasites, self
TLR3	Endolysosome	dsRNA	Virus
TLR4	Plasma membrane	LPS	Bacteria, viruses, self
TLR5	Plasma membrane	Flagellin	Bacteria
TLR6	Plasma membrane	Diacyl lipoprotein	Bacteria, viruses
TLR7 (Human TLR8)	Endolysosome	ssRNA	Virus, bacteria, self
TLR9	Endolysosome	CpG-DNA	Virus, bacteria, protozoa, self
TLR10	Endolysosome	Unknown	Unknown
CLR			
Dectin-1	Plasma membrane	β-Glucan	Fungi
Dectin-2	Plasma membrane	β-Glucan	Fungi
MINCLE	Plasma membrane	SAP130	Self, fungi
RLR			
RIG-I	Cytoplasm	Short dsRNA, 5′triphosphate dsRNA	RNA viruses, DNA virus
MDA5	Cytoplasm	Long dsRNA	RNA viruses (Picornaviridae)
LGP2	Cytoplasm	Unknown	RNA viruses
NLR			
NOD1	Cytoplasm	iE-DAP	Bacteria
NOD1	Cytoplasm	MDP	Bacteria

Table 1.1 Human PRR, their location and ligands (adapted from [10]).

In 1989 Janeway pointed to PRR as a mechanism allowing APC to discriminate between “non-infectious self” from “infectious non-self” therefore triggering inflammatory reaction to microbes [11, 14, 15]. This theory gained considerable support however, in 1994 Matzinger proposed that the immune system is adapted to detecting any type of “danger” regardless whether it is microbial or self-origin [16]. In agreement with the “danger model” many PRR including TLR2,4,7 play dual role and, in addition to detecting PAMP, can also sense molecules released from damaged cells, termed damage-associated molecular patterns (DAMP) (table1) [10, 17]. DAMP include heat shock proteins, uric acid, adenosine triphosphate (ATP), deoxyribonucleic acid (DNA) [18-20]. DAMP are released by cells which are stressed or dying due to infection, injury or irradiation [21].

The TLR, and in particular TLR4, are by far the most studied PRR so far. The engagement of TLR results in activation of APC and promotes their migration to lymph nodes (LMN) [22]. Ligation of TLR stimulates the secretion of pro-inflammatory cytokines such as IL-12, TNFα, IL-15 as well as up-regulation of co-stimulatory molecules including CD80, CD86 and CD40 [23-25]. In addition, TLR engagement augment phagocytosis and bactericidal activity through amplifying mitochondrial ROS production and exert positive control over MHCII Ag presentation by regulating phagosome maturation [26] [27]. Effects of TLR triggering affect all phases of DC-T-cell interactions. Activation of DC with LPS markedly increases Ag loading onto MHCII providing signal 1necesary for T-cell activation [23]. Co-stimulatory molecules such as CD40, CD80 and CD86 which provide signal 2 are rapidly

up-regulated upon TLR activation [23]. Moreover, TLR signalling further promotes DC maturation via stimulation of pro-inflammatory cytokines such as TNFα and IL-15 which also increase the expression of co-stimulatory molecules [23]. Other cytokines triggered by TLR such as IL-1, IL-2 and/or TNFα contribute to T-cell expansion while soluble factors, for example IL-6 secreted in response to CpG or LPS, makes T-cells refractory to suppression by T-reg [23]. On the other hand, induction of IL-12 provides signal 3 promoting Th1 responses [23].

1.1.3 Modulation of T-cell responses

Activation and differentiation of T-cells depends on the delivery of three signals by APC. Signal 1 represents the Ag epitope delivered to the T-cell receptor (TCR) through the engagement of the MHCI/MHCII molecule and conveys information about the molecular identity of the pathogen [28]. Signal 2 which promotes T-cell survival and proliferation is delivered to T-cells through the interaction between co-stimulatory molecules on the surfaces of both APC and T-cells. Signal 2 relies information of the APC-activating potency of the Ag and therefore about its pathogenic potential [28]. The absence of signal 2 results in anergic T-cells [28, 29]. The repertoire of co-stimulatory molecules providing signal 2 is highly versatile and adapts to changes in the tissue environment [30]. Most signal 2 molecules belong to the immunoglobulin superfamily (IgSF) or tumour necrosis factor receptor superfamily (TNFRSF). These can be further divided into families based on their primary amino acid sequence, protein structure and function. The CD28 T-cell receptor which binds to CD80/86 expressed on APC is one of the best

described activating receptors on naïve T-cells [31]. The CD28 and B7 families belong to the IgSF receptors superfamily. Most members of the CD28 family interact primarily with members of the B7 family [32]. However, they exert differential effects on T-cells. While CD28 binding activates T-cells, CD28 homologue, cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), which also binds to CD80/86 competing with CD28, serves as a negative regulator of T-cell activation through inhibition of IL-2 production and cell cycle progression [31]. In addition to CD80/86 intracellular adhesion molecule 1 (ICAM-1) and/or ICAM-2, and OX40 ligand (OX40L) with their respective T-cell counter-receptors, LFA-1 and OX40, represent the principal contact-dependent factors providing signal 2 and stimulating T-cell responses [28, 29]. On the other hand, APC can inhibit T-cell responses by expressing inhibitory molecules, such as the Programmed cell death 1 ligand (PD-L1) which bind to PD-1 receptor expressed on activated T-cells [33]. The interaction of PDL-1 with its receptor leads to T-cell apoptosis and functional exhaustion [34]. Therefore, the strength, duration of T-cell activation and formation of a long-term memory depends on the fine balance of positive and negative signals from APC [34].

Although signals 1 and 2 initiate T-cell activation and proliferation, without a specific cytokine milieu which represents signal 3, T-cells fail to develop optimal effector functions, survive poorly, and are not able to form a memory population [35]. The requirement for a signal 3, and therefore the presence of an inflammatory cytokine allows T-cells that encounter Ag to determine if there is ‘danger’ present, and to respond accordingly [36]. Furthermore, signal

3 is required to orchestrate the adaptive responses by directing T-cell differentiation and conversion towards particular lineage [28, 37]. For example, IL-12 and interferon (IFN) α/β provide signal 3 to CD8+ T-cells while IL-1 may be necessary for CD4+ T-cells [36]. IL-12 could replace the need for an adjuvant in supporting a CD8+ T cell response to peptide Ag during immunisation, while IL-1 did not have an effect [38, 39]. On the other hand, IL-1 substantially enhanced proliferation and differentiation of CD4+ to immunization with peptide Ag in the absence of adjuvant [40, 41]. IL-12 did not exert such an effect on CD4+ T-cells despite its important role in governing CD4+ T-cells polarisation [36]. IL-12 and IFNγ favour Th1 generation, IL-4 and IL-13 stimulate Th2 while IL-6 together with IL-1β and IL-23 drives Th17 generation [37]. On the other hand, transforming growth factor-β (TGF-β) and IL-2 promote T-reg [37]. Importantly a single cytokine is not sufficient in determining the outcome of T-cell differentiation [42]. IL-4 requires another cytokine such as IL-2 which activates STAT5 to polarise T-cells towards Th2 [42]. TGF-β induces distinct T-cell subpopulations in combination with different cytokines: with IL-2 it promotes T-reg, with IL-6 Th17 while with IL-4 induces Th9 cells [42].

Both signal 1 and signal 2 are understood to play important roles in T-cell polarisation thus affecting to a certain extent signal 3. The strength of the interaction of the T-cell receptor with Ag (Signal 1) impacts on the differentiation process in T-cells whereby weak interactions promote Th1 while strong Th2 [43]. Moreover, the binding of co-stimulatory molecules (Signal 2) has been reported to play a role in T-cell polarisation for example

strong CD28 and CD80/86 interactions were shown to favour Th17 development [44].

1.2 Macrophages

1.2.1 The ontogeny of tissue macrophages.

Mφ are found in all tissues and display great heterogeneity in their functions and transcriptional profiles. For many years, Mφ were thought to be derived only from blood Mo entering the tissues [45]. However, recent research demonstrated that first Mφ arise already at embryonic stage before the development of haemapoietic system [45]. Furthermore, lineage-tracing experiments showed that Mφ of different origin prevail in adult tissues albeit at varied proportions. For example, while yolk sac (YS)- derived Mφ are the predominant source of microglia (the resident Mφ of nervous system), they represent a minority (3-5%) of Mφ in adult heart and liver [45],[46],[47],[48],[49],[50]. These and other findings helped to redefine the ontogeny of tissue Mφ as well as revealed their new functions during development [45],[49],[50]. They also posed many new questions as to whether tissue Mφ and Mo-derived Mφ (MoMφ) have similar functions (especially important considering that most *in vitro* experiments are performed using MoMφ) or as to the nature of the relationship between Mo and Mφ.

Current Mφ ontogeny is based mainly on research performed on rodents and describe three lineages of tissue Mφ, each originated at a different developmental stage [45] [47]. Murine Mφ first develop at E8 (embryonic day) from the primitive endoderm of the YS in a process called primitive

haematopoiesis (Figure 1.1) [45],[47]. These Mφ lack a monocytic progenitor and spread through the embryo, including the brain, when the circulatory system becomes established from E8.5 to E10. [45, 51],[47]. These cells retain their high proliferative potential in peripheral tissues and play crucial role in embryogenesis through involvement in the removal of dead cells and tissue maturation [47]. YS Mφ are the main precursors of microglia in the brain and prevail through adulthood free of foetal Mo infiltration [47].

Primitive haematopoiesis is followed by haematopoiesis in the foetal liver (FL) from multi-lineage erythro-myeloid progenitors (EMP) derived from YS which colonise FL from E9.5 and Haematopoietic Stem Cells (HSC) from haematogenic endothelium of the aorto-gonadal-mesonephros region of the embryo. After E11.5 FL becomes the major haematopoietic organ generating all haematopoietic lineages. The definitive haematopoiesis which starts in the FL depends on the transcription factor Myb, in contrast to primitive haematopoiesis in the YS which relies on PU.1 [47, 50]. Mφ derived from FL are generated from Mo and give rise to the majority of adult tissue Mφ [47]. This wave of Mφ travels to all the organs including lung, heart, pancreas and liver superseding YSMφ and is independent of Chemokine (C-C motif) ligand 2/ Chemokine (C-C motif) receptor 2 (CCL2/CCR2) axis [47, 50]. Liver Kupffer cells and lung alveolar Mφ are mainly derived from FLMo [47, 50], [48],[49] while the heart contains different pools of Mφ which persist in adults – derived from YS as well as from FL [47],[48].

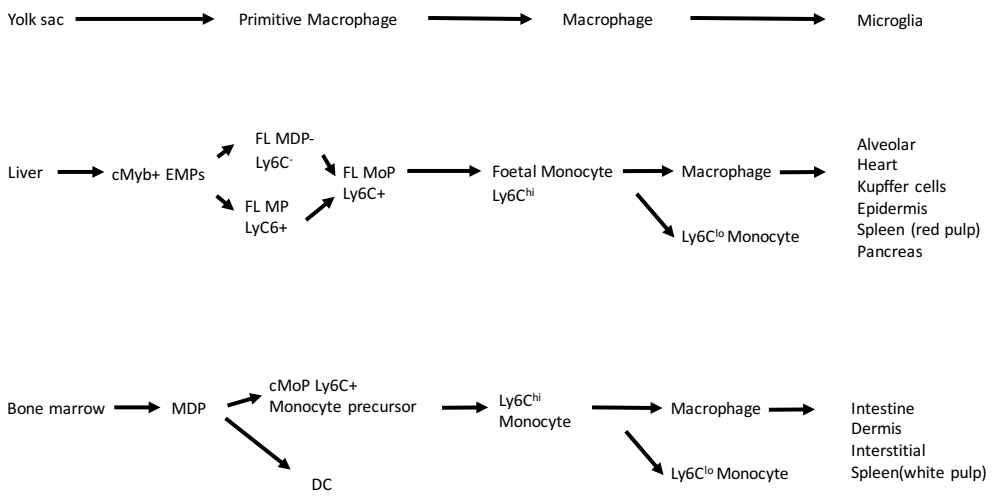


Figure 1.1 Ontogeny of tissue Mφ. Mφ derive from 3 lineages originated at different development stages. First Mφ develop from primitive endoderm in yolk sac in a process of primitive haematopoiesis and spread through embryo through circulatory system. These Mφ are progenitors of microglia. This stage is followed by haematopoiesis in foetal liver which produces Mo-progenitors developing into foetal Mo. Foetal Mo are the precursor of the majority of tissue Mφ. Post-natal haematopoiesis is maintained by BM which similarly to FL haematopoiesis generates Mo which differentiate into Mφ. EMP- erythro-myeloid progenitors , FL- foetal liver , MDP- macrophage/ dendritic cell progenitor, MoP – Mo precursors, DC – dendritic cell

Liver haematopoiesis declines post-natal and is replaced by bone marrow (BM) haematopoiesis from E17.5 [45],[47],[48] (Figure 1.1). For many years' Mφ derived from adult BMMo have been considered as the only source of tissue Mφ [47],[48],[52]. Although this model has now been reconsidered, adult Mo are an important source of tissue Mφ in certain tissues and conditions [47],[48],[52]. Dermis continuously recruits adult Mo and contains both FL- as well as BM-derived Mφ [47],[48]. Similarly, embryonic Mφ are completely replaced by BMMφ in the intestines [47]. The replacement of embryonic Mφ by BMMφ may be connected with decreased self-renewal capacity of embryonic Mφ during adulthood [47]. Adult MoMφ are also key component of the inflammatory milieu [53]. They originate from Mo derived

from haematopoietic stem cells in the BM through definitive haematopoiesis [54].

The Mo-Mφ/DCprogenitor (MDP) is a common link between Mo and DC [54, 55]. It develops into Ly6c+ Mo-specific progenitors (cMoP) which possess proliferative ability [54, 55]. The cMoP progenitors develop into Mo which in mice are composed of 3 subsets: classical Ly6c^{hi} which develop directly from Ly6c+ cMoP, Ly6c^{lo} non-classical Mo differentiated from Ly6c^{hi} Mo and intermediate Mo [54, 55].

Comparison of gene expression demonstrated that Mo subsets in mice closely correlate with human subsets where the Ly6c^{hi} population corresponds with human CD14^{hi}CD16^{-} classical subset and the Ly6c^{lo} population matches the CD14^{dim}CD16^{+} or CD14^{+}CD16^{++}subset [56] (Table 1.2). The intermediate human subset is characterised by low CD16 and high CD14 expression (CD14^{++}CD16^{+} or CD14^{+}CD16^{+}) [57]. Despite the similarities, there are also several important differences between mice and human. While in mice the Ly6C^{hi} Mo are often termed "pro-inflammatory" due to their pro-inflammatory potential, high secretion of TNFα and IL-1, and preferential recruitment to the sites of inflammation, in humans non-classical CD14+CD16+ Mo have been considered as pro-inflammatory because of high levels of pro-inflammatory cytokines such as TNFα and IL-1 released upon stimulation with LPS and higher expression of HLA-DR, CD40 and CD86 comparing to classical and non-classical CD14^{lo}CD16^{+} Mo [58-60]. On the other hand, the classical subset of human Mo preferentially produces IL-10

[59]. In addition, CD16+ve Mo population drastically increase in blood during cancer and many infectious and inflammatory disorders [58, 59]. Their number also raises temporarily during stress and exercise through mobilisation from the marginal pool [61]. On the other hand, therapy with glucocorticosteroids (GCS) depletes CD16+ve Mo while increases the absolute numbers of classical Mo [62].

In mice, the Ly6c^{lo} Mo are thought to remain in the periphery patrolling and removing damaged endothelial cells (EC) [54] while less than 1% may home to un-inflamed tissues in a G-protein-dependent process [59, 62]. Initially research showed that there is no influx of Ly6C^{lo} Mo during early stages of inflammation however Auffray *et al*. demonstrated that Ly6C^{lo} Mo extravasate as quickly as 1-2h after infection with *Listeria monocytogenes* stimulated by expression of CX3CR1 ligands [59, 62]. This triggers early transient inflammatory responses which are overtaken by Ly6C^{hi} Mo at 8 hours after infection [59]. Those LyC6^{lo} Mo seem to initiate differentiation transcription program towards Mφ2 phenotype in contrast to Ly6C^{hi} Mo which promote Mφ1 responses [59, 63, 64]. However, some reported the differentiation of LyC6^{lo} to Mφ1 and Ly6C^{hi} to Mφ2 [65]. On the other hand, during steady state conditions, the Ly6c^{hi} Mo constantly enter tissues from blood and transport Ag to lymph nodes through the lymphatic system while retaining their monocytic characteristics [66]. However, during inflammation, the Ly6C^{hi} Mo are recruited to the tissues through CCL2/CCR2 or CCL1/CCR8 and give rise to Mφ or DC [58, 66]. In humans, the CCL2-CCR2 axis is activated during inflammatory conditions resulting in the infiltration of

classical CD14+CD16- Mo and their differentiation into Mφ under the influence of M-CSF and GM-CSF [62].

The CD16+ Mo do not express CCR2, instead expressing C-X3-C motif chemokine receptor 1 (CX3CR1, Fractalkine receptor) receptors so may be preferentially recruited to anatomic sites reach in CX3CL1 such as brain, intestines and lymph nodes (LMN) [67]. The CD14+CD16+ preferentially differentiate into DC upon trans-endothelial migration or activation with TLR1/2 [68, 69] but are also capable of differentiating into Mφ [62]. During human immunodeficiency virus (HIV) infection, the CD16+ subset expands dramatically and differentiate preferentially into Mφ which stimulate resting T-cells contributing to chronic immune activation [67]. CD16+ subset is also expanded in many tumours including breast and gastrointestinal carcinoma [70, 71]. Furthermore, the expression of CX3CL1is increased in some tumours including glioblastoma [72] while CX3CR1 deficiency was shown to impair metastatic ability of colon cancer [73]. However, the input of CD16+ Mo into the Mφ pool as well as their role during pathology require further investigation and validation.

	Markers	% in whole blood	% of Mo	Chemokine receptors	Main function
Murine					
	Ly6C high	4	40	CCR2-CX3CR1+	Patrolling, tissue repair, differentiation into Mφ, phagocytosis
	Ly6C intermediate				
	Ly6C low		60	CCR2+CX3CR1-	Inflammatory effectors, removal of damaged endothelial cells
Human					
Classical	CD14++CD16-	10%	85	CCR2+CX3CR1-	Phagocytosis, inflammatory effectors, give rise to Mφ
Intermediate	CD14++CD16+		5	CCR2-CX3CR1+	inflammatory effectors
Non-classical	CD14+CD16++		10	CCR2-CX3CR1+	Patrolling, anti-viral role

Table 1.2 Types of human and murine Mo (after [57]).

1.2.2 Environmental programing in homeostasis and disease

Considering the heterogeneous distribution of Mφ derived from YS, FL and BM in various tissues, it is important to understand what factors influence their distribution and why specific precursors play preferential roles in particular organs. One of the potential mechanisms of differential distribution is the availability of the niche and precursor timing. This may be responsible for the poor ability of FL Mo or BM Mo to colonise the brain. Microglia are generated from YS Mo and persist in adulthood [47],[48],[52]. By the time FL Mo are produced, the brain is separated from circulation by a nascent blood-brain barrier (closed around E13.5) making it difficult for Mo to pass. Another

reason for differential distribution of Mφ may be specific signalling received from the tissue and intrinsic capacity of the Mφ precursor [48]. The lack of TGFβ signalling in murine brain results in impaired development of microglia [74]. On the other hand, intestines are colonised by YS and FL Mφ before birth however, during adulthood only Mφ derived from BMMo are presents. The reason may be due to the short 4-6 weeks' life span of intestinal Mφ which rely on constant supply by BMMo dependent on CCR2-CCL2 axis [52].

In light of the differential distribution of Mφ, it is tempting to assume that different Mφ populations are required at different life-stages and in various tissues to perform unique functions. Several groups set out to establish whether Mφ of various origins are unique or equally competent in tissue-specific functions. Van de Laar *et al*. demonstrated that all three pools of Mφ (YS, FL, BM) are able to colonize empty alveolar space which usually is predominantly colonized by FLMφ [48]. Moreover, after 6 weeks in the lungs all Mφ displayed almost identical gene expression profiles [48]. This report demonstrates very well the dominant nature of tissue microenvironment programming in determining the Mφ phenotype. Recently two reports showed that although tissue Mφ share epigenetic structure and gene expression with other myeloid cells and those are modulated by master transcription factors like PU.1, each tissue also drives unique gene expression profile controlled by changes in enhancer landscapes (histone modifications associated with enhancer regions in gene promoters) [75, 76]. In support of this thesis peritoneal Mφ transferred to the lung lost most peritoneal programing and

displayed programing specific for the new tissue [76]. Similarly, although different populations of Mφ exist in myocardium during steady state conditions, after depletion, BMMφ can repopulate all niches [77]. On the other hand, Guntra *et al*. show differences in transcriptional profiles between murine peritoneal Mφ of prenatal origin and BMMφ in response to activation with IL-4 where only BMMφ expressed PDL-2 and CD206 and induced FOXP3 expression in CD4 T-cells [78]. Functional differences correlated with ontogeny were also found in the liver where Kupffer cells – the resident Mφ - demonstrated mainly tolerogenic properties while BMMφ were most likely immunogenic [79]. These findings may suggest that although Mφ of different origin may be able to exert similar tissue-specific properties during homeostasis, during disease or injury they play slightly different functions.

Many tissue Mφ of embryonic origin are characterised by long life-span like murine alveolar which have life-span similar to the animal itself [54, 77, 80-82]. In addition, many tissue Mφ possess the ability of self-renewal by proliferation [54, 77, 80-82] in response to Mφ colony-stimulating factor (M-CSF) [83-85]. Therefore, with the exemption of tissues like skin and gut, during steady-state conditions tissue Mφ are able to replenish their number without the need of additional considerable influx from blood Mo [54, 86]. This situation however changes during diseases when Mφ expand substantially and play critical role in modulating inflammation and healing. Jenkins *et al*. and others demonstrated that during nematode infection, local pool of tissue-resident Mφ expanded through proliferation stimulated by Th-2 derived IL-4 in M-CSF dependent and independent fashion [78, 86, 87].

Moreover, while M-CSF led to influx of MoMφ, IL-4 signalling again triggered their proliferation [86, 87]. Similarly, Granulocyte- Mφ colony-stimulating factor (GM-CSF) was shown to induce murine Mφ proliferation [88, 89]. However, in addition to proliferation, new Mφ are generated from migrating Mo under the influence of M-CSF and GM-CSF [90]. During liver injury, the local pool of Kupffer cells expanded through proliferation, however blood Mo were also massively recruited to the site of injury through CCR2-CCL2 axis and dominated in the Mφ pool [79]. Similarly, the local pool of Mφ during heart infarct expanded dramatically through massive influx of LyC6+high Mo which differentiated into pro-inflammatory Mφ [91].

The local tissue conditioning plays important role during the differentiation of Mφ from Mo. Changes in regulation of MoMφ transformation depending on current tissue state were described in the colon [92]. During steady-state conditions Mo differentiate into regulatory IL-10 secreting Mφ CX3CR1hi in mice and $CD14^{lo}$ in human. However, during inflammation, normal MoMφ differentiation is disrupted resulting in accumulation of pro-inflammatory Mo [92]. While, at the onset of injury both liver and heart BMMφ display pro-inflammatory phenotype, accumulating evidence suggest that during progress of the disease local environmental factors promote the switch to $Ly6C^{lo}$ Mφ supporting resolution of the inflammation [79, 91]. Important findings were recently published by Franklin *et al.* who found that tumour-associated Mφ (TAMφ) in mammary carcinoma form a population distinct from resident mammary tissue Mφ and are not generated by conversion of resident Mφ [93, 94]. Instead TAMφ originated from pro-inflammatory Mo recruited to the site

differentiated towards Mφ. However, the differentiation process was disrupted by tumour microenvironment and resulted in a unique phenotype of TAMφ which accumulated in tumour tissues through proliferation [93, 94]. In support of these findings, Colegio *et al*. showed that tumours secrete lactic acid which is scavenged by TAMφ. Lactic acid activates HIF1α in Mφ which drives expression of a wide array of genes including Vascular endothelial growth factor (VEGF) and Arginase 1 (Arg1) which promote tumour growth and neovascularisation [95]. Movahedi *et al*. confirmed the monocytic origin of TAMφ in mammary murine tumours [96]. Although all TAMφ were generated from Ly6C^{hi}CX3CL1^{lo} Mo, heterogeneous TAMφ populations coexisted in the same tumour depending on the microenvironment. The MHCII^{low} population resided in the normoxic areas of the tumours while the MHCII^{high} TAMφ were located mainly in hypoxic areas and displayed a superior pro-angiogenic activity. These results demonstrate the predominant role of tissue microenvironment for Mφ differentiation and establishing a specific desired phenotype. Furthermore, they provide validation for using MoMφ in *in-vitro* studies.

1.2.3 The diversity of macrophage activation

Mφ were described more than a century ago by Ilya Mechnikov as phagocytes playing key role in innate immunity [97]. During steady state, Mφ are responsible for important homeostatic functions including clearing erythrocytes, apoptotic cells and removal of cellular debris generated during tissue remodelling [98]. These functions are performed by unstimulated Mφ and are accompanied by little or no immune activation [99]. Receptors

involved in these processes include scavenger receptors, the thrombospondin receptor, integrin and complement receptors [100]. Activation of these receptors either fail to transduce signals for induction of pro-inflammatory response or actively produce inhibitory response [100]. In addition, Mφ can detect innate danger signals through a range of receptors including TLR, intracellular PRR and the Interleukin 1 receptor (IL-1R) [101]. Also signals received from other immune cells as well as signals from Mφ themselves can affect their activation status [102, 103]. Upon receiving signals from their environment, Mφ change their morphology and become activated. Mills and Ley suggested that innate immunity controls adaptive immunity and Mφ use T-cells to amplify specific Mφ responses [104]. It may be suggested that Mφ and T-cells form a self-maintaining circle of mutual amplification and maintenance signals.

Originally, Mφ used in *in-vitro* experiments were activated with a combination of TNFα and IFNγ, both products of activated NK and T-cells [100]. This combination results in activated Mφ secreting a broad range of pro-inflammatory cytokines such as IL-12 and TNFα which can efficiently kill intracellular microorganisms [105]. For many years, this population of Mφ was thought to be the only type of activated Mφ [100]. In 1992, another population of Mφ was identified [106]. Signals such as those which are released during tissue injury like IL-4 or IL-13 were also shown to result in activation of tissue Mφ, although the result of this activation was strikingly different. This population of activated Mφ was proposed to be called alternatively activated Mφ in contrast to classical Mφ. Alternatively activated

Mφ produce low amounts of pro-inflammatory cytokines, are less efficient then classically activated Mφ at killing intra-cellular microorganisms and fail to present Ag to T-cells. However, they secrete a range of cellular matrix components and play role in matrix reorganisation and wound healing. With time, other types of alternatively activated Mφ were discovered. Following T-cell literature Mills *et al*. in 2000 proposed to call Mφ with pro-inflammatory properties stimulating Th1 responses as Mφ1 while Mφ stimulating Th2 responses as Mφ2 [107]. Later, other alternatively activated Mφ were joint into a collective Mφ2 group which consists of several separate populations exhibiting various anti-inflammatory properties. The difference between the ratio of IL-12/IL-23 and IL-10 has been suggested as the basis for differentiation between pro-inflammatory Mφ1 which are high producers of IL-12 and IL-23 and Mφ2 which have higher production of IL-10 [106]. However, this division describes cells at the end of their functional spectrums. *In vivo*, a mixed environment exists with lots of various stimuli and this results in Mφ sharing some characteristics from different groups (Figure 1.2) therefore in 2008 a spectrum of Mφ phenotype states was proposed by Mosser and Edwards [106, 108].

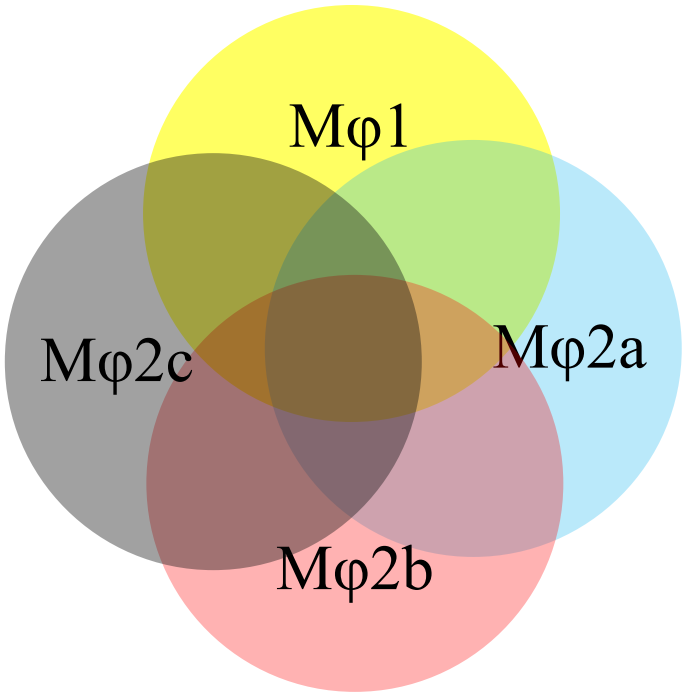


Figure 1.2 Schematic presentation of Mφ activation states. The Mφ1 and various types of known Mφ2 including Mφ2a, Mφ2b, Mφ2c and others, phenotypes represent extremes of the spectrum of Mφ activation (shown here as yellow, blue, red and grey circles) often used for in vitro studies. However in vivo Mφ represent the whole spectrum of activation often expressing mixed and intermediate phenotypes (presented as overlapping colours) [106, 108].

Classically activated or Mφ1 display microbicidal properties and secrete pro-inflammatory cytokines (Table 1.3A) [109]. Their main function is defence against intracellular pathogens directly as well as through activation of adaptive immune responses. [106]. Their generation is initiated by TLR ligation and innate stimuli like GM-CSF, IFNγ and TNFα secreted amongst other by NK cells and maintained by adaptive immune system mainly Th1 and their secretion of IFNγ. Mφ1 are efficient in Ag presentation and promote pro-inflammatory adaptive immune responses through activation of Th1 and Th17 cells [110]. They secrete high levels of pro-inflammatory cytokines, including IL-12, IL-23, TNFα, or IFNγ, and efficiently present Ag as shown by high

expression of HLA-DR and co-stimulatory molecules like CD86 and CD80. [111]. The role of Mφ in priming naïve CD4 responses is still under investigation however, they can efficiently activate memory T-cells as well as naïve CD8 T-cells [112-114]. The combination of activating stimuli, and the timing at which they reach the Mφ is important for their ability to kill the pathogen as can be seen on an example of *Leishmania* spp. infection [115]. These intracellular pathogens replicate primarily in tissue Mφ. Activation of Mφ simultaneously with TNFα and IFNγ before infection results in effective clearance of these parasites [115, 116]. However, stimulation with IFNγ alone results in less efficient clearance of these pathogens as they do not activate TLR receptors and therefore do not trigger detectable TNFα production. The stimulation with TNFα can be replaced effectively by TLR triggering with LPS which promotes endogenous TNFα secretion by Mφ. Interestingly although IFNγ given prior to infection increases the clearance of *Leishmania*, when it is added to Mφ after *Leishmania* infection the intracellular killing is decreased as *Leishmania* interfere with IFNγ signalling [115, 116]. On the other hand, due to high pro-inflammatory profile of Mφ1 their uncontrolled activation plays paramount role in a range of auto-immune disorders like inflammatory bowel disease and arthritis [117].

In contrast to Mφ1, the Mφ2 form a heterogeneous group of differentially polarised cells with unique morphology and functions (Table 1.3B) [118]. Wound healing Mφ or Mφ2a are activated during tissue injury [119, 120]. In response to tissue injury basophils and mast cells start releasing IL-4 which together with IL-13 is later maintained by Th2 [119]. As previously

mentioned in response to those cytokines, Mφ start secreting components of extracellular matrix [100]. They also secrete cytokines like IL-10 and TGFβ which down-regulate adaptive responses [100]. Another important role of Mφ2a is clearing helminths and nematodes infections [121, 122]. IL-4 and IL-13 up-regulate the expression of mannose receptor (MR) and HLA-DR and upregulate the expression of CCL20 and CCL17 [123]. Dysregulation of Mφ2 has been linked to asthma where they may play a role in airway remodelling. They may also be more susceptible to infection with certain intracellular pathogens like *Yersinia enterocolitica, Francisella tularensis* or *M. tuberculosis [100]*. There is a positive correlation between Th2 and Mφ2a and further reports on increased Th17 and iTreg expansion after Mφ2a transfer in murine models [124].

Regulatory Mφ or Mφ2c are generated in response to stress or adaptive responses [125, 126]. Stress activates the hypothalamic-pituitary-adrenal (HPA) axis resulting in release of GCS by adrenal glands [100]. Mφ treated with GCS down-regulate their IL-12 secretion resulting in decreased capacity to induce IFNγ by primed CD4 T-cells while increasing their secretion of IL-4 [127, 128]. Interestingly, short term (2-8h) pulsing of alveolar Mφ with dexamethasone (Dex) before stimulation with LPS lead to increase in their secretion of IL-1β [127, 129]. In contrast, extension of Dex stimulation into a phase of activation with LPS reduced IL-1β secretion by Mφ [129]. Similarly, low dose of Dex increases some of pro-inflammatory functions in contrast to high doses [129, 130]. Other stimuli driving Mφ2c can be provided by late adaptive responses during clearance of infection in the form of IL-10 or TGFβ

secreted by regulatory T-cells (T-reg) [131]. TGFβ and IL-10 produced by T-reg stimulate alternative activation of Mφ characterised by reduced HLA-DR and CD86 expression, increase in CD206 and CD163 and CCL18 expression and increased phagocytosis [131]. In addition, natural T-reg (nT-reg) supress the secretion of pro-inflammatory cytokines like IL-12, IL-1β and TNFα by Mφ stimulated with LPS while enhance IL-1Ra and IL-10 [131]. T-reg are able to inhibit secretion of TNFα or IL-6 in the presence of exogenous IFNγ suggesting their dominance in controlling Mφ phenotype [131]. However, the outcome may be different in the presence of Th1 as IFNγ represents just part of Th1 interactions. During inflammation Mφ2c suppress immune responses and are involved in tissue repair and remodelling [132]. They are also responsible for clearing of early apoptotic cells [133]. Mφ2c effectively reduced glomerulonephritis, tubular atrophy and proteinuria in murine nephrosis demonstrating potential therapeutic efficacy in certain treating kidney disorders [134].

Mφ2b form an exception in the Mφ2 group in that although they conform to the low IL-12, high IL-10 secretion rule, they retain some characteristics attributed to Mφ1 including high level of secretion of other inflammatory cytokines like TNFα and IL-6, the ability to present Ag and expression of co-stimulatory molecules like CD80 [111] or CD86 and HLA-DR [106, 135]. Those Mφ can be generated in the presence of immune complexes (IC) like DNA fragments which are phagocytosed by FcγR or complement receptor [106, 135]. They are involved in immune-regulation and Th2 activation [136]. The Mφ2b contribute to Lupus erythematosus and Lupus nephritis [135, 137,

138] and play a major role in increased susceptibility of burned patients to sepsis [136].

Mφ2d were introduced by Duluc *et al*. as a type of Mφ present in tumours. They are generated in the presence of IL-6, adenosine or LIF (Leukemia Inhibitory Factor) [139]. Mφ2d express high levels of VEGF and IL-10 and low levels of IL-12 and TNFα demonstrating wound healing and pro-angiogenic properties [140]. It is important however to note. that *in vivo,* TAMφ form heterogeneous group often displaying mixed phenotypes and representing a spectrum of Mφ polarization states.

Type	Stimuli	Function	Markers	Pathology
Mφ1	GM-CSF; LPS, IFNγ, TNFα, Th17?	Host-defence against intra-cellular pathogens, mediator of inflammation, drive Th1, Th17 responses	CD68; HLA-DR, CD80, CD86, IL-12, IL-23, TNFα, CCL3 (TNFα, LPS only)	autoimmunity, arthrosclerosis, rheumatoid arthritis (RA), inflammatory bowel disease (IBD)

Table 1.3A Characteristics of most prevalent Mφ activation types [106, 111, 125, 132, 133, 136-139, 141, 142],[135].

Type	Stimuli	Function	Markers	Pathology
Mφ2a	Tissue injury, Fungal and helminth infections? IL-4, IL-13	Wound healing, Drive Th2 responses	HLA-DR, CD200R, CD206, CD163, IL-10, TGFβ, IL-1ra, CCL18, CD86	Contribute to tissue fibrosis during schistosomiasis, asthma
Mφ2b	Immune complexes, LPS+IL-1β, LPS	Stimulation of Th2 responses, angiogenesis, Immunoregulation	HLA-DR, CD86 (DNA), IL-1, IL-10, TNFα, IL-6, CD80 (IL-1β+LPS)	Visceral leishmaniosis, systemic lupus erythematosus (SLE) Increased susceptibility of burned patients to sepsis
Mφ2c	IL-10	Immunoregulatory, Drive Treg responses, Promote vascular and epithelial repair, Clearence of early apoptotic cells	CD163 (-ve in TGFβ, MerTK, IL-10, TGFβ, CD200R, CCL18 (IL-10 only) CD206 (TGFβ only)	Decreased numbers in Lupus erythematosus
	TGFβ			
	Glucocorticoids-stress			
	Treg			
Mφ2d	IL-6, adenosine, LIF (Leukemia Inhibitory Factor)	Wound healing, angiogenesis	IL-10, VEGF, TGFβ	Cancer

Table 1.3B Characteristics of most prevalent Mφ activation types [106, 111, 125, 132, 133, 135-139, 141, 142].

1.2.4 The variability of Mφ phenotypes

Due to the extreme diversity and the multitude of possible polarisation states Mφ defy simple uniform description. Therefore, multiple laboratory methods are usually used for their characterisation including morphologic analysis, expression of surface molecules, secretory profile and gene profiling.

The majority of research on human Mφ is undertaken with MoMφ. However, in contrast to the generation of MoDC (for which there are well-established procedures), there is considerable heterogeneity in the methodology employed for MoMφ generation between research groups. Examples range from the usage of different culture plastic surfaces and medium through the duration of culture and type of stimulus used. For example, Clavel *et al*. polarised MoMφ in polymer inserts [141] while Ambarus and colleagues used plates of normal adherence [143]. In addition, while Clavel *et al*. generated Mφ with respective cytokines for just 4 days, Zizzo *et al*. pre-polarised Mφ with GM-CSF into Mφ1 or M-CSF into Mφ2 before final polarisation with IFNγ for Mφ1 or IL-10, Dex or IL-4 for Mφ2 [133]. In vitro generated Mφ typically display characteristic "fried egg" appearance and are strongly attached to the surface. This typical morphology can however change depending on the type of medium and cytokines used for culture ([144]. Rey-Giraud *et al*. showed that MoMφ can present a typical "fried egg" or spindle-shaped appearance depending on whether they were cultured in RPMI+10% Foetal Bovine Serum (FBS) or X-vivo medium and a in the presence of GM-CSF and M-CSF [144]. The presence of M-CSF or activation signal with LPS/ IFNγ during Mφ

culture leads to the generation of elongated cells while IL-4 promotes circular morphology [145]. On the other hand, stimulation of GM-CSF generated Mφ (GM- Mφ) leads to an increase in detachment from the surface [144]. These results demonstrate the impact of small experimental variations in culture conditions. Although some of these may initially seem insignificant, when combined with the notable plasticity of Mφ they may indeed account for the wide variety of distinct and conflicting outcomes. In an attempt to unify and standardise the field, a recent study contained recommendations for basic generation and reporting standards for *in-vitro* and *in-vivo* research [108].

Diversity is encountered by researchers also *in vivo* when attempting to classify Mφ via flow cytometry or immunohistochemistry in tissues. None of the markers used so far is specific for Mφ and their expression depends on the culture method, species and origin. For many years CD68 (the human homolog of mouse macrosialin) was thought to be a reliable Mφ marker [146] expressed by both Mφ1 and Mφ2. Whilst its function is not fully described, it serves as a scavenger receptor for oxidised low density lipoprotein [146]. In 2004 its specificity for Mφ was challenged by Kunisch and colleagues, who demonstrated its expression in FB [146]. Since then it was found to be present in a wide variety of cells including Mo, DC, EC and some tumour cell lines [146-149]. However, the lack of a better marker which would be specific for all Mφ as well as possible differences between the specificity of distinct antibody clones are the reasons why CD68 is still commonly used in Mφ research. Further characterisation of Mφ and their subsets requires a combination of markers which have higher intensity on some subsets in

comparison to others like HLA DR or which can be triggered only by specific stimulus for example MerTK [133, 143] (Table 1.4 A, B). Other markers may have differential expression by depending on the species used for research. For example, arginase I (ArgI) is a reliable marker for Mφ2 in murine models but is not induced in human Mφ [150, 151]. Molecules involved in Mφ - related activation of pro-inflammatory T-cell subsets - HLA-DR, CD86, CD80, CD40 - are in general up-regulated by T-cell derived cytokines notably IFNγ and IL-4 suggesting a feedback loop between those cells although they are generally higher upon IFNγ polarisation then IL-4 polarisation [143, 145]. In addition, CD80 is also up-regulated by other pro-inflammatory stimuli like TNFα or GM-CSF while it is absent in Mφ polarised with M-CSF or IL-10 making it preferred marker for pro-inflammatory Mφ [141, 143]. On the other hand, CD64, another marker connected with pro-inflammatory Mφ, is strongly up-regulated by IFNγ but two other pro-inflammatory stimuli, TNFα and GM-CSF fail to induce it [143] showing possible oversighted diversity in the population of pro-inflammatory Mφ.

Some commonly used markers connected with anti-inflammatory Mφ are CD163, CD16, CD206, CD200R and MerTK. Both CD163 and CD16 are induced by regulatory stimuli like IL-10, Dex, TGFβ or M-CSF but not by GM-CSF, IL-4 or IFNγ [133, 141, 143, 152] positioning them as good markers for regulatory Mφ (Mφ2c). MerTK is down-regulated by GM-CSF while it is up-regulated by Dex or M-CSF in combination with IL-10 [133].

IL-4 and IL-13 which are cytokines polarising wound-healing Mφ specifically up- regulate CD200R which is not affected by other stimuli like M-CSF, while it is down-regulated by IFNγ [141, 143]. Interestingly CD206 which is a marker traditionally connected with IL-4 in murine models (However, it is important to add that murine dermal Mφ express CD206 without the requirement of IL4 [153]) shows lack of specificity to Mφ2a subgroup in humans as (in addition to being upregulated by IL-4 and -13) it is increased by polarisation with GM-CSF to even higher degree [143, 154, 155]. It is however low on M-CSF, IFNγ or IL-10 stimulated cells [133, 143]. In contrast Zizzo *et al*. [133] found CD206 expression on cells polarised with Dex and M-CSF which may be due to different polarisation protocol.

The distribution of markers across Mφ spectrum changes when two-step polarisation (i.e. firstly with GM-CSF and M-CSF and secondly by T-cell derived cytokines and TLR agonists) is implemented. IFNγ did not increase CD80 and CD64 on GM-CSF polarised Mφ while it significantly increased it on M-CSF polarised Mφ [143]. Although Dex increased MerTK expression in both subsets, its effect was greater in M-CSF Mφ and required lower dose of Dex [133]. In addition, IL-10 required M-CSF to increase MerTK while another Mφ2c stimulus, TGFβ, inhibited MerTK even in the presence of M-CSF [133].

Name	Characterisation	Stimulus	Location	Function	Mφ type
HLA-DR	MHC class II receptor	IFNγ, IL-4	Cell surface	Ag presentation	Mφ1, Mφ2a, Mφ2b
CD86	Cluster of Differentiation 86 (B7-2)	IFNγ, IL-4	Cell surface	Co-stimulatory signals to T-cells. Ligand for CD28 and CTLA-4.	Mφ1, Mφ2a
CD80	Cluster of Differentiation 80 (B7-1)	IFNγ, GM-CSF, TNFα, IL-1β+LPS	Cell surface	Co-stimulatory signals to T-cells. Ligand for CD28 and CTLA-4.	Mφ1, Mφ2b
CD40	Cluster of Differentiation 40	IFNγ/LPS	Cell surface	Co-stimulatory protein activating APC. Binds to CD154 (CD40L) on Th cells	Mφ1
CD64	Cluster of Differentiation 64. Fc receptor	IFNγ	Cell surface	High affinity receptor for IgG Ab	Mφ1
CD206	the macrophage mannose receptor MMR	IL-4, GM-CSF, TGFβ	Cell surface	phagocytic receptor mediating the binding and ingestion of micro-organisms displaying glycans on their surface. Clearance of glycoproteins from circulation produced during inflammation and wound healing	Mφ1, Mφ2a, Mφ2c

Table1.4A Characterization of surface markers typically expressed on Mφ1 [111, 133, 141-143, 145, 156-160].

Name	Characterisation	Stimulus	Location	Function	Mφ type
CD163	RM3/1, haemoglobin scavenger receptor (HbSR), M130, or p155	IL-10, M-CSF, Dex, TGFβ	Cell surface	scavenger receptor binding Haemoglobin:Haptoglobin (Hb–Hp) complexes, receptor for gram+ and gram – bacteria	Mφ2c,
CD16	FcγRIII	IL-10, Dex, M-CSF	Cell surface	Low affinity Fc receptor. Binds to Fc portion of Igg antibodies and activated NK cells	Mφ2c
MerTK	member of Tam (Tyro3, Axl, Mer) receptor subfamily	Dex, IL-10+M-CSF	Cell surface	Removal of apoptotic cells	Mφ2c
CD200R	Receptor	IL-4, IL-13	Cell surface	Receptor for CD200. Involved in immunosuppression	Mφ2a
CD206	the macrophage mannose receptor MMR	IL-4, GM-CSF, TGFβ	Cell surface	phagocytic receptor mediating the binding and ingestion of micro-organisms	Mφ1, Mφ2a, Mφ2c,

Table 1.4B Characterization of surface markers typically expressed on Mφ2 [111, 133, 141-143, 145, 156-160]

The division of Mφ into various subtypes is further supported by their secretory profile. This seems to be less interchangeable in response to single stimuli then phenotypic markers. In general, Mφ1 are characterised by prominent secretion of pro-inflammatory cytokines notably IL-12, IL-23, TNFα, while the major Mφ2 cytokine, independent of their subset, is IL-10 with TGFβ or VEGF also being expressed in particular Mφ2 subsets [161]. The exception is presented by Mφ2b which retain high level of secretion of TNFα and IL-6 [111]. In addition to phenotypic and secretory profiles recent research has identified transcription factors and genes involved in differentiation of Mφ phenotype i.e. SOCS1 and IRF4 in IL-4 stimulated MoMφ and IRF5 and pSTAT1 in MoMφ activated with LPS or IFNγ [108]

1.3 Dendritic cells in immune system

DC are professional APC which sample Ag and initiate adaptive immune responses [162]. DC were first described by Ralph Steinman and Zanvil Cohn in 1973 as a novel immune cell type discovered in murine spleen [163]. They were given the name dendritic derived from the Greek word, Dendron, meaning a tree, due to their characteristic appearance of tree-like branching cells in phase-contract microscopy. In 1982, a rare population of cells (<0.1% of mononuclear cells) with features similar to murine DC (for example, high expression of MHCII and the ability to activate T-cells) was found by Steinman and colleagues in human blood [164]. Since then DC were found to form a heterogeneous population of haematopoietic cells present in both blood and tissues [165-169].

1.3.1 Dendritic cells subsets

DC can be divided into various populations based on their body distribution, ontogeny and performed functions. According to body distribution DC can be divided into cells circulating in the blood compartment or tissue resident. Circulating DC are divided into myeloid DC (myDC) otherwise known as conventional DC (cDC) and plasmacytoid DC (pDC) based on their expression of CD11c (a myeloid lineage marker) and CD123 (IL3R) (pDC marker). Although both markers are used as the main receptors to identify DC population, it is worth to keep in mind that they are non-specific with CD11c expressed also on Mo and Mφ [170, 171] while CD123 reported at high levels on basophilic granulocytes and at low levels on Mo, MyDC and certain subsets of haematopoietic progenitor cells [172].

The CD11c+ MyDC are currently divided into cDC1 expressing CD141 (thrombomodulin, BDCA3) and cDC2 expressing CD1c (BDCA1). The cDC2 co-express CD11b and high levels of CD11c while cDC1 lack CD11b and express low levels of CD11c. The CD141 is not a specific marker and can be expressed by a range of other cells. However, the cDC1 can be differentiated from other cells by low or absent expression of other markers such as CD14, CD1c and CD11b [173]. The cDC1 represent 0.1% of mononuclear cells and around 4% of CD11c+ MyDC which makes their studying challenging due to difficulties with obtaining sufficient numbers [174, 175]. In contrast, the cDC2 subset is more numerous representing 10-25% of Cd11c+MyDC [174].

Both types of human MyDC have been identified in blood as well as in lymph node, spleen, tonsil, bone marrow and non-lymphoid tissue, skin, liver and lung [173, 176-178]. The expression of MHCI as well as the capacity of both MyDC types to present Ag is similar [178, 179]. Moreover, both MyDC promote Th1 polarisation depending on specific TLR stimulation [180]. However, both subtypes differ in their expression of TLR receptor. While cDC2 express all TLR apart from TLR9, the cDC1 also lack the expression of TLR4 -5 and -7 while have higher expression of TLR3 and -10 (Table 1.5) [180, 181].

Human pDC can be identified by unique expression of BDCA2 (CD303, a C-type lectin receptor family member) and BDCA4 (CD304, Neuropilin-1 (NRP1)). pDC selectively express TLR7 and -9 and secrete large amount of type I interferons I response to viral infection [180, 182].

In addition to MyDC and pDC subtypes of DC such as Langerhans cells (LC) and Sulpho LacNAc DC (slanDC) can differentiate from Mo in inflammatory conditions and in certain tissues. In addition, *in vitro* generated human MoDC are widely used as a functional model of DC behaviour [183]. However, genome-wide expression profiling demonstrated that MoDC are more similar to Mo than blood DC subsets [184]. The slanDC subset are highly inflammatory and thought to be of monocytic origin [185, 186]. Due to their transcriptional overlap with CD16+Mo they are sometimes classified as a Mo subset rather than DC [186]. Apart of Sulpho LacNAc (a modified PSGL-1) slanDC also express FcγRIII (CD16) and CD11c. Blood slanDC produce high levels of Th17-polarising cytokines such as IL-23, IL-1β and IL-6 and are linked to several autoimmune diseases including psoriasis and multiple sclerosis ([187, 188]. Phenotypic maturation of slanDC in blood is inhibited by erythrocytes through a CD47-dependent mechanism ([189]).

LC are resident DC population in the epidermis however they have also been found in other squamous epithelia such as bronchus and oral and genital mucous membranes [186, 190]. Human LC contain high levels of the C-type lectin langerin and the epithelial cell adhesion molecule (EpCAM) [191]. LC constantly migrate to skin-draining LMN and preferentially induce Th2 responses [177, 192]. In addition, LC possess the ability to cross-prime CD8 T-cells [192].

1.3.2 Dendritic cell ontogeny

DC derive from bone marrow from common myeloid progenitor (CMP) which gives rise to Mφ/DC progenitor (MDP) (Figure 1.3). The MDP further differentiate to common Mo precursors (cMOP) and common DC precursor (CDP) [193]. The common DC precursor (CDP) gives rise to cDC as well as pDC. Although the CDP retains low potential to differentiate into Mφ *in vitro* it lacks this capacity *in vivo* [194]. The CDP differentiate *in vitro* into DC in the presence of Flt3 ligand alone and do not survive or differentiate in the presence of M-CSF [194]. During homeostasis, nearly all DC differentiate from CDP ([194]). Originally, Mo have been considered as the main precursors of DC. Subsequent studies demonstrated that Mo differentiate into cDC mainly during inflammation [195, 196]. However, recently it was established, that Mo also contribute to DC pool in specific tissues such as gut, spleen or lung during steady state conditions [196-198].

LC are characterised by a unique developmental process where haematopoietic progenitors from YS and FL establish in the skin before birth allowing LC to self-renew during homeostasis [199]. However, during inflammation LC are being replenished by Mo-derived cells [195].

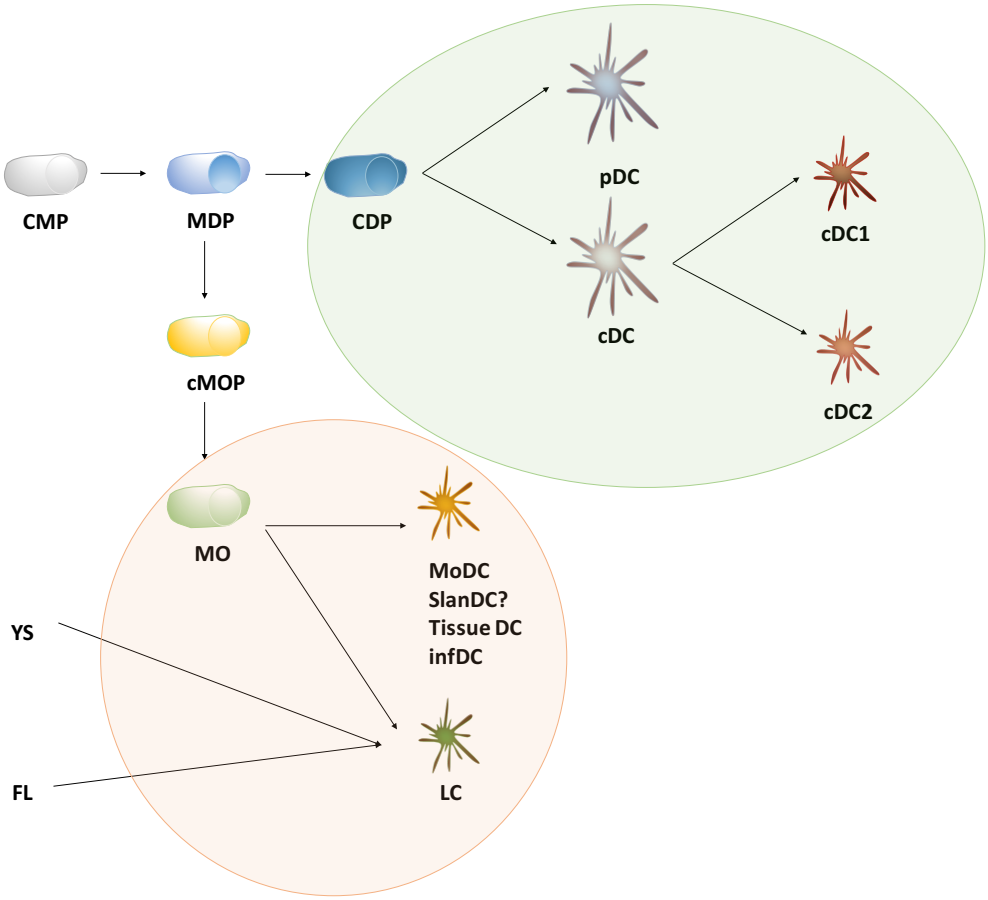


Figure1.3 Classification of DC subsets based on ontogeny and phenotype. All DC derive from CMP in bone marrow. CMP further differentiate into MDP common precursor of DC and Mφ which can differentiate into CDP unique for DC only or cMOP generating Mo. Upon inflammation Mo can give rise to inflammatory DC. Proposed nomenclature based on ontogeny where cell derived from CDP should be called DC (light green box) and cells derived from Mo should be called Mo-derived cells ([49]). DC – dendritic cells, CMP- common myeloid progenitor, MDP- Mφ/DC progenitor, CDP- common DC precursor, pDC – plasmacytoid DC, cDC-conventional DC, cMOP- common Mo precursors, MO - monocyte, MoDC - Mo-derived DC, infDC – inflammatory DC, YS-yolk sack, FL- foetal liver, LC-Langerhans cells.

1.3.3 Dendritic Cell function

Although DC comprise a heterogeneous population of cells, the prevalent unifying feature is their capacity to efficiently capture, process and present Ag inducing *the novo* Ag-specific activation of CD4+ and CD8+ T-cells [200-

206]. Moreover, DC can also directly induce Ag-independent proliferation of CD8+T-cells in a Th-independent manner [207, 208]. In addition to priming T-cell response, DC also possess the ability to activate B-cells and NK-cells. Moreover, DC play an important role in immune-homeostasis through presentation of self-Ag. Following Ag uptake in the presence of PAMP or DAMP, DC enter program of maturation which comprises the blockade of the MHCII-peptide complexes on their surface, cytoskeletal changes and up-regulation of chemokine receptors homing to secondary lymphoid organs [209].

The migration of DC from tissues towards draining LMN occurs via afferent lymphatics. CCL21 expressed on lymphatic EC interacts with CCR7 on DC surface which becomes up-regulated upon activation, guiding DC towards and into the lumen of initial lymphatic vessels through haptotaxis [210]. Inside the afferent lymphatic vessels, DC are passively transported by the lymph flow into the subcapsular sinus (SCS) of LMN. Here they enter the lymph node parenchyma in a process dependent on the interaction between CCR7 and CCL19, CCL21 produced by fibroblastic reticular cells of the T-cell zone stromal compartment [211]. The positioning of DC within the lymph node is closely linked to their function [210]. Although Ag-specific interactions between T-cells and DC occur in the T-cell zone, mice of the paucity of lymph node T cells strain 3, lacking the CCR7 ligands CCL19 and CCL21, were found to have normal or enhanced CD4+ T-cell responses [25, 212]. Interestingly, Leon *et al*. found that after infection with an intestinal nematode, Ag-bearing DC and CD4+ T-cells upregulated the chemokine

receptor CXCR5 and localized together outside the T-cell zone compartment by a mechanism dependent on the chemokine CXCL13 [83]. These findings show that T-cell differentiation can initiate outside T-cell zone compartment. It is important to note that due to technical requirements of functional studies on cell migration such as uninterrupted blood flow and intact lymph vessels and LMN, most of current knowledge on this topic is derived from animal models [210].

Upon reaching T-cell zone compartment in LMN DC activate or inhibit CD4+ and CD8+ T-cells. The engagement of distinct PRR during Ag uptake plays crucial role in the regulation of the three signals required for T-cells activation [23]. Different subsets of DC express different sets of TLR (Table 1.5) which may explain certain diverse effects of DC activation [22, 23]. The exact expression of various TLR has been difficult to elucidate. Most methods rely upon detection of mRNA which does not necessary transfer into the functional protein expression [22, 23]. The use of pure TLR agonists further expanded the knowledge of the functional TLR expression profiles on DC. Additionally, various types of DC can react differently in response to signalling from the same TLR. Indeed, Ito *et al*. demonstrated that human pDC stimulated with TLR7 agonists secreted type 1 IFN, whilst myDC stimulated with the same agonist secreted IL-12p70 [213]. This data needs to be treated with caution as R848 and Imiquimod used in this study can also trigger TLR8 which is not present on pDC and may therefore explain the difference in IL-12p70 secretion [213, 214].

TLR/Cell type	CD11c	pDC	MODC
TLR1	+++	++	++
TLR2	++	+/-	++
TLR3	+++	-	++
TLR4	++	-	++
TLR5	++	+/-	+/-
TLR6	++	+	++
TLR7	+	++	-
TLR8	+	-	++
TLR9	-	+++	-
TLR10	++	NA	NA

Table 1.5 TLR expression patterns in human DC subsets (adapted from [22, 23]).

In addition to activating T-cells, DC are important for the maintenance of central and peripheral tolerance. Thymic CD11c+ DC are involved in the deletion of self-reactive CD4 T-cells and to lesser extend CD8 T-cells as well as the induction of Foxp3+ T-reg therefore demonstrating their role in central tolerance [215-217]. In the periphery, immature DC play important role in maintaining self-tolerance by presenting T-cell an Ag without signal 2 and 3 which leads to T-cell anergy and elimination [218]. However mature DC can also trigger immune-tolerance. MoDC stimulated with TNFα and PGE2 display high expression of co-stimulatory molecules and pro-inflammatory cytokines characteristic for maturation however, they suppress T-cell activation through factors like indoleamine 2,3 deoxygenase (IDO) and IL-10 [219]. DC can also exert inhibitory effect on T-cell function though up-regulation of molecules such as PDL1 or TGF-β [220, 221]. In summary, regulatory DC (DCreg) play important role in self-tolerance and reduction of their functions has been linked to autoimmune diseases such as systemic lupus erythematodes (SLE), RA, or psoriasis [222]. Moreover, the induction of DCreg during infections such as Listeriosis or tuberculosis may protect the

host from dissemination of infection due to granuloma destruction by T-cells [74]. On the other hand, DCreg are often found in tumours and play an important role in promoting tumour growth through the development of tumour immune-evasion [221].

1.4 IL-23

1.4.1 IL-12 cytokine family

IL-23 is a heterodimeric cytokine which together with IL-12, IL-27 and IL-35 belongs to IL-12 cytokine family [223]. All cytokines in this family consist of two subunits (α-chain: p19, p28 or p35 and β-chain: p40 or EBI3 (Epstein Bar virus–induced protein 3) which expression is regulated independently. IL-23 and IL-12 share a common p40 β-chain which associate with IL-12p35 to form IL-12 or IL-23p19 to form IL-23 (Figure 1.4) [224]. Similarly, EBI3 heterodimerises with p28 or p35 to form IL-27 and IL-35 respectively [225, 226].

Members of the IL-12 family employ common receptor chains and signalling pathways. IL-12 utilises IL12Rβ1 and IL12Rβ2 which transfers signals onto Signal transducer and activator of transcription (STAT) 4 while IL-23 signals through IL12Rβ1 and IL23R onto STAT3 and STAT4 [224, 227-230]. IL-35 signals via gp130 and IL12Rβ2 but can also use gp130-gp130 and IL12Rβ2-lL12Rβ2 homodimers to transfer signal onto STAT1 and STAT4 [231]. On the other hand, IL-27 signals through gp130 and WSX-1 onto STAT1 and STAT3 [232-234]. In contrast to IL-12, IL-23, and IL-27 that are primarily secreted by activated APC, IL-35 production is restricted to T-reg [235].

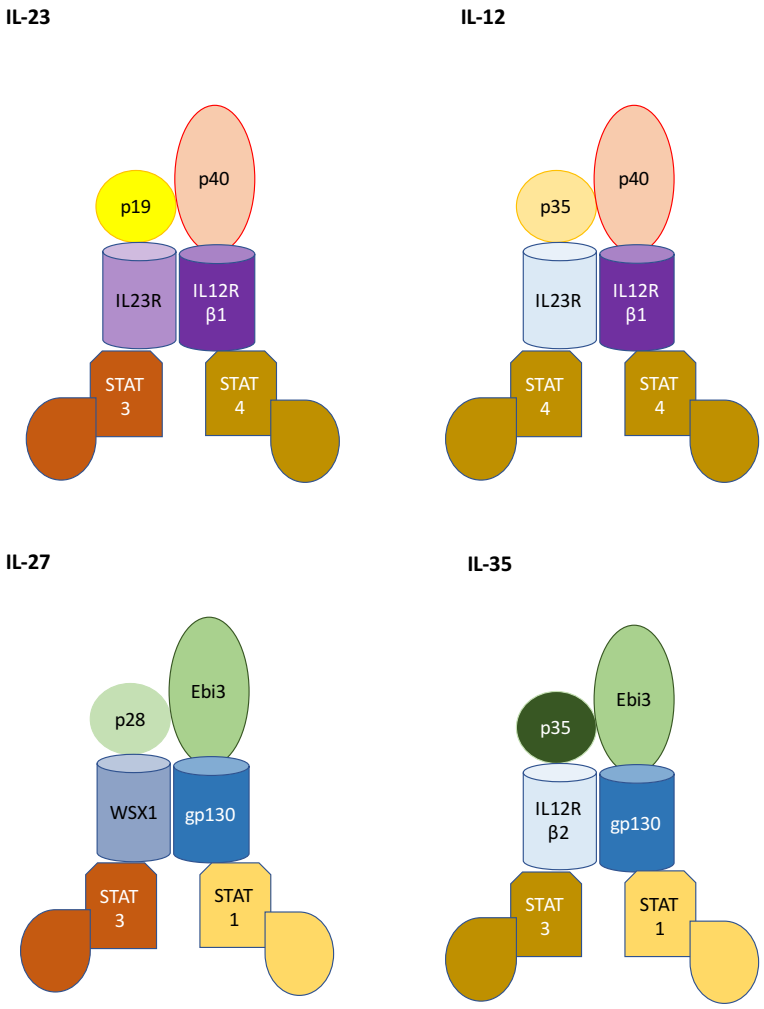


Figure 1.4 Schematic of IL-12 family cytokines, their receptors and signalling partners (from [223]). Cytokines in the IL-12 family consist of two subunits - α-chain: p19, p28 or p35 and β-chain: p40 or EBI3. IL-23 and IL-12 share a common p40 β-chain which associate with IL-12p35 to form IL-12 or IL-23p19 to form IL-23. Similarly, EBI3 heterodimerises with p28 or p35 to form IL-27 and IL-35 respectively. EBI3-Epstein Bar virus–induced protein 3.

Despite similarities in their structure, receptors and downstream signalling components the cytokines exert unique, often contrasting biological functions. Both IL-12 and IL-23 are pro-inflammatory however, while IL-12 stimulates anti-microbial resistance through Th1 responses, the IL-23 supports Th17 and promotes chronic inflammation [236, 237]. Due to its pro-inflammatory functions IL-12 is considered to be important for the development of

autoimmune diseases [238-240]. Treatment with anti-IL-12p40 monoclonal antibody significantly reduced disease in experimental autoimmune encephalomyelitis (EAE) and blocked relapses [241]. However subsequent studies demonstrated that antip40 antibodies in fact inhibit the IL-23-driven Th17 cells, responsible for EAE progression [235]. Further studies confirmed the key role of IL-23 in autoimmune diseases [242, 243]. In contrast to other cytokines from the IL-12 family, IL-35 exerts potent anti-inflammatory functions mainly through promotion of T-reg expansion and function [244-246].

IL-27 displays both pro- and anti-inflammatory properties. IL-27 can induce Th1 generation from naïve T-cells and trigger their secretion of IFNγ in *in vitro* settings. Moreover, IL-27 enhances the secretion of IFNγ in the presence of low levels of IL-12, while is redundant in the presence of high concentration of IL-12 [247, 248]. Several studies suggested that the positive effect of IL-27 on Th1 responses may be caused by inhibition of Th2 responses [233, 249, 250]. However further research on murine models *in vivo* demonstrated that effective Th1 responses can occur without IL-27 [251, 252]. Moreover, studies utilising *M. tuberculosis* infection suggested that IL-27ra deficiency results in more efficient bacterial clearance [251, 253]. Remarkably, the knockout animals produced highly effective Th1 responses but also displayed signs of uncontrolled immune stimulation suggesting regulatory properties of IL-27 [254, 255]. Indeed, IL-27 exerts anti-inflammatory effects by suppression of pro-inflammatory cytokines such as IL-6 and IL-17 and inhibition of the development of Th17 [256-258].

Moreover IL-27 enhances Foxp3 T-reg functions and induces IL-10 production by CD4 effector and Tr1 cells although, it can inhibit the development of T-reg and suppresses Foxp3 induction [259, 260]. Furthermore IL-27 can enhance CD8+ T-cells memory and cytotoxic activity of NK cells [261, 262]. Overall it can be postulated that the effect of IL-27 depends on the context of disease, location and timing.

1.4.2 Signalling pathways involved in regulation of IL-23 secretion.

Initially described by Oppman and colleagues, IL-23 is secreted by activated MoDC [224]. In 2008 Roses *et al*. reported secretion of IL-23 by MyDC upon stimulation with TLR agonists namely TLR2, 4 and 7/8. Interestingly, none of the single TLR ligands induced IL-12 secretion showing differential regulation of these two cytokines despite sharing a common subunit with IL-12 [263]. IL-23p19 expression has been demonstrated in human MoMφ stimulated with *Escherichia Coli* LPS [264]. In addition, Mφ secrete high levels of IL-23 but not IL-12 upon mycoplasma infection [265]. Furthermore, while Sendai virus induces IL-23 secretion by Mφ, influenza virus fails to do so [266, 267]. IL-23 production also occurs in human Mo infected with *Francisella* and in the microglia of patients with multiple sclerosis (MS) [268-270]. Moreover IL-23p19 expression was shown in synovial FB from patients with RA however, no secretion of active IL-23 has been demonstrated [271-273]. In addition to TLR ligands other molecules such as PGE2 and TNFα/IL-1β can also induce IL-23 secretion [272, 274, 275].

Major signalling pathways linked with regulation of IL-23 production include Zymozan and Dectin1/TLR2 Syk-CARD (caspase recruitment domain family)-NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells) pathway, the Pi3K/Akt pathway and the p38 MAPK/Erk/ NFκB pathway [268, 276, 277]. Zymozan is a preparation of cell wall of yeast *Saccharomyces cerevisiae* containing β-glucan [278]. Induction of IL-23 by zymozan is mediated via DC-associated C-type lectin-1 (Dectin-1) alone or with TLR2 [279, 280]. Both Dectin-1 and TLR2 recognise components of fungal cell wall [278]. The phosphorylation of ITAM (immunoreceptor tyrosine-based inhibitory motif)-like motif recruits spleen tyrosine kinase (Syk) [281]. Syk recruitment is required for the assembly of a scaffold complex consisting of CARD9, BCL-10 (B cell lymphoma 10) and MALT1 (mucosa-associated lymphoid tissue lymphoma translocation gene 1). The CARD9-BCL-10-MALT1 complex stimulates activation of canonical NFκB pathway however, the mechanism of this activation remains unclear [282].

The PI3K/Akt pathways seem to play a positive role in IL-23 secretion in contrast to their inhibitory effect on IL-12 [268]. Butchar *et al.* demonstrated a positive role of PI3K and NFκB in *Francisella* infected human Mo [268]. Furthermore, PI3K inhibitor, wortmannin, significantly reduced IL-23 secretion by DC [283]. Blockade of PI3K in Mφ also inhibited Il-23 secretion. Moreover, PI3K has been shown to mediate up-regulation of IL-23 secretion by CCR7 ligands in murine DC [284].

TLR signalling triggers the MAPK (Mitogen activated protein kinase) signalling pathways consisting of Erk, JNK (c-Jun-N-terminal kinases) and p38 [276]. Increased phosphorylation of Erk1/2 and p38 leads to up-regulation of IL-23 secretion by human cord-blood derived DC activated with LPS and IL-12 [285]. On the other hand, inhibition of p38 in activated Mφ enhanced IL23p19 expression [286]. Furthermore, evidence suggests a role for JNK in IL-23p19 expression by LPS-stimulated Mφ. The apparent disparities in the role of MAPK pathways observed by different groups may be explained by differential regulation of cytokine secretion in distinct cell populations. Moreover, further post-transcriptional modifications may be involved in the regulation of cytokine secretion. This point is well illustrated in the case of synovial FB from patients with RA. Up-regulation of IL-23p19 mRNA is seen in synovial FB upon stimulation of PI3K/Akt, NFκB and p38 MAPK pathways [272]. However, this is limited to transcription as these cells do not secrete IL-23. In brief, the regulation of cytokine expression and function occurs at several discrete levels and may vary widely between cell types.

Several molecules play negative roles in IL-23 secretion. Protein phosphatase 2A (PP2A) inhibits IL-23 secretion by TLR-stimulated DC through inhibition of NFκB activation [287]. PP2A associates with IKKβ which terminates IκB kinase (IKK) phosphorylation. IKKα and β form complex which mediates phosphorylation and degradation of IκB which is necessary for NFκB activation [288]. B-cell leukaemia 3 (Bcl3) is suggested to negatively regulate the IL-23p19gene [288]. BMDC responded with increased secretion of IL-23 upon stimulation with LPS in Bcl3 knock-down mice while Bcl3 over-

expression was connected with decreased secretion of IL-23 by LPS-activated BMDC in IL-10–/– mice [288]. Similarly, inhibition of the Ras-related C3 botulinum toxin substrate 1 (Rac1) increased expression of IL-23p19 through enhanced NFκB activity [286].

Transcription factors including positive regulators such as c-Rel, RelA, Activator protein 1 (AP-1), SMAD-3, the cAMP response element binding protein (CREB) and Activating transcription factor 2 (ATF-2) as well as negative regulator Interferon regulatory factor 1 (IRF- 1), modulate IL23 production through transcriptional control of IL23p19 [289]. The role of NFκB for IL-23 was demonstrated by several groups. Carmody *et al*. showed that p19 gene expression in DC requires c-Rel. Moreover, short interfering RNA against RelA decreases p19 transcription in a human Mφ cell lines [286]. In support of this, the proximal κB site in the IL-23p19 promoter is necessary for the RelA- and c-Rel-dependent induction of p19 transcription in murine BMMφ [290]. AP-1 is involved in the transcription of p19 in activated murine DC and Mφ [291]. Secretion of IL-23 by slanDC triggered by autocrine TNFα and IL-1β is mediated through AP1 and NFκB [263]. Prostaglandin E2 (PGE2) has been shown to increase IL-23 secretion through CREB transcription factor [289, 292]. CREB induced by Protein kinase A (PKA) interacts with EPAC (exchange factor directly activated by cAMP)-induced C/EBPβ (CCAAT-enhancer-binding protein) to up-regulate p19 expression in BMDC and Mφ1 [289]. Interestingly, simultaneous CREB activation inhibits the expression of other pro-inflammatory cytokines by Mφ such as IL-12 while it leads to increase of IL-10 secretion by Mφ2.

Furthermore, an inhibition of NFκB activity by CREB due to competition for CBP (CREB binding protein)/p300 has been suggested [293, 294]. Therefore, rather than being expressed as a consequence of a single transcription factor, it is likely that the balance between CREB and NFκB regulates IL-23 expression.

1.4.3 IL-23 function

IL-23 receptors are mainly expressed on activated/memory T-cells and NK cells, and are expressed at lower levels by Mo, Mφ and DC in humans [229]. In addition, IL-23 receptors are expressed by some tumour cells including lung and colorectal carcinoma as well as melanocytes [295-297]. Murine IL-23 receptors were detected on T-cells, BMDC, and Mφ1 [229, 242].

The majority of studies concerning IL-23 function focused on its role in Th17 responses. IL-23 is required for the expansion and maintenance of Th17 cells while IL-1β (TGFβ in mice) and IL-6 induce their development [298-300]. However, IL-23 can exert profound direct inflammatory effects, irrespective of Th17. Early reports suggested a degree of redundancy of IL-23 effects with regards to IL-12 [224]. Similarly to IL-12, IL-23 increased human T-cell production of IFNγ, although to a lower levels than IL-12 [224]. However, later reports suggested that murine CD4 and CD8 T-cells do not respond to IL-23 with secretion of IFNγ [301-303]. Moreover, IL-23 can negatively regulate IL-12-induced secretion of IFNγ by murine CD4 and CD8 T-cells [303]. While IL-12 stimulates the proliferation of both naïve and memory human T-cells and naïve murine T-cells, IL-23 increases the proliferation of human and

murine memory T-cells while not having an effect on naïve T-cells [224, 304]. Based on these findings it was suggested thet IL-23 acts predominantly on memory T-cells [304]. IL-23 synergistically with IL-18 or IL-1β induces the secretion of IFNγ and up-regulates CD56 expression by NKT-cells but not NK cells [305, 306]. In a positive feedback loop, IFNγ induces IL-23 production [306].

As described earlier, IL-23 receptors are expressed by a range of tumours including breast, lung and colorectal [295, 307-310]. IL-23 directly promotes proliferation of lung cancer and squamous carcinoma cells of the oral cavity [295, 307]. Furthermore, IL-23 may be involved in the transition from adenomatous polyps to colorectal carcinoma [308]. IL-23 is also suppressed NK-cell dependent control of lung metastases [311]. On the other hand, IL-23 was indispensable for DNA repair in primary melanocytes thereby preventing their tumorigenesis [297].

IL-23 regulates tumour development indirectly via IL-17 secreting cells. Tumour associated Th17 cells occur coincidentally with increased IL-23 levels and promote angiogenesis and growth [312, 313]. Furthermore, increased levels of IL-17 correlate with poor prognosis [314-316]. Th17 were also indicated as the source of tumour-induced T-reg [317]. Hinrichs *et al*. described that adoptively transferred IL-17-secreting CD8+ T cells enhanced antitumor immunity in B16 melanoma through conversion into IFNγ-producing effector T-cells [318]. Moreover Muranski *et al*. reported the ability of tumour-specific Th17 to reduce B16 melanoma through IFN-γ production

[319]. These studies suggest high plasticity of Th17 and demonstrate the importance of local factors in regulating outcomes of Th17 actions.

The IL-23/Th17 axis is important for protective immunity to bacterial and fungal infections including *Listeria monocytogenes*, *Cryptococcus neoformans, Mycobacterium tuberculosis* and *Klebsiella pneumoniae* [320-324]. Furthermore, Th17 cells provide protection against muco-cutaneous and systemic candidiasis through stimulation of DC, Mφ and NK-cells functions [280, 325]. However, IL-23/IL-17 can exacerbate pathogen-induced inflammation, promotint tissue damage through up-regulation of IL-6, IL-1, TNFα and MMP9 and influx of inflammatory myeloid cells like granulocytes, Mφ and neutrophils [320, 326-328].

IL-23 and IL-17 play indispensable roles in autoimmune diseases such as EAE in mice and MS in humans, psoriasis, RA and spondyloarthritis (SpA) due to their role in the development and maintenance of chronic inflammation [242, 329, 330]. Therapeutic antibodies against IL-23 or IL-17 are highly efficacious in psoriasis and many other autoimmune disorders. However, it is important to remember that both cytokines also display unique properties influencing the possible outcomes of therapeutic interventions. An interesting dichotomy between IL-23 and IL-17 was observed in Crohn's disease [331] where inhibition of IL-23 attenuated inflammation by increasing T-reg accumulation, whilst inhibition of IL-17 exacerbated inflammation due to breakdown of the intestinal epithelial barrier.

1.5 Innate memory

Immune responses were traditionally classified as innate, characterised by lack of specificity or memory, and adaptive, which are slower to develop but highly specific to the encountered pathogen and result in a long-lasting memory. This view has been challenged in recent years based on a review of immune mechanisms governing resistance to reinfection in plants and invertebrates (which lack adaptive immune system) and in mammalian vaccination models. Furthermore, discovery of PPR demonstrated a certain degree of specificity in the innate immune system. Based on these findings, Natea *et al*. proposed a term "trained immunity" which is characterised by increased response of innate immune cells to secondary infection by the same or different pathogen (cross-protection) [332] . The main characteristics of innate memory in vertebrates include the ability of innate immune cells to raise differential (enhanced or inhibited) responses to secondary insult and low specificity which contrasts with adaptive immune responses. Furthermore, innate immune memory is regulated by epigenetic reprogramming in opposition to permanent genetic changes such as recombination involved in adaptive memory. Finally, trained memory has a shorter duration (months) comparing to adaptive memory.

Compelling evidence suggesting the presence of innate memory in vertebrates comes from studies showing T-cell independent protection against *Candida albicans, Salmonella, Listeria monocytogenes* or *Schistosoma mansoni* following immunisation of mice with bacillus Calmette Guerin (BCG) vaccine [333-336]. The protection was mediated by tissue Mφ [337] . Furthermore,

increased levels of IFNγ resulting from latent infection with herpesvirus stimulated systemic Mφ activation leading to enhanced resistance to *Listeria monocytogenes* and *Yersinia pestis* [332]. Similarly, immunisation with attenuated PCA-2 strain of *C. albicans* induced protection against *Staphylococcus aureus* which was once again dependent on activated Mφ [338]. Importantly, this protection was also present in athymic mice demonstrating lack of dependency on T-cells [339]. In agreement with these studies a recent report demonstrated that infection with *Nippostrongylus brasiliensis* induces long-lasting effector Mφ phenotype maintaining protection from reinfection in a T- and B-cell independent manner [340].

Other cells of innate immune system are also involved in conferring innate memory. Transfer of CpG activated Thy1.2^{+} DC was required for protection against *Listeria monocytogenes* lethal infection in naïve mice [341]. Flagellin induced protection against rotavirus in a DC-dependent manner via DC-derived IL-18 which stimulated secretion of IL-22 by epithelial cells [342]. Following infection with murine cytomegalovirus, Ly49H+ NK cells proliferated and remained in organs ready to undergo secondary expansion and rapid activation upon reinfection.

Multiple epidemiological studies demonstrate that many vaccines such as BCG or measles affect immune responses towards unrelated pathogens. Vaccinia recipients had reduced risk of asthma and malignant melanoma and the injection has been associated with reduced mortality in adults (It need to be mentioned that no randomised trials for effectiveness of Vaccinia on

mortality have been conducted) [343]. Similarly, observational studies suggest lower all-cause mortality in children who received measles vaccine[344]. Although observational studies are prone to selection bias there are no studies contradicting these results. Furthermore, recent randomised trial suggested that BCG-vaccinated children demonstrate lower incidence of eczema [345].

One of the most important characteristics of adaptive immunity is its exquisite specificity. Although trained memory involves changes in a number and function of various innate immune cells resulting in increased protection from a broad range of secondary infectious agents, the discovery of PRR which recognise particular PAMP and DAMP and convey specific activation patterns implies a degree of specificity of innate immune cells [12, 346]. TLR agonists (LPS) can influence bone marrow hematopoiesis by stimulating granulopoiesis preferentially over lymphopoiesis [347]. Stimulation of the common lymphoid progenitors (CLP) with the TLR9 ligand CpG ODN promotes their ability to generate DC while limiting their differentiation to B-lineage cells [348]. Circulating hematopoietic stem and progenitor cells (HSPC) stimulated with different TLR-agonists promote development and differentiation of specific myeloid subsets, for example while TLR7/8 ligands promote differentiation of CD34+ cells into CD11c+ CD14− DC, stimulation with TLR2 ligands skews the differentiation towards CD11c+ CD14+ Mo [349]. Therefore, HSPC can orchestrate the development of innate memory during infection by influencing generation of immune cells during infection. The importance of HSPC in forming of innate immunity is clearly seen while considering trained immunity in Mo versus their lifespan. While Mo have

short life-span of up to one day, trained Mo were found in the circulation of BCG-vaccinated patients 3 months after vaccination suggesting reprogramming of progenitor cells [350, 351]. One of the main components of the membrane of Gram negative bacteria, LPS, which binds to TLR4, exerts diverse effects on innate responses depending on the dose and frequency of application [352]. While primary exposure leads to activation of immune system and production of various cytokines, subsequent exposure induces long-lasting tolerance due to genomic reprograming of Mφ [353]. LPS-tolerant phase in patients suffering from sepsis remains at least 15 days post septic shock [354]. In contrast, administration of very low doses of LPS (<100 pg/ml) during priming enhances secondary responses by immune cells [355, 356]. Therefore, innate memory may increase organism resistance to reinfection or protect organism from debilitating effects of sustained inflammation.

The molecular mechanisms behind trained immunity are only starting to be elucidated and include epigenetic changes such as histone modification and chromatin folding triggered by activation of various immunological (ie. cAMP- PKA or STAT1 activation) and metabolic (aerobic glycolysis) pathways [357]. miRNAs such as miR-146a or miR-208 have been implicated in the development of innate memory due to their high metabolic stability (miR-208 half-life in murine heart is estimated for over 12 days) [358]. Furthermore, DC and Mo can secrete miRNA and transfer them to neighbouring cells thus effectively propagating the memory state [359-361].

On the other hand, the role of long noncoding RNA requires further examination.

Although characterised only recently, innate memory plays paramount role in the defence mechanisms of vertebrate immune system. Future work should elaborate further on the effects of innate memory in aim to utilise the findings in designing improved vaccines, tumour therapy and treatment of chronic inflammatory states. Furthermore, further delineation of molecular mechanisms governing the development of innate memory will likely lead to new improved therapies combining modulation of both innate and adaptive memory.

1.6 The role of stroma in governing immune responses

Immune cells are in a constant crosstalk with their environment which consists of stromal cells such as FB, epithelial and endothelial cell as well as extracellular matrix. All these components have the potential to influence the functions of immune system and the eventual direction of immune responses.

Due to their location, stromal cells are the first to encounter stress caused by microbes entering the tissues and bloodstream, local cell death or tissue damage. It is tempting therefore, to assume that these cells act as sentinels for local homeostasis and relay the information to immune system. Indeed, multiple reports demonstrated expression of functional TLR and NOD receptors by connective tissue cells. Epidermal keratinocytes (KC) possess TLR 1-6 and 9 [362-367] and their activation promotes distinct immune

responses [368]. Furthermore, increased TLR 1, 2, 4, 5 and 9 expression by KC occurs in psoriatic plaques [365, 369-371]. KC activated in the presence of double stranded (ds) RNA and inflammatory cytokines induce maturation of DC towards a Th1 polarising phenotype [372]. In contrast, triggering of TLR3 and TLR9 on KC (by poly I:C and CpG , respectively) promoted an influx of memory T-cells and production of type I IFN [362]. FB also express multiple TLR whose engagement induces cytokine secretion [373, 374]. Activation of FB via TLR is implicated in hypertrophic scarring and increased Th1 and Th17 responses in RA [374, 375]. Similarly, activation of endothelial cells (EC) through PRR and cytokines leads to structural changes including increased permeability, upregulation of adhesion molecules and secretion of IL-1, -6, -8, -10 or monocyte chemotactic protein-1 (MCP-1) [376-378].

A number of stromal cells such as EC, KC, FB, astrocytes and adipocytes express MHC II molecules either constitutively or upon stimulation. Such cells are classified as non-professional APC [379-386]. The ability of stromal cells to effectively present Ag is established in both murine and human models. Acting as non-haematopoietic APC, intestinal myoFB efficiently present allo-Ag to T-cells and are sufficient to induce lethal acute graft-versus-host disease in the absence of professional APC [387]. Transgenic mice expressing autoAg under KC control develop autoimmunity mediated by CD4 T-cells [383]. Furthermore, KC pulsed with excessive peptide are targets for cytotoxic T-cells [384]. Immortalised human brain microvascular hCMEC/D3 endothelial cells (HBEC) expressing MHCII, CD40 and inducible costimulator-ligand (ICOSL) supported proliferation of CD4 and CD8 T cells suggesting their ability to process and present Ag. EC are also implicated in

allograft rejection though expression of MHCII, activation of CD4 responses and induction of MHCI-specific circulating memory CD8 T cells [380]. MHCII positive astrocytes were found in the lesions in MS [388, 389] while the expression of MHCII on FB and chondrocytes occurs in RA [390-392] and associates with disease progression.

In addition to a role in Ag presentation, expression of MHCII on stromal cells serves alternate functions. When combined with absent or low expression of CD80/86, MHCII expression is suggested to induce tolerogenic T-cell responses and therefore play an important role in maintaining immune tolerance [382, 393, 394]. Furthermore, it was suggested that periodic low-affinity interactions between TCR and endothelial MHC promote memory T-cells survival while high-affinity engagement initiates a localized recall response [381]. EC can prevent, delay or promote T-cell diapedesis via MHCII interaction. Tay *et al.* and others demonstrated that endothelium can stop Ag-specific memory T-cell transmigration via endothelial-derived MHCII-T-cell interaction which is overcome in inflammatory conditions such as pre-treatment with IFNγ [395-397]. The complexity of MHCII functions was well demonstrated with FB. Although FB express MHCII upon stimulation with IFNγ, they fail to effectively trigger the proliferation of CD4+ T-cells. However, binding of MHCII on activated FB up-regulates their secretion of IL-6, IL-8, MCP-1, PGE2 and RANTES (CCL5) [398] suggesting that MHCII acts as a receptor for FB rather than activating receptor for T-cells. Although FB were not able to activate CD4 cells in direct interactions through MHCII, stimulation of FB through HLA-DQ promoted Th2

polarisation of CD4+ responses and this was mediated through secreted cytokines [399].

In addition to MHCII, stromal cells commonly express a range of co-stimulatory (i.e. ICAM-1, vascular cell adhesion molecule -1 (VCAM-1), CD40, ICOSL) and inhibitory molecules (i.e. Programmed death (PD) -L1 and PD-L2) through which they can affect the direction of T-cell response [400-407]. IFNγ-activated KC effectively present Ag to both Th1 and Th2 T-cell lines in a CD54 (ICAM-1)-dependent manner [408]. On the other hand, KC promote tolerance via expression of PDL1 [409, 410]. Furthermore, gastric epithelial cells secrete TGFβ and express PD-L1 upon infection with *H. pylori* leading to Th1 hypo-responsiveness and promoting T-reg and Th17 differentiation and therefore chronic inflammation [411-413]. These complex findings may reflect different functions of stromal cells under various homeostatic or inflammatory conditions. Keir and co-workers showed that PD-L1/PD-L2 expression on non-lymphoid cells is sufficient to protect against diabetes [407] while PD-L1 expression on EC was found necessary for control of immune-mediated myocarditis [401].

Stromal cells act as sentinels of local conditions but also specifically modulate their behaviour in response to immune cues [414]. Crosstalk between stromal and immune cells therefore leads to unique inflammatory profiles. Disruption of these interactions has been implicated in multiple autoimmune diseases such as psoriasis, arthritis, atopic dermatitis (AD) as well as the development and progression of cancer. Purwar *et al*. showed that IL-13-activated KC

promote preferential enrichment of Th2 cells in the skin of AD patients suggesting a positive feedback loop between T-cells and KC [415]. The authors further suggested the cooperation between IL-13 and IFNγ stimulate KC to recruit mixed T-cells phenotype thus contributing to chronic eczema [415, 416]. While activation of KC with dsRNA in the presence of IL-4, -13 and/or TNFα up-regulated Thymic stromal lymphopoietin (TLSP) secretion by KC, thereby favouring Th2 responses, stimulation with dsRNA alone promoted Th1 [417]. Furthermore, stimulation of IFNγ-activated KC with IL-18 triggered CXCL9, 10 and 11 secretion and increased expression of MHCII resulting in IFNγ production by activated CD4+ T-cells [418, 419]. Similarly, co-culture of synovial FB with Tcells stimulated IDO expression by FB resulting in reciprocal inhibition of Th1 but not Th2 or Th17 proliferation [420, 421]. Furthermore, FB convert FOXP3+Tregs into pathogenic Th17 in autoimmune arthritis via IL-6 [422]. FB derived IL-15 is implicated in T-cell cytokine secretion in RA through [423, 424] and reciprocally, T-cells stimulate IL-15, IL6 and IL-8 secretion by FB therefore creating a persistent feedback loop maintaining chronic inflammation [424]. This loop can be disrupted by Methotrexate which interferes with T-cell-FB adhesion [424]. Therefore, RA-FB exert dual action on T-cells potentiating the potency of T-reg whilst simultaneously promoting pro-inflammatory CD4 responses and ultimately supporting a chronic pro-inflammatory state.

Only recently has an appreciation emerged of the roles stromal cells play in immune responses. Therefore, whilst this field is in its infancy, it is imperative to understand how they contribute to immunity for infectious,

autoimmune and malignant disease. A more complete understanding of the immune-modulatory role of stromal cells has the potential to provide novel immunotherapeutic targets immune-related disorders.

1.7 Inflammation in the tumour environment

Inflammation is believed to play an important role in the development and progression of tumours. The correlation between chronic inflammation and cancer was first observed at the end of 19th century by Virchow [425]. However later studies revealed that, although chronic inflammatory diseases are frequently associated with increased risk of carcinogenesis, acute inflammation contributes to the regression of tumours [426]. Considering the involvement of the immune system in tumour development and the ability of leukocytes to eradicate tumours, numerous studies have addressed the mechanisms governing the interactions between these cells. Results from animal models suggest that carcinogenesis associated with inflammation is a gradual process sustained by tissue damage and damage-induced proliferation [426]. Furthermore, chronic inflammation may stimulate recruitment of BM-derived cells which have increased malignant potential [427]. However, transformed cells are often highly immunogenic and thus readily eliminated. Therefore, tumours employ mechanisms which facilitate immune evasion, such as antigen shedding, impairing DC maturation and promoting T-reg responses. On the other hand, tumours often maintain low levels of inflammation for example, through recruitment of Mφ, which promote angiogenesis and invasion by structural changes in tumour microenvironment [425, 426].

Although much research has focused on interactions between leukocytes and tumour cells, a growing body of evidence suggests that cross-talk between leucocytes and tumour associated FB (TAF) is important, independently shaping tumour progression and clinical outcome. In many tumours, specific leukocyte subsets localise to FB-rich stromal compartments of the tumour. While immature DC are found in tumour beds, mature DC occupy the tumour stroma. In lymph node metastases from melanoma patients, NK cells were detected adjacent to FB surrounding metastatic lesions [428]. In lung tumours the number of T-reg in the stroma was higher than in the tumour nest and correlated with poor outcome [429]. In contrast, elevated numbers of CD4+ T cells in the cancer stroma, but not within cancer core, correlated with survival in Non-small-cell lung carcinoma (NSCLC) [430]. The discrepancy in regards to prognosis between these reports is likely to be due to the fact that Kinoshita specifically focused on T-reg while Wakabayashi looked at whole CD4+ cohort. Similarly, thymic carcinoma tumour-infiltrating lymphocytes were generally more abundant in the stroma [431]. The patients with low CD4+ and low CD20+ lymphocytes within tumour stroma showed poor survival [431]. The differential clinical outcomes correlated with compartmentalisation of leucocytes to tumour beds or stroma and this highlights the importance of leucocyte-stromal interactions for tumour progression.

The presence of activated pro-inflammatory FB was recently demonstrated in early pre-neoplastic lesions in skin carcinogenesis model. This pro-inflammatory phenotype was initiated by immune cells and further maintained by tumour. Furthermore, activated FB were necessary for malignant

transformation of skin lesions and their ablation significantly inhibited the number and progression of papillomas [432]. While TAF strongly promote tumour growth, normal resting FB may inhibit tumour development. The contrasting role of TAF and resting FB was shown in xenograft models where tumour cells were implanted into immune-deficient mice with normal or tumour-associated FB. Tumours that developed from carcinoma cells injected with TAF demonstrated increased growth compared to those with normal FB [433]. TAF promote tumour growth by remodelling the extra-cellular matrix (ECM) and providing high energy metabolites for cancer cells through the "reverse Warburg effect" [434]. In addition, TAF may harness other cells to create favourable environment for tumour growth. For example, Mφ recruited to tumours by TAF stimulate angiogenesis and tumour invasion.

1.7.1 Immune-mediated mobilisation and activation of FB as crucial step in carcinogenesis.

Local immune infiltration is a hallmark of cancer and plays an important role in tumour promotion and progression [435, 436]. Chronic inflammation caused by infections and autoimmune diseases correlates with increased risk of cancer due to stimulation of proliferation and enhancement of genetic instability in epithelial cells [437, 438]. However, recent evidence suggests another important mechanism by which leucocytes promote carcinogenesis, namely inflammation-induced mobilisation and activation of FB which in turn potentiate inflammatory signalling cascades. Moreover, inflammation, although necessary, may not be sufficient to induce the progression of pre-malignant tissue to neoplasia without FB cross-talk [439]. Indeed, the presence of activated pro-inflammatory FB occurs in incipient pre-neoplastic

lesions in murine skin carcinogenesis [440]. FB activation was mediated by leukocyte-derived IL-1β which stimulated expression of pro-inflammatory gene signature in FB in an NFκB dependent manner.

The rate-limiting role of TAF in malignant transformation was shown in chemically-induced skin carcinogenesis [432]. 12-dimethylbenz-(a)anthracene/12-O-tetradec-anoylphorbol-13-acetate (DMBA/TPA) is a strong inducer of chronic inflammation [441]. However, the application of carcinogen resulted also in a massive accumulation and proliferation of FSP+ FB which encouraged Mφ recruitment through secretion of CCL2 [432]. Selective depletion of FB from skin lesions decreased numbers of infiltrating Mφ and reduced the incidence of skin papillomas and their progression to carcinomas. Similarly enhanced accumulation and proliferation of FB in AOM/DSS (azoxymethane/dextran sulfate sodium) induced colitis preceded transformation of colitis to carcinoma [429]. FB accumulation was regulated by CCL3 which acted on CCR4 receptor present on FB. Steinhauser *et al.* showed that in a self-perpetuating process the adhesion of FB to Mφ through CD54 stimulated respiratory burst which was necessary for further up-regulation of CCL3 secretion by Mφ [442]. Interestingly the depletion of only Mφ or FB from inflammatory lesions resulted in reduced metastatic transformation, demonstrating the importance of both cell types [429, 443]. The relevance of the CCL3-CCR4 axis in tumour development was confirmed by Wu *et al.* who showed that constitutive expression of CCL3 on murine intrapulmonary Mφ, regulates intra-tumour trafficking of leukocytes and FB and subsequent metastasis [444].

Tumours are often described as “wounds that don’t heal” [445] and the close relationship between Mφ and FB throughout carcinogenesis is not surprising considering their roles in wound resolution. During the initial, pro-inflammatory phase of wound healing, Mφ1 prevail in the tissue while later phase is correlated with immunosuppressive Mφ2 [446]. Similarly, during the early neoplastic changes Mφ1 dominate while Mφ2 prevail at later stages of tumour development. However, recently both Mφ types as well as cells of mixed phenotype were shown to be present in tumours at the same time at various locations [447]. In agreement with their function in wound healing Mφ1 activated the pro-inflammatory phenotype in FB while Mφ2 reversed this but promoted FB proliferation [446]. Therefore, retention of Mφ1 in sites of chronic inflammation can prevent resolution of lesions by secreting mixture of signals from Mφ1 and Mφ2 which in turn stimulate secondary expansion and activation of FB. Interestingly Mφ2 were as effective as Mφ1 in activation of FB in a recent study of prostate carcinoma [448]. Apparent inconsistencies between studies may be attributed to tissue-dependent differences in FB or disparity in culturing methods. Moreover, FBS has the potential to induce αSMA expression in FB [449]. It is therefore possible that specific culture conditions may prime FB towards distinct responses to various stimuli. Similarly, initial activation of FB by immune cells may influence their further responses to signals from epithelial cells during tumour development.

1.7.2 TAF-dependent regulation of trafficking and function of monocytes and macrophages.

TAMφ are a common component of the inflammatory stroma and play crucial role in tumour development. Although both Mφ1 and Mφ2 types are present in

the tumour mass and can promote carcinogenesis, Mφ2 correlate with worse prognosis suggesting their role in supporting tumour growth [450]. Recruited blood Mo which differentiate into Mφ upon entering tissues serve as the main source of TAMφ [451]. Although non-classical Mo comprise only 5-10% of the total pool of circulating Mo in healthy individuals, they are dramatically expanded in cancer [452-454] and associate with poor prognosis [453]. On the other hand mobilisation of inflammatory Mo in pancreatic cancer inversely correlates with survival [455]. These two Mo types vary in their expression of chemokine receptors suggesting their differential homing into tissues. While the pro-inflammatory type has high CCR2 expression, non-classical monocytes demonstrate low CCR2 and high CXCR4 [456]. It is inconclusive which of those types represent the major source of TAMφ with some studies indicating the classical while others the non-classical subset. Therefore, the preferential recruitment of Mφ precursors may depend on the tumour type and its microenvironment [457]. Both tumours and TAF express chemokines that chemoattract Mo and Mφ [440, 448]. Moreover, TAF isolated from prostate carcinoma recruit human Mo as potently as tumour cells [448]. However, TAF-dependent recruitment was mainly mediated by SDF1 (CXCL12) which acts on CXCR4 receptor expressed by non-classical monocytes while tumour-derived effect depended on CCL2 which mainly recruits CCR2 expressing classical type. This suggests preferential recruitment of different Mo types into various compartments of tumours. Indeed in another study, TAF displayed increased expression of CXCL14 (BRAK) versus tumour cells *per se* [458]. Interestingly, CXCL14 is down-regulated in cancer due to its inhibitory effect on tumour growth while it is often up-regulated in infiltrating leucocytes and

TAF [459, 460]. Moreover TAF-derived CXCL14 potently induced Mo migration, acting preferentially on the CD16+ subset. This is in agreement with Kurth *et al*. who showed that CXCL14 recruits mainly blood Mo and Mo activated by c-AMP up-regulating agents: PGE_2 and forskolin [461]. Therefore, it was suggested that BRAK-responsive Mo enter the tissues where they become target for PGE_2 producing TAF thus maturing into Mφ in inflammatory-independent manner [461].

1.7.3 Role of COX2 and PGE_2 in cancer

Prostaglandins are the product of arachidonic acid which is released from plasma membrane by phospholipase A-2. The process is mediated by COX enzymes which produce unstable PGE G_2 converted to PGE H_2 which is a precursor for several prostaglandins including PGE_2, PGD_2, $PGF_{2\alpha}$ and thromboxane-A2. Two COX enzymes are currently described: COX-1 which is constitutively expressed in most tissues including kidney, stomach and colon and is located in endoplasmic reticulum and COX-2 acting mainly in the nuclear membrane which is not normally expressed in tissues with the exception of kidneys, seminal vesicles and central nervous system [462, 463]. However, COX2 can be induced by cells such as FB and Mo during inflammatory and tumorigenic conditions. Following COX activation cell-specific synthases complete the synthesis of prostanoids,
PGE2 is produced by various cell including Mφ, FB and tumour cells. It binds to G protein–coupled, rhodopsin-type EP receptors (EP1-4) [464].
EP1signalling stimulates phosphatidylinositol hydrolysis resulting in Ca2+

influx which activates the p38 and ERK pathways. EP2 and EP4 stimulate cAMP production by adenylate cyclase which activates cAMP-dependent protein kinase (PKA) and in effect phosphorylates CREB while inhibits MAPK activation [465, 466]. In contrast, EP3 signalling suppresses cAMP and as a result activates MAPK. However, recent studies suggest that different splice variants of EP3 may activate cAMP [467]. EP3 signalling have been shown to play a role in tumour development and angiogenesis via reduction of VEGF expression in tumour stroma [468].

Considering the opposing effects of receptors triggering on cell function it is not surprising that PGE_2 exerts pleiotropic functions in cancer and inflammation. During the onset of inflammation PGE_2 promotes vasodilation and increases vascular permeability stimulating the influx of immune cells including Mφ and neutrophils [469, 470]. Furthermore, PGE_2 promotes the activation of Th17 cells directly as well as via stimulating IL-23 secretion by DC, therefore favouring chronic inflammation. PGE2-mediated activation of Th17 have been attributed to play a significant role in the development and maintenance of inflammatory diseases such as IBD as well as cancer [471, 472]. On the other hand, PGE_2 selectively inhibits IFNγ and IL-12 production by CD4+ T-cells and promotes T-reg responses [473][474, 475]. Although, PGE_2 enhances CCR7 expression in DC it also inhibits CCL-19 secretion and reduces the ability of DC to attract naïve T-cells [476]. The effect of PGE_2 on DC depends on the site of encounter – while in peripheral tissues PGE_2 promotes DC activation, in LN it exerts inhibitory effect. As mentioned earlier TAF affect Mo differentiation into Mφ via secreted PGE_2. Furthermore, Mφ

activity can be directed towards Mφ 2 indirectly via Th2-type cytokines as well as directly through activation of c-AMP-CREB pathway [477]. Additionally, PGE_2 inhibits Mφ maturation and phagocytosis [478, 479].

PGE_2 is often found at increased concentrations in tumours comparing to neighbouring tissues [480]. Furthermore, to its role in modulating anti-tumour immune responses PGE_2 can directly promote tumour survival and progression. In addition to inhibiting cell apoptosis it induces cell proliferation stimulating tumour growth [481]. PGE_2 promotes angiogenesis and increases cell migration supporting cancer's metastatic potential [482]. The pro-cancerous effects of PGE2 have been supported by a substantial body of evidence from studies in rodents and humans using nonsteroidal anti-inflammatory drugs (NSAID) which were found to have a protective effect against the development and progression of multiple types of cancer. Interestingly, recent report suggested inhibitory effect of PGE_2 on the growth of pancreatic ductal adenocarcinoma cells which is mediated via EP4 receptors [488]. This report supports earlier study showing that administration of 16,16-dimethyl-PGE_2 (a stable PGE_2 analogue), for 8 weeks to Apc^{Min} mice resulted in a decrease in the size and number of intestinal tumours [489]. The potential inhibiting role of PGE_2 in carcinogenesis requires further validation. However, such hypothesis seems plausible considering the variety of PGE_2 receptors, their differential expression and unique signalling pathways.

1.8 Radiotherapy

1.8.1 Ionising radiation

RT is one of the pillars of cancer treatment and is received by an estimated 50% of cancer patients for survival or palliative benefits [490]. It is highly cost-effective amounting for about 5% of the total cost of cancer therapy [491]. The first reported use of x-rays in cancer was in 1896, just 7 months after their discovery by Wilhelm Roentgen [492]. Since then the efficacy and safety of RT greatly improved due to technical advances and increased knowledge of radiation biology [492]. RT utilises ionising radiation (IR) to exert cell death of cancer cells. (Table 1.6).

Type	**Source**	**Type of ionisation**	**Characteristics**
UV	10-125 nm	Indirect	
X-rays	$< 10^{-9}$m	Indirect/direct	Produced outside the nucleus High penetration
γ-rays	$< 3x10^{-11}$m	Indirect/direct	Produced inside the nucleus High penetration
α	helium nuclei – two protons and two neutrons	direct	Low penetration depth (few cm in air)
β	Electrons/positrons	direct	Low penetration but higher than alpha (few m in air)
neutron	neutrons	indirect	High penetration
protons	protons	Indirect/direct	Highly specific energy distribution allowing targeting defined area

Table 1.6 Types of ionizing radiation. Ionising radiation is a type of radiation (the emission of particles or waves) where electromagnetic waves or particles carry enough energy to remove an electron from an atom or a molecule thereby causing their ionisation. Types of ionising radiation include ultraviolet radiation, X-rays, γ-rays, α- particles, β-radiation and neutron radiation. Non-ionising radiation include microwave, radio-wave, UV, infrared and visible light.[493]

The biological effects of IR occur primarily due to DNA damage. The effect on a cell can be direct or indirect [491, 494]. Direct ionisation occurs when charged particles interact directly with a cell molecule and remove an electron [494]. During indirect radiation, non-charged particles, such as photons, interact with molecules like water, producing free radicals which in turn ionise other molecules or atoms (Figure 1.5) [491, 494]. Since cells contain up to 80% of water both types of radiation can cause significant damage to the RNA and DNA [491]. About 60% of X-ray and γ-ray effects is generated by indirect radiation where the effect of created free radicals on cell molecules is amplified by their reactions with lipids, membranes and oxygen species [491, 494].

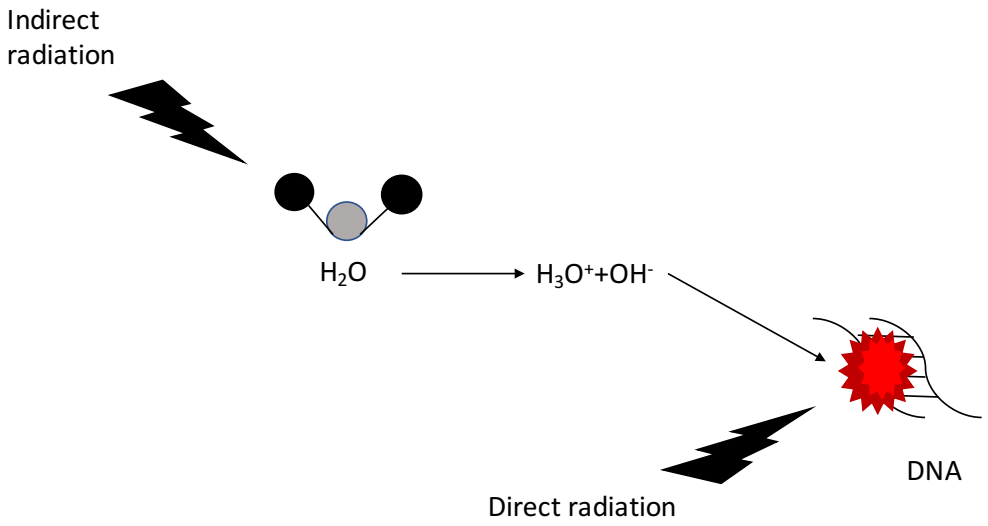


Fig.1.5 Direct vs Indirect IR. When IR is absorbed into a cell, the photon is assimilated and its energy is utilised to produce fast electron. The electron may interact directly with DNA causing damage (direct radiation) or may react with water present in the cells creating free radical, particularly the hydroxyl radical (OH^-) which diffuses into DNA and causes single or more damaging double-strand breaks [491, 494].

IR can have a stochastic or deterministic effect on a living organism [495]. Stochastic effects have no threshold and the severity of the result does not increase with the dose however, the risk of an effect to occur increases in linear fashion to the dose increase [495, 496]. Stochastic effects are usually connected with lower exposures to IR and are involved in carcinogenesis and certain hereditary disorders [495-497]. Deterministic effects occur only when the threshold of exposure is exceeded and their severity increases according to the dose of IR received [495, 497, 498]. Deterministic events are usually connected with higher-dose exposure and except for radiation-induced cataracts, are caused by cell killing [495-499]. Examples of deterministic effects include erythema or radiation sickness [497, 498]. Both stochastic and deterministic effects affect cancer RT.

The aim of RT is to kill tumour cells while sparing normal tissues [494]. To achieve this, radiotherapists commonly employ a fractionated regimen with doses of 2-6Gy delivered daily over several weeks to a cumulative dose of 50-80Gy (fractionated RT) [500] (Figure 1.6).

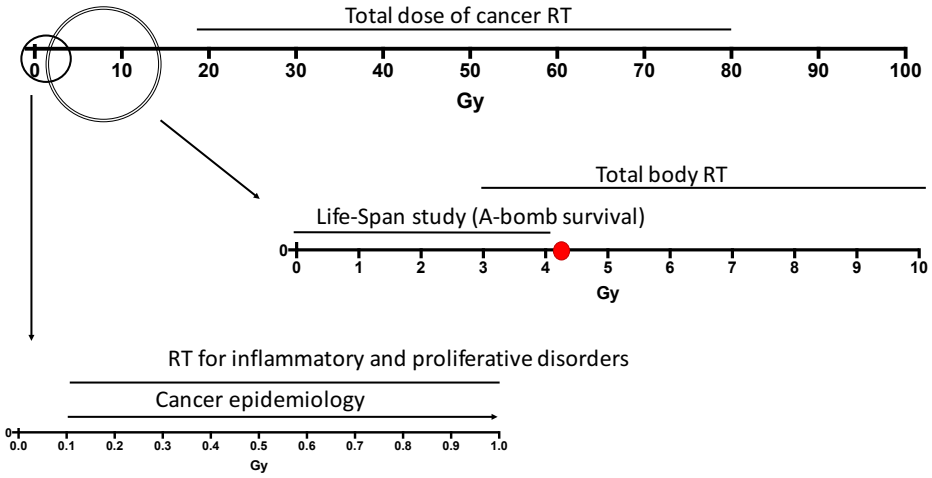


Figure1.6 Comparison of exposure ranges of IR after [500-503]. During RT cancer patients receive doses of up to 80Gy usually infractionated doses ovr several weeks. During total body irradiation for ablation of bone marrow in haematogeneous malignancies patients typically receive 3-10Gy. The LD50 for humans is 4.25Gy (red circle). Life-span study on A-bomb survival who received up to 4Gy has been conducted. Epidemiological studies suggest increased risk of certain malignancies in people who received doses grater then 0.5Gy. Low-dose radiotherapy (LD-RT) (single dose ≤ 1·0 Gy) has been used in the treatment of degenerative, hyperproliferative and inflammatory diseases such as keloid, calcaneodynia, and osteoarthritis.

This method allows to spare normal tissues by enabling them to repair sublethal damage during interfraction interval in contrast to tumour cells which often have impaired repair mechanisms [492, 504]. Moreover, fractionation increases the damage to the tumour by reoxygenation and repopulation of irradiated sites with healthy cells [504, 505],[492]. Oxygen is critical for radiation-induced cell death [492, 505, 506]. The presence of oxygen during irradiation increases cell death by 2.5-3 fold by fixing free radicals which induce DNA damage [507, 508]. On the other hand, hypoxia induces Hypoxia-inducible factor 1α (HIF1α) which leads to up-regulation of genes involved in cell survival [508]. Furthermore hypoxia can increase tumour radioresistance by accelerated removal of free radicals through activation of autophagy [509].

1.8.2. Biology of cancer radiotherapy

Fractionation of RT allows for realigning of proliferating cells from radio-resistant S-phase of cell cycle to radio-sensitive G_2-M phase [492, 504]. IR delays the normal progression through the cell cycle with the most delay in G2 and significant delays in G1 and S phase [510]. These delays result from induction of DNA damage by IR and activation of DNA damage response (DDR) pathways [511]. DNA damage is recognised by sensor proteins such as

BRCA1, 53BP1, Mre11 and RAD50 [512]. Sensor proteins which directly recognise DNA damage, present the information to transducers such as ATM, ataxia telangiectasia and Rad3-related (ATR), Checkpoint kinase (Chk) 1 and Chk2. Transducers, with the help of mediators facilitating phospohrylation events, amplify and rely the damage signal to effector proteins, D-type cyclines and cyclin E,-in association with regulatory kinases Cyclin-dependent kinase (CDK) 4 and CDK2 which govern cell cycle progression [512]. At the effector stage ,DNA damage checkpoints interfase with cell cycle arrests leading to cell repair or death (Figure 1.7) [512].

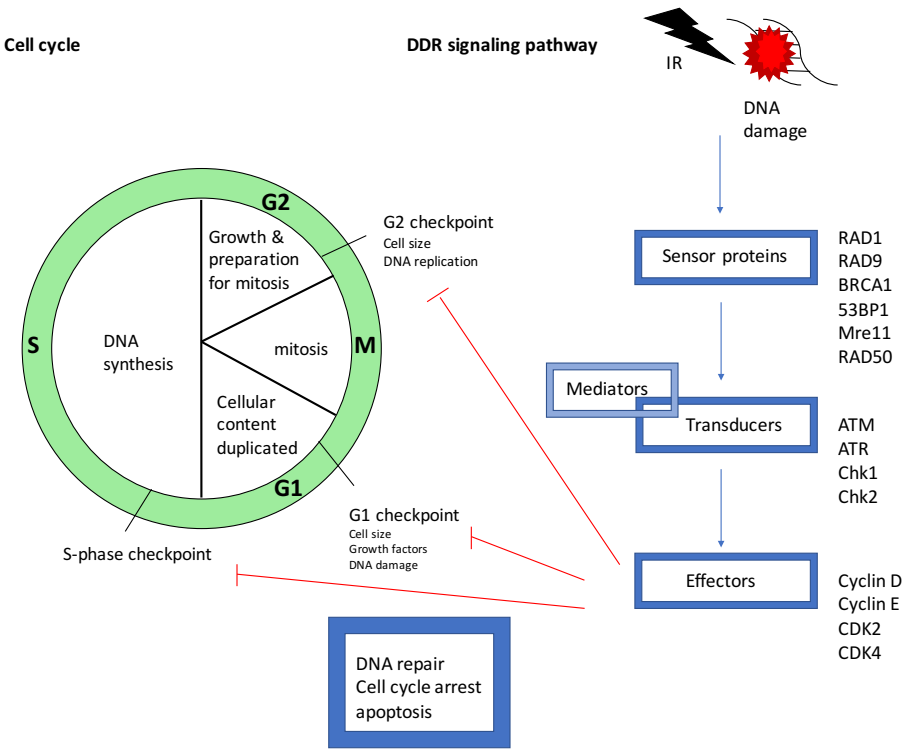


Figure1.7 Cell cycle and DNA repair mechanisms after [512]
DDR signalling pathway. The DDR pathway is activated by DNA damage which is directly recognised by sensor proteins convey the signal onto transducers. The transducers (kinases including ATM/ATR and their down-stream kinases) further amplify and rely these signals with the help of mediators (proteins facilitating phosphorylation). Effectors are downstream proteins involved in processes leading to DNA repair, cell cycle arrest or apoptosis.

The initial step of sensing DNA damage is still not well understood and requires further investigation [512]. Many proteins acting as DNA damage sensors play pleyotropic role in DDR being also transducers or effectors. Proteins such as ATM or Mre11-RAD50-NBS1 complex recognise DNA double strands breaks (DSB) [513, 514]. DSB are considered as the most dangerous type of DNA damage as both strands of DNA are being simultaneously damaged. DSB can be repaired by error-free homologous recombination (HR) or error-prone non-homologous end-joining (NHEJ) [515]. Highly proliferating cells like cancer cells are particularly susceptible to DNA damage because during replication single strand break (SSB) can be coverted into DSB which, in the absence of intact HR pathways, lead to chromosome rearrangement and ultimately genetic instability [515]. NHEJ is responsible for DSB triggered by IR predominantly in G0 and G1 phase of the cell cycle [516, 517].

DSB are repaired by HR during the S and G2 phases of the cell cycle and involve activation of DDR by kinases ATM and ATR which activate effectors BRCA2 and RAD51 [515]. Phosphorylation of ATM results in activation of various effectors governing the G1-S cell-cycle checkpoint through p53, G2/M checkpoint through Chk2 at Thr68; and the intra-S cycle checkpoint SMC1 [518, 519].

1.8.3 ATM kinase

The ATM serine/threonin protein kinase together with ATR, DNA-dependent protein kinase catalytic subunit (DNA-PKcs), mammalian target of rapamycin

(mTOR)/FKBP-rapamycin-associated protein (mTOR/FRAP), and ATX/SMG belongs to the family of phosphoinositide 3-kinase-like protein kinases (PIKK) [520]. ATM is a large protein (370kDa) which structure has not yet been fully revealed [521]. The kinase domains are located near the carboxyl terminal neighbouring conserved FAT (FRAP-ATM-TRRAP) and FAT carboxy-terminal (FATC) domains [522]. The FAT and FATC domains are regulatory domains preventing ATM auto-phosphorylation in the absence of DNA damage [523]. Internal and amino-terminal regions of ATM contain numerous α-helical HEAT (Huntington-elongation factor 3-protein phosphatase 2A-TOR1) repeats [522]. ATM is a critical regulator of cell response to IR and radiomimetic agents inducing DSB [524].

In undamaged cells, ATM exists as an inactive non-covalent dimer/tetramer with its kinase domain attached to an internal domain containing Serine (Ser) 1981 of an adjacent ATM molecule [520]. This inert state is maintained by constitutive interaction with PP2A. Upon DNA damage, ATM is rapidly activated and recruited to DSB by Mre11-Rad50-NBS1 (MRN complex) proteins binding to the C-terminal of NBS1 via specific HEAT repeats [525-528]. You et al. demonstrated that the recruitment of ATM to the DSB can precede ATM auto-phosphorylation and the interaction with MRN complex is necessary for activation [527]. Once bound, ATM rapidly auto-phosphorylates at Ser-1981 causing dissociation of active ATM monomers. However, following irradiation, the binding between ATM and PP2A rapidly dissociate in ATM-dependent fashion possibly due to ATM auto-phosphorylation or ATM-mediated phosphorylation of PP2A [529, 530]. This allows for

accumulation of active ATM monomers in the nuclei. Interestingly IR not only causes DSB but also accumulation of free radicals which can directly auto-phosphorylate ATM and trigger its migration to DSB sites [531].

Following activation, ATM triggers a cascade of DDR events. While bound by MRN complex, ATM reciprocally phosphorylates several NBS1 residues triggering S-phase arrest [532]. ATM-dependent phosphorylation of NBS1 is also required for activation of several ATM substrates such as Chk2 and SMC1 [519, 533]. Therefore, NBS plays a dual role as a sensor of DNA damage in an MRN complex and an effector of ATM signalling. In addition, ATM phosphorylates the histone variant H2AX over large chromatin domains which is required for accumulation of many DNA repair proteins and chromatin-remodelling complexes around DSB. The activation of H2AX recruits BRCA1 and 53BP1 which both enhance ATM activity [534]. Both these proteins are involved in the maintenance of ATM activation also serving as ATM substrates [529, 534, 535]. BRCA1 phosphorylation by ATM at Ser1387 activates it as a regulator of the intra-S-phase checkpoint, while the phosphorylation on Ser1423 mediates the control of G2–M checkpoint [536, 537]. ATM further promotes BRCA1 activity by phosphorylating its inhibitor CtIP [538]. In addition, ATM stimulates BRCA1 activity in an indirect manner through activation of Chk2 kinase which phosphorylates BRCA1 at Ser988 therefore releasing BRCA1 from its inhibitor hCds1 [539]. The example of BRCA1 regulation illustrates the precision and target specificity of handling the effector by ATM. The same principle is illustrated by activation and stabilization of p53 by ATM. ATM directly activates p53 through

phosphorylation at Ser15 and other sites [540, 541] while at the same time inhibits degradation of p53 by phosphorylation of Mouse double minute 2 homolog (MDM2) [542, 543]. Furthermore, Chk2 phosphorylates p53 at Ser20 interfering with MDM2 interactions [544]. Through the accumulation of transcriptionally active p53 in the nucleus ATM promotes apoptosis [545-547]. Apart of its function in cell cycle checkpoints control, ATM is also involved in HR through the phosphorylation of the RAD51 and RAD52 proteins [548, 549].

One striking feature of the ATM pathway is the use of versatile protein kinases able to affect several downstream substrates simultaneously. One such example is Chk2, that phosphorylates CDC25A and CDC25C therefore preventing activation of CDK1 and CDK2 which drives the cell cycle progress through several checkpoints [549-552].

The Ca^{2+}/cAMP response element-binding protein (CREB) represent another target for the ATM pathway. CREB is a basic leucine zipper family transcription factor promoting cell proliferation, differentiation and survival [553]. The canonical CREB activation pathway involves phosphorylation of CREB at Ser-133 by PKA or other Ser-133 kinases which promotes the interaction between the kinase-inducible domain (KID) of CREB with the transcriptional coactivator CREB-binding protein (CBP) [544, 554]. The recruitment of CBP to CREB-loaded promoters triggers histone acetylation and transcriptional activation which results in increased expression of CREB target genes [544, 555]. This interaction is negatively modulated by

phosphorylation at several inhibitory sites residing within the KID including Ser-142, Thr-100, Ser-111, and Ser-121 [555]. Shi *et al*. demonstrated that ATM regulates the activity of CREB by phosphorylation of Thr-100, Ser-111, and Ser-121 in Ser-133 phosphorylation-dependent manner [555]. Of those 3 inhibitory residues, Ser-121 is restricted to severe DNA damage and dependent on ATM signalling [544].

1.8.4 ATM kinase functions in immunity

ATM kinase is paramount for the DDR response. However, due to the variety of its targets, ATM also plays a role in a range of other cellular processes including neuronal development and regulation of immune responses. Ataxia-telangiectasia (A-T) patients characterised by autosomal recessive mutations in the ATM gene on chromosome 11 display progressive neurodegeneration, ocular and cutaneous telangiectasia, premature aging and immunodeficiency [556]. Although immunodeficiency does not affect all A-T patients, when present it is characterised by low Immunoglobulin (Ig) G4 (65% of patients), IgA (63%), IgG2 (48%), IgE (23%), and IgG (18%). Lymphopenia affects 71% of patients, with reduced B-lymphocyte number in 75%, CD4 T lymphocytes in 69%, and CD8 T lymphocytes in 51% [556, 557]. The extent of immune defects is correlated with the particular ATM mutation and hence the remaining level of kinase activity. It has been suggested that even 5% of ATM kinase activity is sufficient to provide relatively normal immune functions [557].

The involvement of ATM in immune regulation is multifactorial. ATM controls DNA DSB repair and stabilisation not only in response to radiation damage but also during VDJ recombination. It therefore plays a key role in the generation and maintenance of T- and B-cell Ag receptor diversity [558]. Furthermore A-T has been associated with defective thymus resulting in low thymic output which can be responsible for T-cell lymphopenia [559, 560]. Genotoxic stress and stalled DNA replication upregulate DNAM-1 and NKG2D ligands under the control of ATM, ATR and Chk1 [561, 562]. Many of the known ATM targets are involved in immune regulation. ATM controls expression of IFN-γ and IFN-α genes in T-cells via NFκB induction [563] and is activated in response to LPS in murine BMMφ [564]. The actions of NFκB are inhibited by CREB which blocks the binding of CBP to the NF-kB complex, therefore limiting pro-inflammatory responses [565]. CREB promotes FoxP3 expression in T-reg cells ([566]). Moreover, CREB activation leads to zymozan-induced secretion of IL-10 by DC [567], while it decreases IL-12 [568]. Furthermore, a CREB-C/EBPβ cascade induces Mφ2 phenotype characterised by induction of *Il10, Il13ra*, and *Arg-1* genes expression [569, 570]. Interestingly, PKA-mediated phosphorylation of CREB on Ser133 in Mφ promotes TNFα suppression and IL-10 induction, whereas the same phosphorylation stimulated by LPS and mediated by MSK1 is non-functional for transcriptional modulation [571]. These findings demonstrate the complexity of CREB regulation and the importance of up-stream pathways. Overall, the data suggests that ATM kinase exert pro-inflammatory functions via promotion of NFκB and inhibition of CREB activation.

1.9 Hypotheses and aims

1.9.1 Hypotheses

1. Macrophages exhibit limited plasticity and their final properties depend on signals received during differentiation from monocytes.

2. Macrophages possess the ability to regulate the expression of specific cytokines independently of their polarisation status.

3. Ionising radiation modulates immune responses in part by regulating cytokine secretion by APC.

4. Stromal cells such as FB support the function of APC in the presence of IR.

1.9.2 Aims and objectives

The aim of this study was to identify the influence of environmental factors on APC function and their response to therapeutic interventions. Furthermore, we intended to deepen our understanding of the cross-talk between FB and APC and its effects on immune responses to RT. This study was conducted with a view to provide basis for novel approaches in immunotherapy alone or in combination with RT.

Chapter 2 Materials and methods

2.1 Regents

All reagents were endotoxin-free. Recombinant human GM-CSF and TNFα was purchased from Peprotech, IL-2, IL-4 and IFN-γ from R&D Systems. Ultrapure TLR4-agonist (*Salmonella Minnesota* LPS) and TLR7/8 agonist (R848) were purchased from InvivoGen. Recombinant human IL-1β and M-CSF were from ImmunoTools. IL-23, mouse anti-human CD4-PECy7, mouse anti-human CD45RA-FITC, mouse anti-human CD14-Pe-Cy5.5 and matching isotype controls were from e-Bioscience. PGE2, Indomethacin, forskolin, FBS, DMSO, EDTA and Sodium azide were from Sigma-Aldrich. C-Ci (2-Naphthol-AS-E phosphate) was from Merk-Millipore. Mouse anti-human CD4-PE and CD4 activation, mouse anti-human CD28 were from BD Biosciences. CD3 (OKT3) was produced in-house. Phosphate buffered saline (PBS) was from Gibco, Life Technologies. AnnexinV/PI staining kit was obtained from BD Bioscience

2.2 Primary cell isolation

2.2.1 Isolation of CD14+ve monocytes from blood

Ethical approval to take blood from healthy donors was obtained from Nottingham University Ethics Committee. Each donor signed a written informed consent form before phlebotomy. Donors with significant medical problems, currently on immunosuppressive medication or with anaemia were asked not to volunteer, although no specific screening were performed or any

data recorded. Whole blood (buffy coats and cones) was either obtained by venepuncture or purchased from National Blood Service (Sheffield)

Heparinised whole blood or buffy coats were diluted with PBS as needed, layered without mixing over Histopaque-1077 (Sigma) and centrifuged at 400g for 30 minutes at room temperature with zero deceleration. Peripheral blood mononuclear cells were collected from the plasma/Histopaque-1077 interface, washed three times with PBS to remove platelets, and washed once in cold MACS buffer (PBS + 1% FBS, 2ml 0.5M EDTA pH-8.0) before proceeding to magnetic separation. For buffy coats, an extra step of red cell lysis was performed by adding 18ml DNase RNase free water (Gibco) to cell pellet for 20 seconds followed by the addition of 2ml 10xPBS.

2.2.2 Isolation of monocytes

CD14+ve cells were isolated using magnetic microbeads (Miltenyi Biotec). 100-150µl CD14 microbeads (Miltenyi) with 200-400µl MACS buffer were added to the re-suspended cell pellet depending on cell number and incubated at 4°C for 15 minutes. Cells were washed in cold MACS buffer and CD14+ve cells selected using an LS column. Purity was routinely >90% as assessed by FACS analysis with CD14 antibodies (Miltenyi Biotec).

2.2.3 Isolation of slanDC

SlanDC were isolated from fresh peripheral blood. After washing in cold MACS PBMC were incubated with M-DC8 hybridoma supernatant (received from prof. Mark Shmitz from University of Dresden) 10 µg/ml of Ab for 15 min at 4 °C. Next cells were washed in MACS buffer and incubated with anti-

mouse IgM microbeads (Miltenyi) for another 15min at 4 °C. SlanDC were positively selected through LS column (Miltenyi). Cell suspension was then eluted onto a second MS column to increase purity. Purity was routinely >95% as assessed by FACS analysis with anti-SLAN antibodies (Miltenyi Biotec). Following isolation SlanDC were rested for 6 h before experiments to allow for phenotypical maturation.

2.2.4 Naïve T-cells

Naïve T cells (CD4+CD45RA+) were isolated using CD4+ T cell isolation kit II followed by CD45RA microbeads (both Miltenyi). PBMC were washed in cold MACS buffer and incubated with biotinylated antibody cocktail at 4^{o}C for 10 minutes in MACS buffer. Next Anti-biotin microbeads were added and cells were incubated at 4^{o}C for 15 minutes. After washing in MACS buffer, CD4+ cells were negatively selected using an LS column, washed and incubated in MACS buffer with CD45RA+ microbeads for 15 minutes at 4^{o}C. After washing, CD4+CD45RA+ cells were positively selected using an MS column.

2.3 Generation of MoMφ

CD14+ Mo were cultured in RPMI1640 medium containing 10% FBS and 1% sodium pyruvate in low adherence 6-well plates (Corning Costar) at concentration 5x10^{6} cells per well per 5mL of medium for 5-7 days. For generation of GM-Mφ, GM-CSF was added at a concentration 20U/mL while for generation of M-Mφ, M-CSF was added at 10ng/mL. Additional complete medium with respective cytokines was added on day 4. Immature Mφ were collected by incubation on ice for 20 min in cold PBS and gentle pipetting,

washed and plated in normal adherence cell culture plate (Nunclon) for experiments. Typical yield ranged between 25-40% of the initial cellular input.

2.4 Generation of MoDC

For the generation of moDC, freshly isolated monocytes were cultured in DC medium with 1000u/ml rhGM-CSF and 1000U/ml rhIL-4 at a concentration of 0.5-1.0x10^6 cells/ ml in T75 or T175 flasks. On day 4, the cells were fed with half volume of the initial amount of the medium with cytokines. The cells were then harvested at day 5-6 and plated in tissue culture plate in DC medium with rhGM-CSF for further experiments.

2.5 Culture of BJ6 and primary FB

The human dermal FB cell line BJ6 was obtained from Dr. Lloyd Hamilton (University of Nottingham). Primary dermal FB were kindly donated by Dr. Anja Saalbach (University of Leipzig). All cultures were tested free of mycoplasma infection. Frozen vials of FB were defrosted from liquid nitrogen stores by warming up in the water bath at 37^{o}C. As soon as the cell suspension was defrosted, it was transferred to a falcon tube and warmed FB medium (DMEM with 10% FBS with 1% L-glutamine) was added dropwise whilst mixing the cell suspension. The cells were then centrifuged at 180xg for 5 minutes and resuspended in fresh BJ6 medium for culturing in T25 flask. Once the cell density reached the maximum as judged by microscopic examination, the cell suspension was aspirated and pelleted in a falcon tube. Then the cells were passaged at 1:3 for routine culture. BJ6 were used up to 12th passage while primary FB were used up to 4th passage.

2.6 Freezing FB

After harvesting from flasks, cells were washed once in complete medium, counted and resuspended at 3-5x10^6 cell /ml in freezing medium (40% cell culture medium, 50% FCS and 10% DMSO). Cells were then quickly aliquoted as 1ml per tube and placed in a cryopreservation vessel filled with isopropanol. Cells were then cooled to -80^{o}C at no more than 1^{o}C/minute for 24 hours before transferring into liquid nitrogen. Before freezing, culture supernatant was taken for mycoplasma testing and routinely found to be negative.

2.7 Stimulation of Mφ for phenotypic characterisation and secretory profile

Mφ were counted, washed and seeded in 96-well normal adherence tissue culture plates in full Mφ medium without GM-CSF or M-CSF at concentration of 5x10^4 cells/well. Next Mφ were rested for 2h in 37^{o}C and treated with respective drugs. Unless otherwise stated GM-Mφ were activated LPS (500 ng/mL and IFN-γ (1000 U/mL), while M-Mφ with LPS (500ng/mL) only. Supernatants were collected after 24h and stored in -20 ^{o}C for analysis of cytokine secretion by ELISA. For flow cytometry, cells were detached by scraping or trypsinisation in 0.5% trypsin in PBS.

2.8 Stimulation of MoDC and SlanDC for phenotypic characterisation and secretory profile

MoDC or SlanDC were plated in 96-well tissue culture plates at 5x10^4 or 1x10^4 cells /well respectively in triplicates in 100µl DC medium. After resting for 1 hours (MoDC) or 6h (Slan DC), cells were treated with LPS (500ng/mL) in a final volume of 200µl with or without IFNγ (1000U/ml). After 24 hours,

cells were microscopically confirmed to be free of microbial infection before 175µl of culture supernatant was collected and stored at -20^{o}C for subsequent cytokine secretion analysis by ELISA.

2.9 Co-culture of FB with APC

For co-culture experiments FB were seeded in flat-bottomed 96 well plates and rested overnight. APC were added to FB next day and rested for 1h before treatment. MoDC were added to FB at proportion 4:1 ($8x10^6$ DC: $2x10^6$ FB), SlanDC at proportion 1:2 ($1x10^6$ SlanDC:$2x10^6$ FB) and MoMφ at proportion 2.5:1 ($5x10^6$ Mφ:$20x10^6$ FB). Co-cultures were incubated for 24h in 5% CO_2, 37^{o}C. Supernatants were collected and stored at -20 ^{o}C awaiting analysis. All experimental conditions were performed in biological triplicate and on multiple donors unless otherwise stated.

2.10 Irradiation of cells

Cells were irradiated in tissue culture plates immediately prior to DC/Mφ activation with LPS (500ng/ml, InvivoGen) and/or IFNγ (1000U/ml, R&D). For experiments with indomethacin MoDC were activated 3 hours before irradiation and adding to FB to reduce the effect of COX2 inhibitor on DC maturation. Radiation (0-6Gy of 195kVp X-rays, 0.87Gy/min, 0.5mm Cu filter, 48.4 cm FSD) was delivered using a Gulmay Xstrahl cabinet irradiation facility within the department. Cell morphology was examined by phase-contrast microscopy (x40) following radiation and after a further 24hrs of culture and viability at 24h was determined by trypan blue staining. Apoptosis and necrosis was detected using AnnexinV/PI kit from BD bioscience. Flow

cytometry was performed using Beckmann Coulter FC500 flow cytometer and analysed with FlowJo software

2.11 Generation of Th17 responses

DC monocultures or DC-FB co-cultures were treated as previously described for 12 hrs before 90% of the supernatant was discarded and replaced with fresh media thus preventing LPS/IFNγ affecting T-cells. It was previously shown that IL-23 is not produced until >12rs after TLR stimulation [572]. Isolated T-cells were cultured in 48 well plates at $2x10^5$ cells per well. T-cells were cultured in a 1:1 ratio of fresh proliferation medium and DC monoculture or DC:FB co-culture supernatants for 5 days in the presence of anti-CD3 and anti-CD28 microbeads. As controls, T-cells were also treated with recombinant cytokines (IL-1β, IL-6, IL-23) instead of co-culture supernatants. Next T-cells were rested for 2 days and re-stimulated with anti-CD3/anti-CD28. For FACS assessment of IL-17 T cells were treated with Brefeldin A (BD). Cells were collected for FACS 24 hours after re-stimulation and for ELISA 48h after re-stimulation.

2.12 Determination cytokine secretion in supernatants

The cytokine content of supernatants was determined using commercial ELISA kits according to manufacturer's instructions. Human IL-23 (Bioscience) had sensitivity 15pg/mL, IL-12p70 (BD Biosciences) 3.9pg/mL, IL-10, IL-1β, TNFα, IL-17, IL-27 and PGE2 were measured with DuoSet assays (R&D Systems) and had sensitivities 15.6, 3.9, 15.6, 7.8, 156 and 30.9pg/mL respectively.IL-6 (ImmunoTools-Friesoythe) had sensitivity 3.9pg/mL. Samples were diluted between 1/2 and 1/200 as needed and assayed

alongside duplicate serial dilutions of protein standard. Absorbance was measured at 450nm using Tecan microplate reader.

2.13 Flow Cytometry

2.13.1 Characterisation of APC phenotype by flow cytometry

Mφ were detached by scraping or trypsinisation in 0.5% trypsin in PBS. FB were detached by trypsinisation. DC and Mo were collected using gentle pipetting. Next cells were stained in FACS buffer (PBS with 1% FBS and 0.1% sodium azide) with required antibodies and matched isotypes (table 2.1). Samples were acquired on MACS Quant Flow Cytometer and analyzed using FlowJo software (Treestar Inc.). In some cases, samples were fixed in 2% formaldehyde (Sigma) and stored in the dark at 4^{o}C until acquisition.

Name	Type	Clone	Fluorophore	Source
HLA-DR	IgG2a	AC122	FITC	Miltenyi
CD86	IgG1	FM95	Vioblue	Miltenyi
CD80	IgG1	2D10	APC	Miltenyi
CD40	IgG1	5C3	FITC	BD Bioscience
CD83	IgG1	HB15e	PE	BD Bioscience
IgG2a isotype		S43.10	FITC	Miltenyi
IgG1 isotype		IS5-21F5	Vioblue	Miltenyi
IgG1 isotype		IS5-21F5	APC	Miltenyi
IgG1 isotype		MOPC-21	FITC	BD Bioscience
IgG1 isotype		MOPC-21	PE	BD Bioscience

Table2.1 Antibodies used for flow cytometry.

2.13.2 Determination of Viability

Mφ and FB were detached by scraping or trypsinisation in 0.5% trypsin in PBS. MoDC were collected by gentle pipetting. Cells were washed twice with cold PBS and then resuspended in 1x binding buffer at a concentration of 10^6 cells/ml. 100 µl of the solution (10^5 cells) were transferred to a 5ml FACS tube. Then FITC-labelled Annexin V (5µl) and Propidium Iodide (PI) (5µl) were added to each tube. The cells were mixed by gentle vortex and incubated for 15 minutes at RT in the dark. Cells were then resuspended in additional 400µl of 1x binding buffer and acquired within 1h. All samples were acquired on MACS Quant Flow Cytometer within 1h and analyzed using FlowJo

software. As positive control a sample treated for 24h with 0.25% DMSO was used.

2.13.3 Intracellular staining for CD68

Intracellular staining for CD68 was performed according to manufacturer instructions (Miltenyi Biotec). Mφ were detached as above. Cells were fixed with Inside Fix (Miltenyi) and incubated for 20min in the dark at room temperature. After washing by centrifugation (180g, 5min) cells were incubated 10 minutes at room temperature with CD68 antibody or isotype in Inside Perm (all Miltenyi). After another wash samples were resuspended in FACS buffer and acquired on MACS Quant Flow Cytometer.

2.13.4 Intracellular staining for phospho-ATM

Cells were harvested with 2% cold methanol, washed (180g for 5 minutes) and incubated for 15 minutes at RT in 500µl of 2% formaldehyde. Next the cells were fixed in 1ml methanol for 15 minutes on ice and kept overnight at 4^0C or proceeded to the next step. Permeablization was carried out by incubating with 50µl 0.5% saponin (in FACS buffer) for 30 minutes on ice. Next cells were stained with primary antibody in 0.2% saponin (in FACS buffer) for 1h at RT. Non-specific binding was blocked with 500 µl 5% FBS in FACS buffer for 15 minutes and then stained with secondary antibody in 0.2% saponin (in FACS buffer) for 30 minutes on ice. Cells were washed following every incubation and finally resuspended in FACS buffer for acquisition. Binding of primary antibodies mouse monoclonal anti-human ATM phospho-Ser1981 (Millipore) was detected with a FITC-conjugated secondary antibody (polyclonal rabbit anti-mouse IgG F(ab)2-FITC, Dako).

2.14 Quantitative PCR

DC ($1x10^6$ cells) were plated in DC medium in 12-well plates. Cells were irradiated and activated with LPS and IFNγ as per protocol. At 12h cells were harvested into cold PBS, RNA was isolated using Nucleospin RNA ll Extraction kit (Macherey-Nagel) and cDNA was prepared (GoScript Reverse Transcription System, Promega).

Quantitation of mRNA levels of p19

Quantitative PCR was carried out for IL23p19 withTOP1 as a housekeeping gene using Taqman primers (Applied Biosystems, TOP1 Hs00243257_m1, IL-12p19 Hs00372324_m1). The Master Mix used was from Primer Design (Southampton, UK) and the assay was carried out on Stratagene MX3005P. Primers and probes were used at 15 pmol/µl in a final volume of 20µl and the reaction was carried out in triplicates. PCR conditions for all primers were 10min/950C of 1 cycle, then 50 cycles of 15s/950C and 60s/600C. Data analysis was undertaken with Stratagene software. Quantification was carried out using δδCT method where δCT= (gene of interest CT)-(TOP1 CT), δδ CT was calculated with mDC as the reference condition and then fold change in gene expression = 2-δδCT. Amplification efficiency was 90-110%.

2.15 Western blotting

MoDC and MoMφ were plated in 12-well tissue culture plate at 10^6 cells in 1ml. After resting, cells were irradiated followed by activation with LPS/IFNγ (MoDC, GM-Mφ) or LPS (M-Mφ). Cells were harvested in cold PBS, washed and lysed in RIPA buffer (Sigma) containing protease inhibitor cocktail,

phosphatase inhibitor cocktails 2 and 3 and benzonase endonuclease (all Sigma) on ice for 1h. Residual lysis buffer was removed following centrifugation and samples stored frozen at -20^{0}C until use.

Proteins were resolved in 10% Tris/glycine gels (made in house) or 4-12% Tris/glycine gradient gel (Novex) by electrophoresis. Proteins were then transferred onto nitrocellulose membranes and membranes blocked with 5% milk in PBS-0.1% Tween-20 for 1h and probed with primary antibodies overnight at 4^{0}C. Following washing, the binding of primary antibodies was detected with appropriate secondary antibodies in the dark for 1h at room temperature in 1% milk (w/v) in PBS-0.1% (v/v) Tween-20. Membranes were washed repeatedly after each antibody with PBS-0.1% 9 (v/v) Tween-20 to remove any unbound milk or antibodies. The primary antibodies used were mouse monoclonal anti-human ATM phosphoSer1981 (Clone 10H11.E12, mouse IgG1κ) from Millipore, polyclonal rabbit anti-human DNA-PKcs phosho-S2056 (rabbit IgG) from Abcam, anti-human phospho-eIF2α, mouse anti-human CREB phosphoSer121 from Cell Signalling and mouse anti-human β (Sigma). Infrared-dye conjugated secondary antibodies: donkey anti-rabbit-800 and donkey anti-mouse-600 were obtained from Li-COR. The membranes were scanned on a Li-COR Odyssey infrared reader and the images were analysed by the software Li-COR® Odyssey-version 3.0. The intensity of the target protein was normalized to the intensity of the corresponding β-actin band for each sample.

2.16 β-galactosidase assay

Cells were washed 2x with PBS and fixed for 10min at room temperature in 4% formaldehyde. After 2 washes with PBS cells were incubated for 48h in

freshly prepared β-galactosidase in DMSO containing 1mg/ml 5-bromo-4chloro-3-indolyl β-D-galactopyranoside (X-Gal), 5mM potassium ferrocyanide, 5mM potassium ferricyanide, 150mM NaCl, 2mM MgCl2, 40mM citric acid (all sigma) at pH 6.0.

2.17 Statistical analysis

Results were statistically analyzed using GraphPad® Prism software and figures annotated as follows: *:0.05>p≥0.01, **:0.01p≥0.001, ***:0.001>p≥0.0001, ****:p<0.0001. Results are presented as mean ± standard deviation (SD). Non-parametric unpaired t-test was performed for data from one experiment in aim to compare biological triplicates between different treatments. Data from multiple donors containing two sets of treatments was tested by non-parametric paired t-test. Data from multiple donors for comparing effect between treatments were tested with one-way ANOVA for one variable or two-way ANOVA if two variables applied. The difference between two groups was then tested by unpaired non-parametric t-test with Mann-Whitney test. Results were presented as the mean ± standard deviation (SD), unless otherwise stated.

Chapter 3 Optimisation of culture conditions for macrophages

3.1 Introduction

Mo isolation procedures were well established in the laboratory. In contrast, protocols for Mφ from purified Mo required optimisation due to low number of recovered MoMφ and mixed donor-specific phenotype. Mφ are extremely plastic cells highly sensitive to culture conditions which require consistent laboratory procedure for reproducible results. The type of medium and plastics used as well as cytokines added during generation and activation process vary greatly between laboratories and often impact on the outcomes of experiments [133, 141, 144]. Therefore, it was important to establish optimal culture conditions for MoMφ.

3.2 Results

3.2.1 Isolation of CD14+ve cells

The purity of isolated Mo was routinely checked by flow cytometry assessing CD14 expression and was above 90% (Figure 3.1). In rare cases where CD14+ fell below 90% cells were re-purified through magnetic columns or discarded from further use.

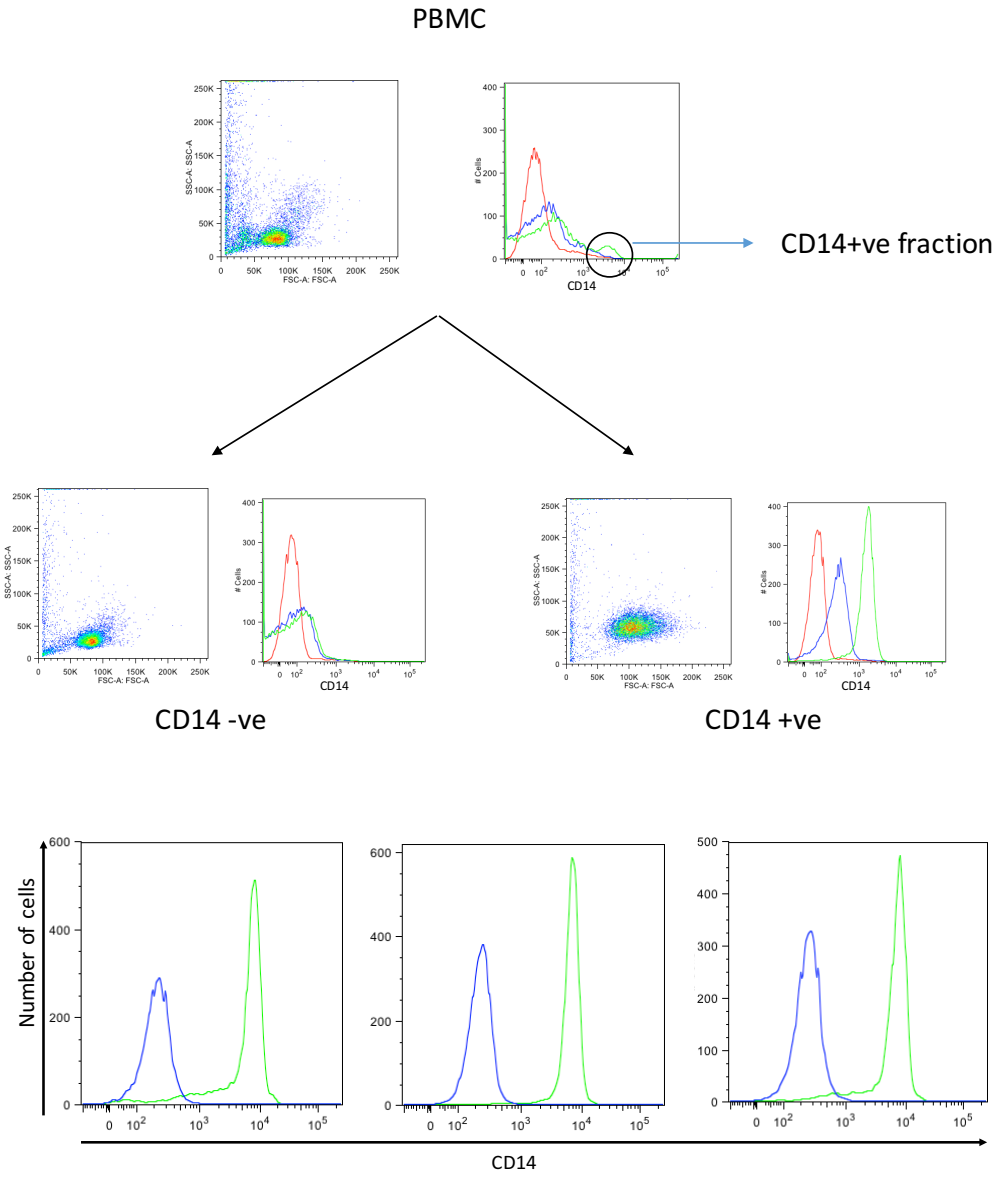


Figure 3.1 Isolation of CD14+ve population from PBMC. PBMC acquired from buffy coats or fresh blood through gradient centrifugation were incubated with CD14 magnetic beads and run through a magnetic column. Cells were stained with anti-CD14 Ab for flow cytometry. Red line- unstained, green line- antibody, blue line – matched isotype control. Graphs show an example of CD14 expression by cells before and after running through magnetic column on 1 donor and the purity of isolated Mo in 3 representative donors.

3.2.2 The effect of FBS type on cytokine secretion

The source and lot of FBS can affect immune cells function and cytokine secretion [573] therefore, the effect of FBS type on MoMφ during moving to a new lot was routinely examined. The effect of FBS on cytokine secretion was found to be donor specific therefore testing was performed on multiple donors. Figure 3.2 shows an example of differential secretion of cytokines under the same culture condition with the exception of different FBS added to the media. IL-12 secretion was lower in GM-Mφ in both donors in FBS A while IL-10 secretion was affected only in donor 2. Based on those results FBS A was not used in further experiments.

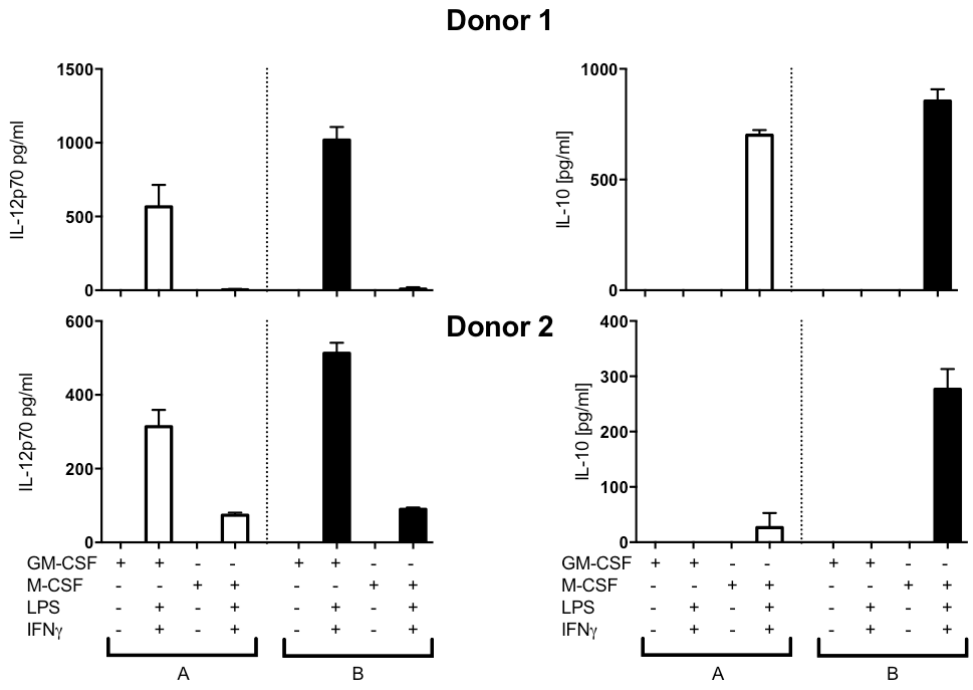


Figure 3.2 Determination of cytokine secretion depending on the type of FBS in media. MoMφ generated and cultured in medium with specific FBS (A, B) were activated with LPS/IFNγ (Mφ1= GM-Mφ) or LPS (Mφ2= M-Mφ) and cytokine secretion was assessed by ELISA 24h later. Shown are means + SD of triplicate stimulation.

3.2.3 Characterisation of macrophages differentiated on normal vs low adherence plates

Typically, Mφ are strongly attached to the surface and reported MoMφ - generation protocols commonly utilise low-attachment containers for Mφ culture. Cells were seeded into normal or low-adherence plates and their morphology was assessed by microscopic examination. Mφ, which were generated in normal adherence plates displayed typical previously described morphology of GM-Mφ (round shape) and M-Mφ (spindle shape) [145] while both GM-Mφ and M-Mφ growing on low adherence plates presented similar round "fried egg" appearance (Figure 3.3).

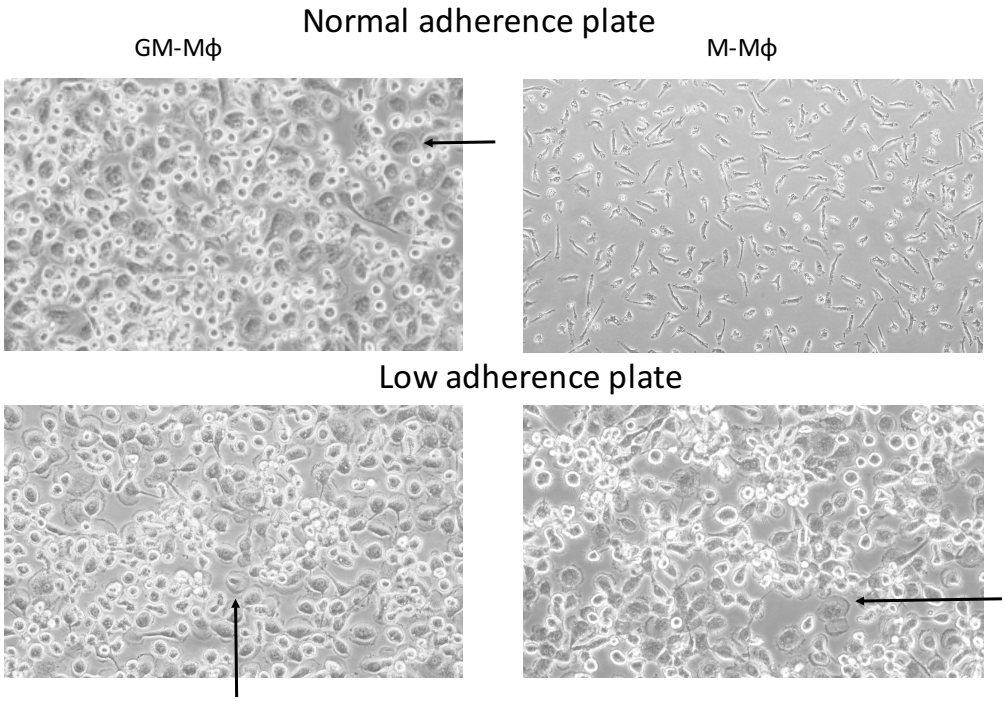


Figure 3.3 Morphology of MoMφ differentiated on normal vs low adherence plates. Freshly isolated Mo were differentiated for 7 days in normal adherence (NA) or low adherence (LA) plates in the presence of GM-CSF or M-CSF. Photographs were taken under 10x magnification. Arrows show typical "fried egg" appearance of Mφ1 (GM-Mφ). Shown a representative donor.

Next, we assessed cytokine secretion by Mφ. Due to low numbers of M-Mφ collected from normal adherence plates, cytokine secretion was compared

only in GM-Mφ (Figure 3.4). IL-12 secretion, one of the main pro-inflammatory cytokines, from GM-Mφ generated in normal and low adherence plates and next cultured for 24h on normal adherence plates was assessed in 3 donors. Resting Mφ did not secrete IL-12 regardless of the type of plate used for generation while, stimulation with LPS only gave minimal secretion of IL-12, less than stimulation with LPS/IFNγ. Activation of Mφ with LPS/IFNγ resulted in the release of IL-12 by Mφ generated in low and normal adherence plates. The secretion of IL-12 was significantly higher in low adherence plates in 2 donors ($p<0.0005$) while it was lower in 1 donor ($p<0.005$). We therefore used low-adherence plates for further research.

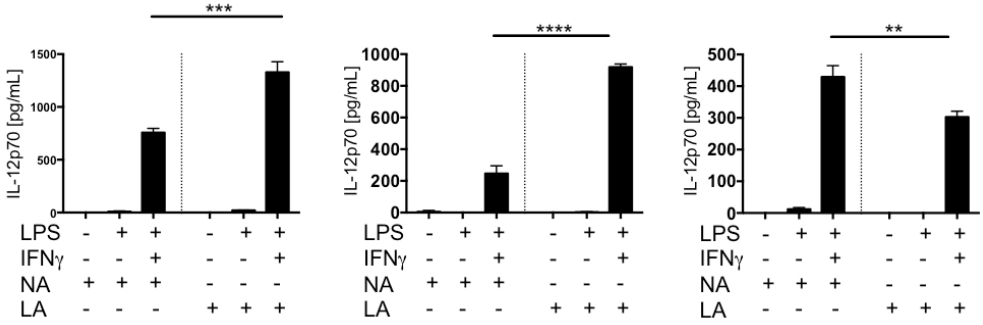


Figure 3.4 Comparison of IL-12p70 secretion by GM-Mφ generated in normal (NA) or low (LA) adherence plates. Freshly isolated Mo were differentiated for 7 days in normal or low adherence plates in the presence of GM-CSF. On day 7 Mφ were counted, washed and seeded in 96-well plates. Cells were rested for 2h before activation with LPS/IFNγ. IL-12p70 secretion was measured at 24h by ELISA. Data shown for 3 donors tested. Statistically significant differences were determined by the unpaired student's t-test.

3.2.4 Time-course of MoMφ generation

Having established the effect of different plastics on MoMφ generation we set out to determine the optimal time required for differentiation of Mo into MoMφ. In the literature, most commonly Mφ are used after 5-7 days' culture

although some sources advise 14 days of differentiation [133, 141, 143-145]. We compared IL-12 and IL-10 secretion on day 0,1,5 and 7 in Mo incubated with GM-CSF or M-CSF and stimulated with LPS/IFNγ.

Freshly isolated Mo (day 0) activated with LPS secreted small amounts of IL-12 which was abrogated by addition of IFNγ. After 1 day of incubation with GM- or M-CSF cells lost their ability to respond to LPS only with regards to IL-12 secretion (Figure 3.5A). However, cells from 2 out of 3 donors stimulated with LPS and IFNγ in the presence of GM-CSF started producing small amounts of IL-12. Cells from the 3rd donor as well as cells from all donors incubated in the presence of M-CSF did not respond to the addition of IFNγ to LPS. IL-12 secretion upon stimulation with LPS/IFNγ significantly increased on day 5 and remained high on day 7. While in the presence of GM-SF IL-12 seems to reach the maximum level on day 5, M-Mφ increased significantly secretion on day 7. The cells remained un-responsive to LPS alone with regards to IL-12 secretion.

IL-10 was readily secreted by freshly isolated Mo activated with both LPS and LPS/IFNγ (Figure 3.5B). The secretion was reduced after 24 h of incubation with GM-CSF in all 3 donors while incubation with M-CSF resulted in increase of IL-10 upon stimulation with LPS only. On day 5 and 7 Mφ secreted IL-10 regardless of GM- or M-CSF presence.

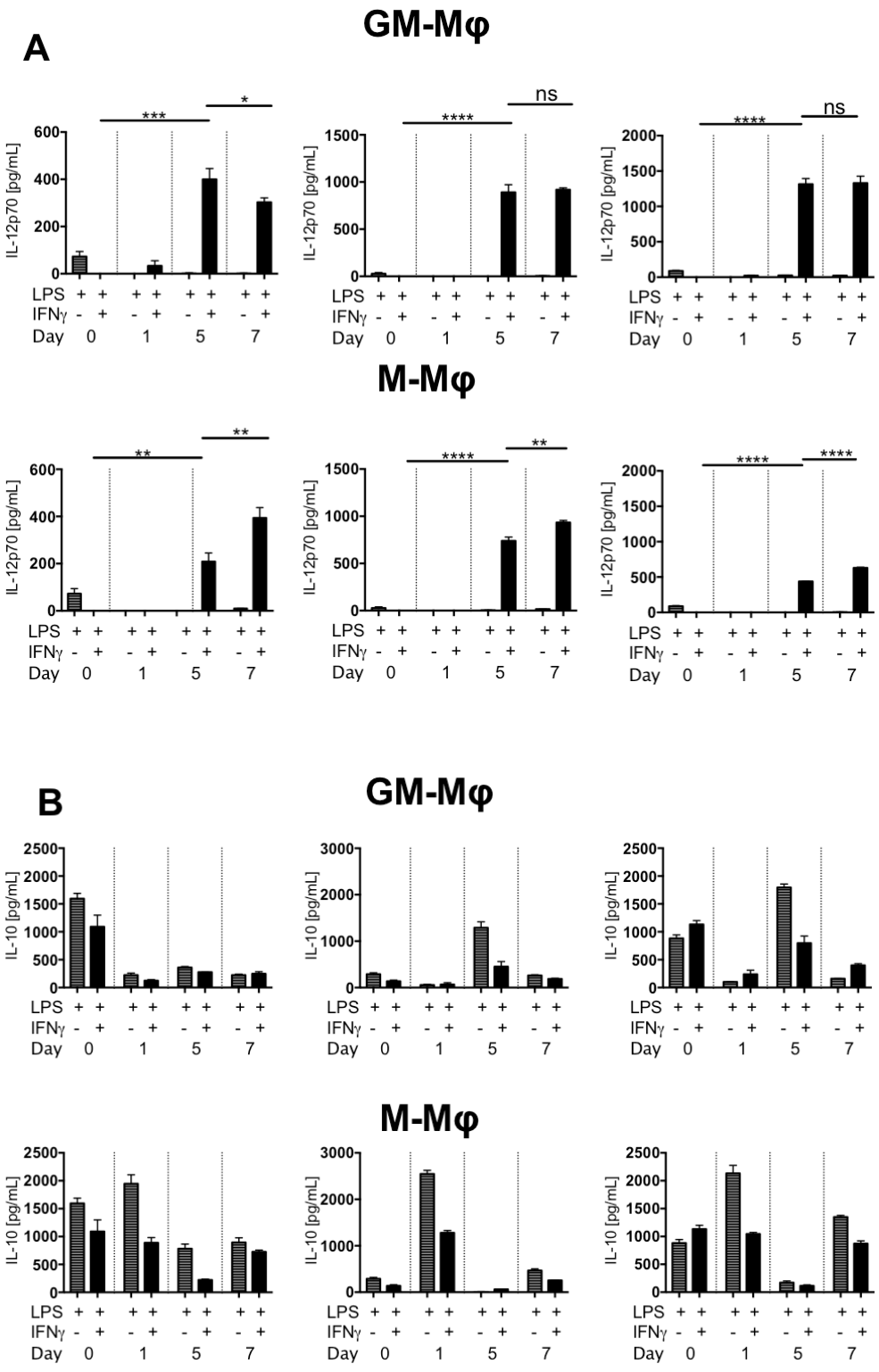


Figure 3.5 Time-course of Mφ generation. Mo differentiated with GM-CSF or M-CSF for 0-7 days were counted, washed and rested for 2h before activation with LPS/IFNγ. IL-12 (**A**) and IL-10 (**B**) secretion was assessed in 3 donors at 24h by ELISA. Shown are means + SD of triplicate stimulation. Statistically significant differences were determined by the unpaired student's t-test.

3.2.5 Requirement for GM-CSF and M-CSF during activation

As described earlier Mφ were generated by incubation for 5-7 days with GM- or M-CSF. However, after this time cells were washed to remove remaining cytokines and activated with LPS and IFNγ without the presence of GM- or M-CSF. Therefore, the need for addition of GM- or M-CSF during activation was assessed.

Firstly, we determined whether incubation of generated Mφ in the presence or absence of cytokines can affect their viability during short-term (24h) culture. Mφ were generated according to standard protocol and on day 5-6 were collected, washed and seeded in normal adherence plates in the presence or absence of GM-CSF or M-CSF. After 24h viability was assessed by FACS using PI staining. PI is a fluorescent agent which binds to DNA and RNA by intercalating between the bases of nucleic acid binding to the nucleotide pair of guanine and cytosine [296, 574]. While it is bound, its fluorescence increases 20-to 30- fold. PI is impermeant which means it cannot pass through an intact membrane of live of early apoptotic cells. However, cells undergoing late apoptosis and necrosis are characterised by increased permeability of both the plasma and nuclear membranes which allow PI to enter the cell and nuclei and bind to DNA or RNA causing red fluorescence which can be measured by FACS [296, 575]. Prior to FACS analysis debris was gated out on FSS/SCS (Figure 3.6A). There was little effect of the addition of GM-CSF to GM-Mφ or M-CSF to M-Mφ and in all donors, more than 88% of cells remained viable despite of the presence or absence of the cytokines (Figure 3.6 B, C).

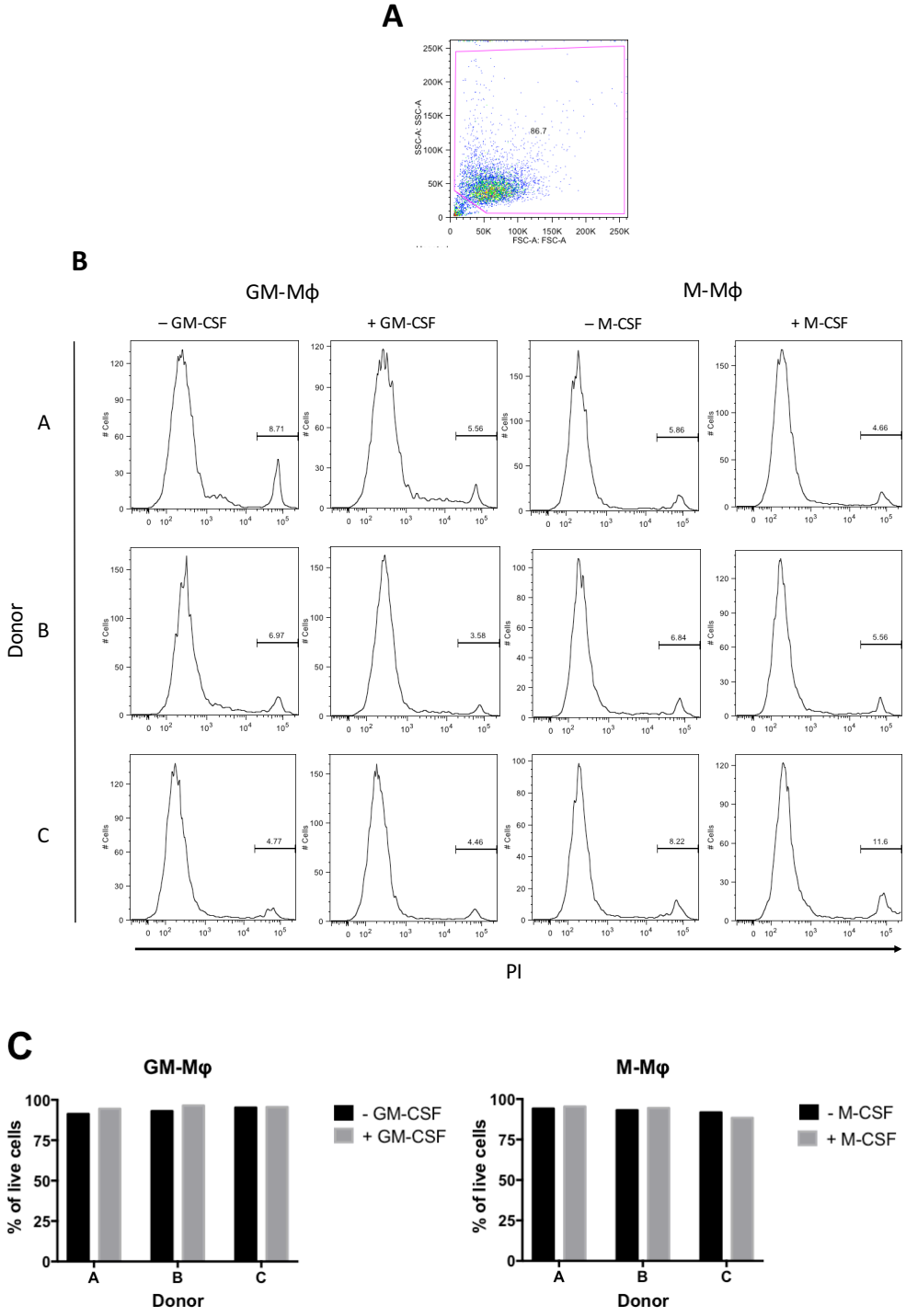


Figure 3.6 Effect of GM-CSF and M-CSF on the viability of Mφ. MoMφ generated in the presence of GM-CSF and M-CSF were washed and reseeded for 24h with or without indicated cytokines. After 24h of incubation MoMφ were detached by trypsinisation, stained with PI and assessed by FACS. **A**- Graph shows gating strategy for analysis of cells and debris exclusion. **B**- PI staining of MoMφ is shown for 3 donors. **C**- Percentage of live cells is shown for all tested donors.

Next, we determined cytokine secretion of MoMφ cultured for 24h in the presence or absence of GM- and M-CSF. There was no secretion of IL-12, IL-23 and IL-10 by GM-Mφ or M-Mφ without stimulation with LPS/IFNγ in all donors tested (Figure 3.7). No significant difference was detected in the secretion of IL-12 by GM-Mφ and M-Mφ activated with LPS and IFNγ in the presence or absence of GM-CSF and M-CSF respectively in 2 out of 3 donors tested. In 1 donor, there was a small but significant ($P<0.05$) increase in the secretion of IL-12 in the presence of the respected cytokines by both types of Mφ. The presence of GM- or M-CSF did not affect the secretion of IL-23 and IL-10 by LPS/IFNγ activated Mφ in all donors tested. GM-Mφ did not secrete IL-10 regardless of the presence of activation stimuli.

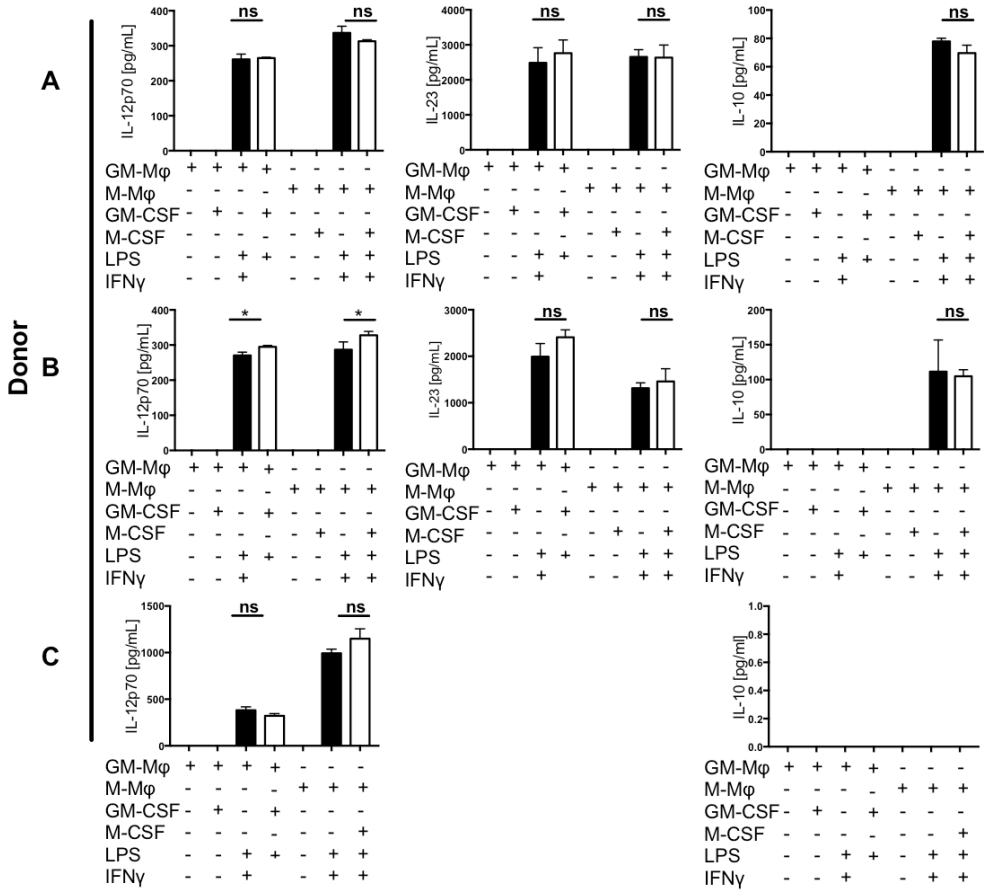


Figure 3.7 Effect of the presence of GM-CSF/M-CSF on cytokine secretion by MoMφ. MoMφ were activated or left non-activated in the presence or absence of GM-CSF/M-CSF. The secretion of IL-12p70 and IL-10 was measured at 24h by ELISA in 3 donors and IL-23 in 2 donors. Shown are means + SD of triplicate stimulation. Statistically significant differences were determined by the unpaired student's t-test.

Finally, we assessed the expression of HLA-DR and CD86 (Figure 3.8 A, B). The number of HLA-DR+ve GM-Mφ raised dramatically after stimulation with LPS/IFNγ and was unaffected by culturing cells in the absence or presence of GM- or M-CSF. The expression of HLA-DR in M-Mφ in 2 out of 3 donors increased slightly upon addition of GM-CSF while it was not affected by M-CSF or LPS. In one donor, the number of positive cells dropped

sharply upon addition of GM- or M-CSF comparing to cells in media alone. This effect was reduced by activation of cells with LPS.

The number of CD86+ve cells increased upon activation of GM-Mφ in all donors and in M-Mφ in 2 out of 3 donors (Figure 3.8 A, C). The addition of M-CSF to GM-Mφ increased the expression of CD86 in 1 donor (Donor C) while there was no effect in other donors. Culture of M-Mφ in the presence of GM-CSF resulted in slightly increased number of CD86+ve cells in 2 out of 3 donors regardless of their activation status while it increased CD86 expression only by resting GM-Mφ. There was no effect of continuous addition of GM-CSF on activated GM-Mφ.

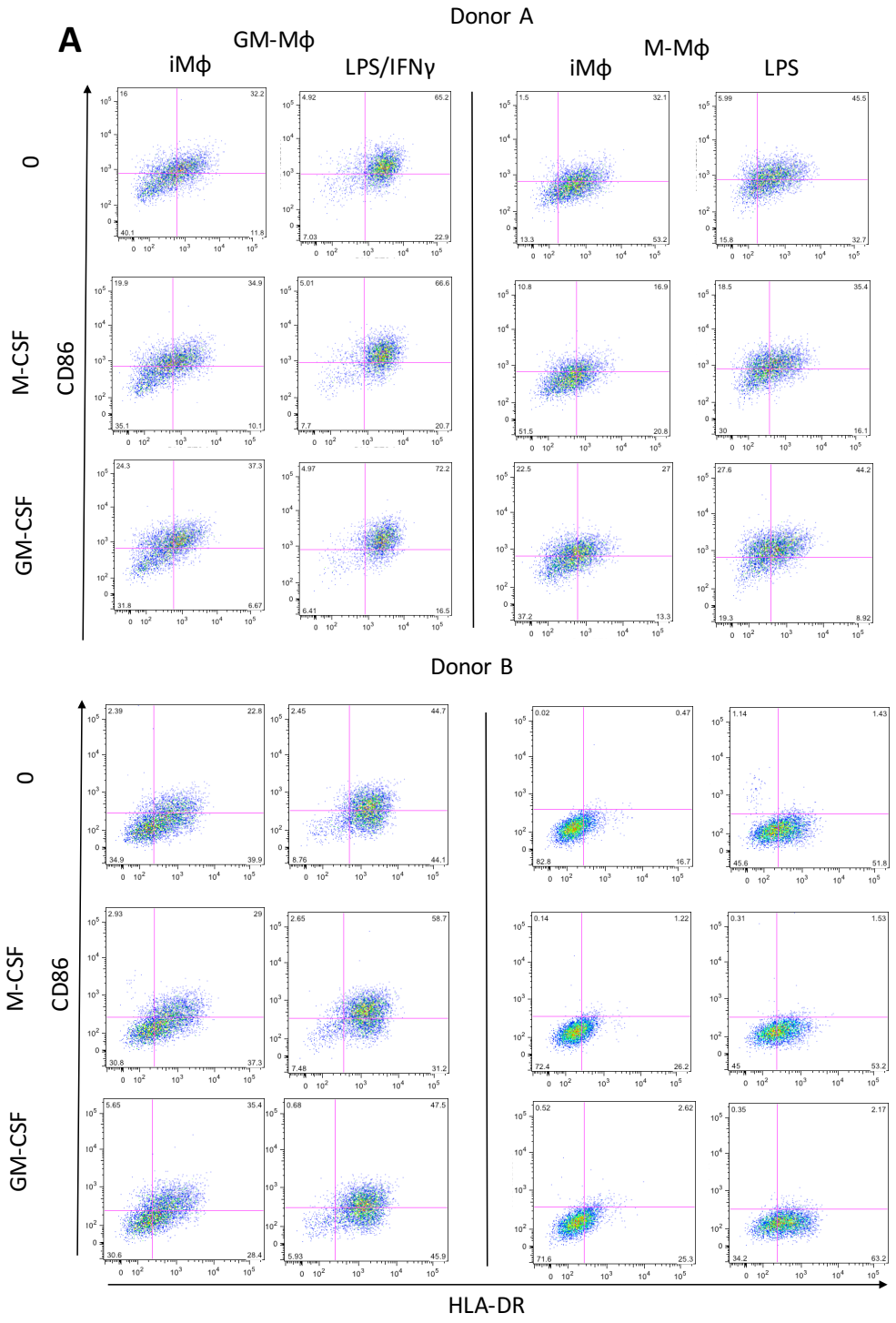


Figure 3.8A. Effect of GM-CSF/M-CSF presence on HLA-DR and CD86 expression by MoMφ. MoMφ were activated or left untreated in the presence or absence of GM-CSF/M-CSF. Cells were collected at 24h, stained with HLA-DR and CD86 antibodies with matched isotype controls and acquired by flow cytometry. Donors A,B

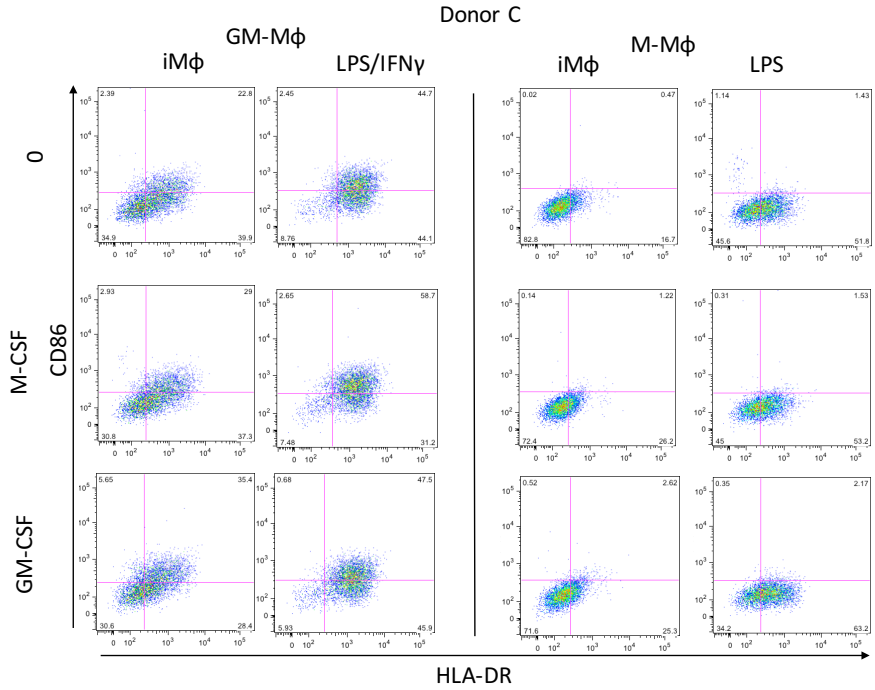


Figure 3.8A. Effect of GM-CSF/M-CSF presence on HLA-DR and CD86 expression by MoMφ. MoMφ were activated or left untreated in the presence or absence of GM-CSF/M-CSF. Cells were collected at 24h, stained with HLA-DR and CD86 antibodies with matched isotype controls and acquired by flow cytometry. Donor C

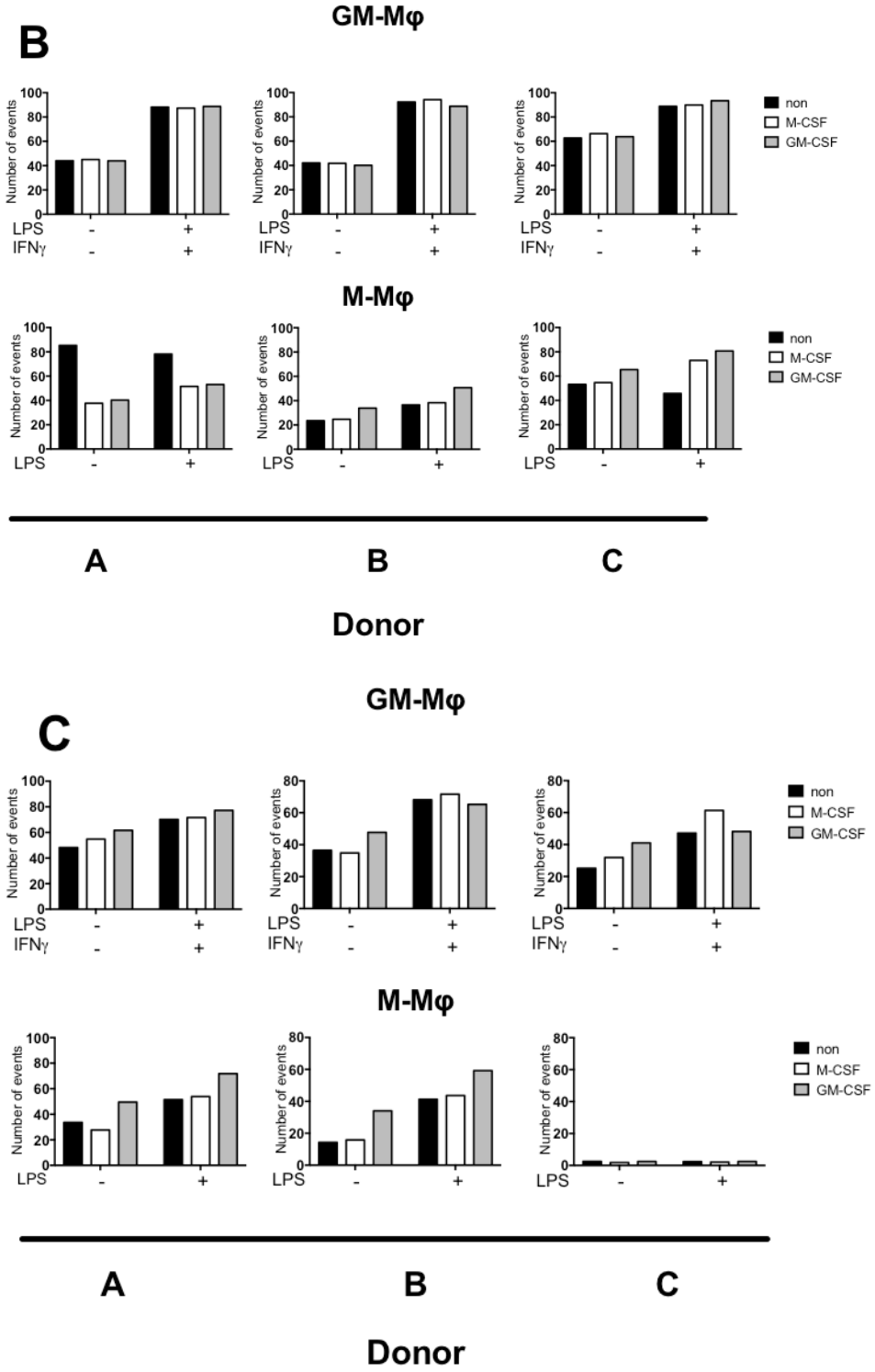


Figure 3.8B, C Effect of GM-CSF/M-CSF presence on HLA-DR and CD86 expression by MoMφ. MoMφ were activated or left untreated in the presence or absence of GM-CSF/M-CSF. Cells were collected at 24h, stained with HLA-DR and CD86 antibodies with matched isotype controls and

acquired by flow cytometry. The summary figures represent the total number of cells positive for HLA-DR (B) and CD86 (C) in 3 donors.

3.2.6 MoMφ phenotype

CD68 is a widely accepted universal Mφ marker therefore, firstly we assessed its expression on MoMφ. Both GM-Mφ and M-Mφ demonstrated similar expression of CD68. (Figure 3.10A). In the majority of published studies, Mφ are characterised as pro-inflammatory Mφ1 and anti-inflammatory Mφ2 types with characteristic phenotypes [124, 154, 574]. In agreement with these reports, generation of MoMφ in the presence of GM-CSF resulted in immature phenotype with no secretion of IL-12, -23, -6, TNFα, IL-1β and IL-10 (Figure 3.9) while expressing medium expression of HLA-DR, CD86 and CD80 (Figure 3.10 B,C). In contrast, there was no expression of CD40. Activation of GM-Mφ with LPS and IFNγ further increased the expression of HLA-DR, CD80 and CD86 and induced the expression of CD40. The secretion of IL-12, -23, -6, IL-1β and TNFα was also induced while there was no or low secretion of IL-10. On the other hand, MoMφ generated in the presence of M-CSF displayed lower expression of HLA-DR and CD86 (Figure 3.10 B,C) and no secretion of IL-12, -23, -6, IL-1β, TNFα or IL-10 consistent with Mφ2 phenotype. Upon stimulation with LPS M-Mφ only modestly increased their expression of HLA-DR and CD86. The expression of CD80 on immature as well as activated M-Mφ was donor dependent varying from lower to comparable to GM-Mφ while CD40 remained negative irrespective of activation. This effect was assisted by induced secretion of IL-10 while IL-12, -23 remained absent or low. The secretion of IL-6, IL-1β and TNFα was induced by stimulation with LPS although remained lower then GM-Mφ.

Resting MoMφ did not express CD83. Activation of GM-Mφ with LPS and IFNγ stimulated low expression (1.5-2 fold increase above isotype control) of CD83 in 4 tested donors while M-Mφ activated with LPS remained negative.

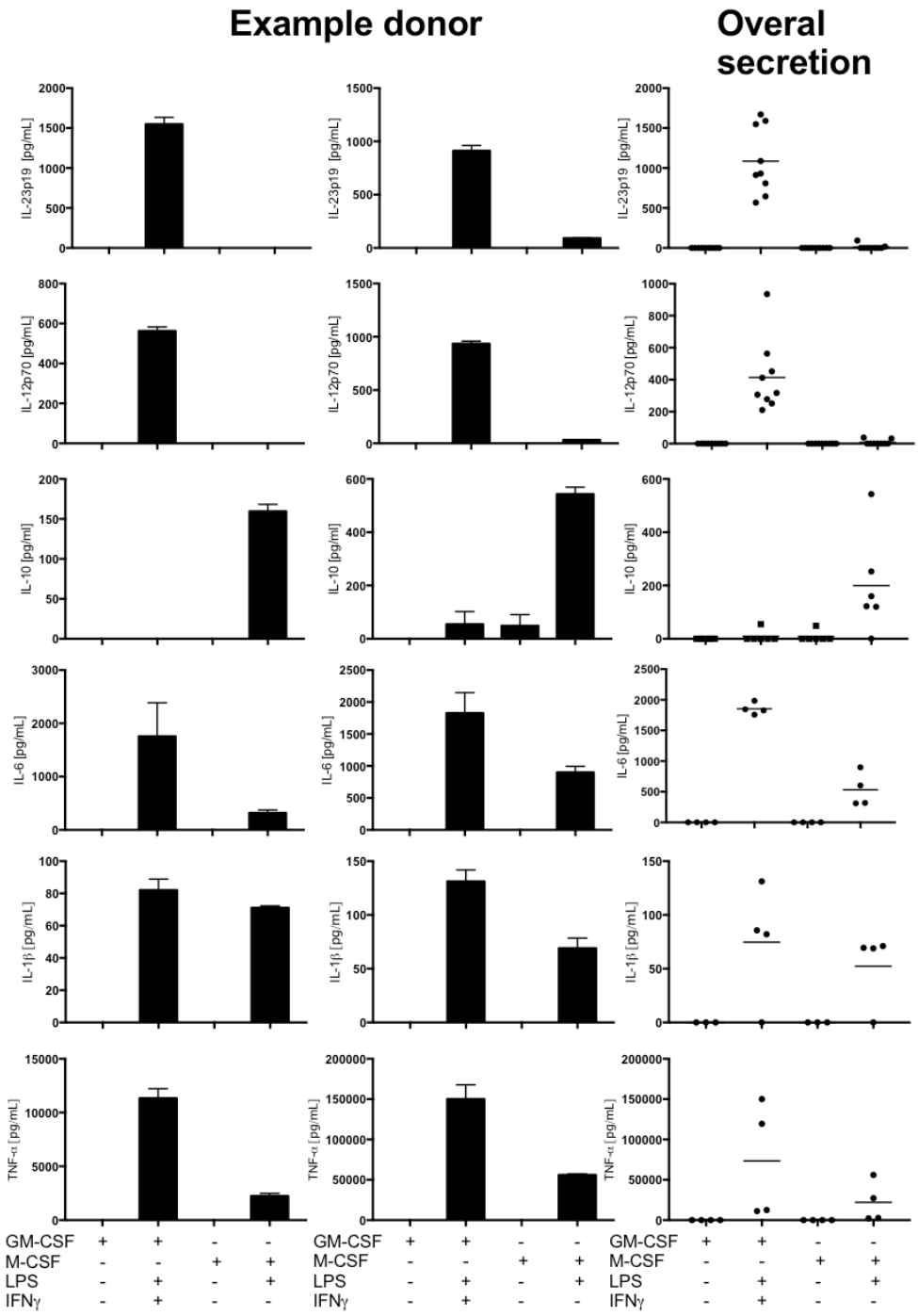


Figure 3.9 Secretion of cytokines by MoMφ. MoMφ were generated in the presence of GM-CSF or M-CSF for 5-7 days and activated with LPS and IFNγ, or LPS alone. Secretion of cytokines was analysed by ELISA in supernatants collected 24h after stimulation. Shown are data from 2 individual

donors (mean +/-SD), and a summary of all donors (n=4) with mean values indicated.

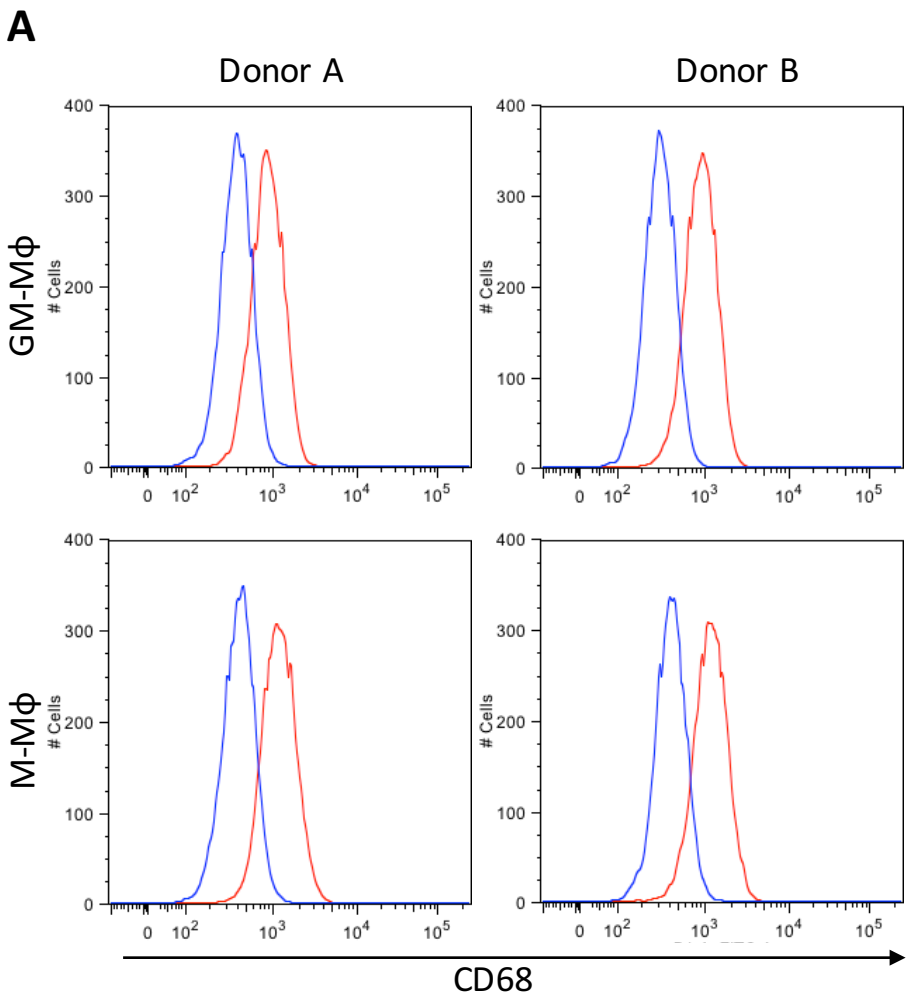


Figure 3.10A Phenotype of Mo-Mφ. **A**- MoMφ were generated in the presence of GM-CSF or M-CSF for 5-7 days. Upon collection cells were washed, fixed and permeabilized. Next cells were stained with CD68 antibodies, resuspended in FACS buffer and acquired by flow cytometry. Shown are two representative donors out of 6. **B, C**- MoMφ resting or activated for 24h were collected by scraping and gentle pipetting and the expression of HLA-DR, CD80, CD86, CD83 and CD40 was measured by flow cytometry. Shown are two representative donors out of 6.

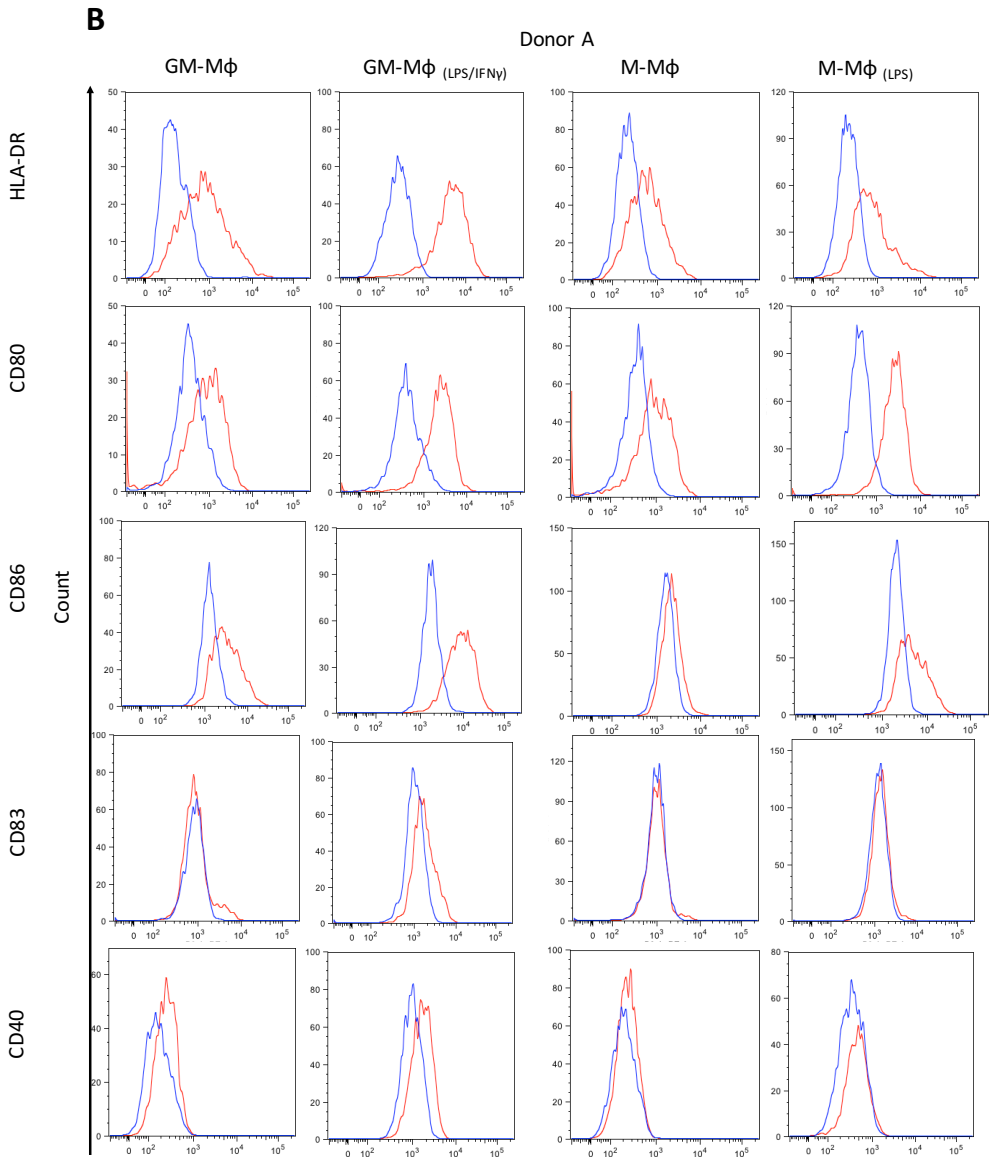


Figure 3.10B Phenotype of Mo-Mφ. **A-** MoMφ were generated in the presence of GM-CSF or M-CSF for 5-7 days. Upon collection cells were washed, fixed and permeabilized. Next cells were stained with CD68 antibodies, resuspended in FACS buffer and acquired by flow cytometry. Shown are two representative donors out of 6. **B, C**- MoMφ resting or activated for 24h were collected by scraping and gentle pipetting and the expression of HLA-DR, CD80, CD86, CD83 and CD40 was measured by flow cytometry. Shown are two representative donors out of 6.

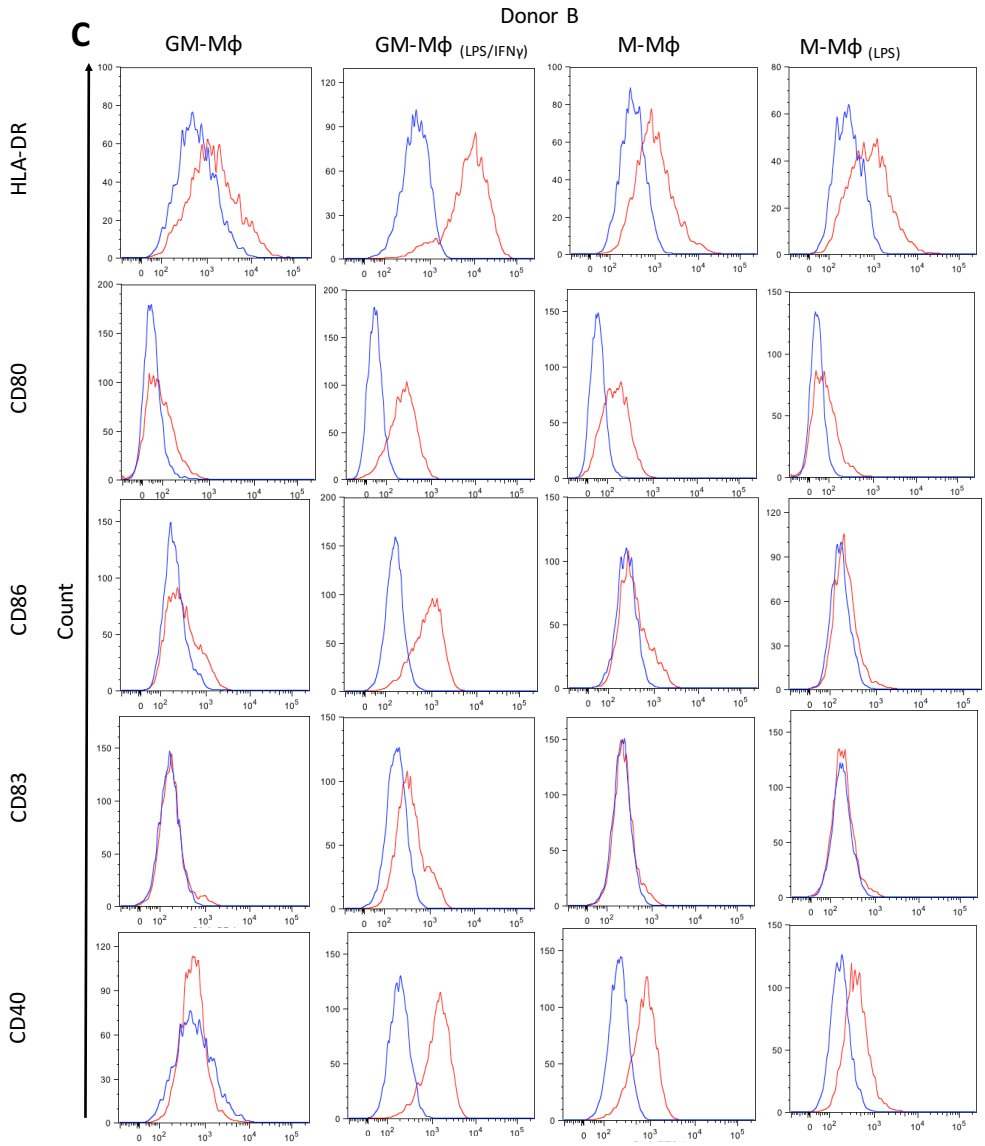


Figure 3.10 C Phenotype of Mo-Mφ. **A**- MoMφ were generated in the presence of GM-CSF or M-CSF for 5-7 days. Upon collection cells were washed, fixed and permeabilized. Next cells were stained with CD68 antibodies, resuspended in FACS buffer and acquired by flow cytometry. Shown are two representative donors out of 6. **B, C**- MoMφ resting or activated for 24h were collected by scraping and gentle pipetting and the expression of HLA-DR, CD80, CD86, CD83 and CD40 was measured by flow cytometry. Shown are two representative donors out of 6.

3.3 Discussion

Differentiation of Mo into Mφ *in vivo* and *in vitro* can be affected by many exogenous stimuli resulting in a tissue- and condition-specific final Mφ phenotype. These stimuli include TLR ligands, cytokines, cell-cell contact and

surface attachment [144, 576-578]. The protocols for generating MoMφ vary greatly between laboratories with some utilising low-attachment plastic surfaces while others using normal-attachment plates or flasks [141, 143]. Since Mφ strongly adhere to the surface and can be damaged during collection for further experiments, the low-attachment containers provide researchers with distinct advantages. However, cell contact and surface attachment provide important signals during differentiation of Mo to Mφ in tissues which affect Mφ phenotype and activation status [576, 577].

Comparing morphology and cytokine secretion of MoMφ generated in normal and low-adherence plates provided an initial description of the effect of different surfaces on Mφ behaviour. Normal adherence vessels are usually made from treated polystyrene which becomes hydrophilic and negatively charged once medium is added [579, 580]. This allows for cell attachment proteins (vitronectin and fibronectin) from serum in culture medium to adhere to the bottom of the plate, providing a better surface for cells to attach. Furthermore, plate surface is usually coated with extracellular matrix or attachment and adhesion proteins, such as collagen, laminin and fibronectin [581]. On the other hand, low attachment can be achieved by using untreated polystyrene culture vessels due to their hydrophobic properties [579]. However, there can be still certain amount of binding through hydrophobic interactions as well as in certain cell types. In 1990s Corning® introduced Ultra-Low Attachment plates coated with hydrophilic and neutrally charged hydrogel layer [579]. Since attachment is mediated through either hydrophobic or ionic interactions, this hydrogel surface naturally inhibits cell

attachment. Furthermore, this hydrogel surface is biologically inert and prevents the activation of macrophages [578]. Typically, MoMφ generated in the presence of GM-CSF display a characteristic “fried-egg” morphology in microscopic examination while MoMφ generated with M-CSF are presented as “spindle-shaped “cells [144]. Our results confirmed these findings in normal-attachment plates. Interestingly M-Mφ generated in low-attachment plates adopted “fried-egg” shape so distinctive for GM-Mφ. The difference of Mφ morphology depending on attachment surface may suggest the dependency of especially M-Mφ on the additional signalling from the surface to perform specific functions.

The comparison of cytokine secretion between MoMφ generated in normal vs low-adherence plates showed higher IL-12 secretion in low-attachment plates in 2 out of 3 donors. The reason for decreased IL-12 secretion in normal-attachment plates may be the inadvertent damage of cells during de-attachment procedure. However, higher levels of IL-12 secretion may be caused by more pronounced activation status in cells generated in low-attachment plates and allowed to attach immediately before activation in comparison to cells attached throughout generation and activation. Schakel et al. demonstrated that slan-DC respond with higher levels of IL-12 secretion when cultured for 3h in vitro before activation and hypothesised that leucocytes may require this short period of maturation in vitro before fully responding to LPS [189]. It may be similarly hypothesised that MoMφ which were attached during their generation process may have partially lost their sensitivity to attachment signalling which may amplify LPS activation.

Next, we investigated whether MoMφ generated in the presence of GM-CSF or M-CSF display a stable phenotype or require constant influx of these haematopoietic cytokines from their environment. *In vivo,* Mφ are constantly exposed to low-levels of M-CSF which is required to maintain Mφ viability and promotes Mφ2-like polarisation state [582-585]. GM-CSF is also involved in Mφ viability but its levels raise during inflammatory conditions and it skews Mφ phenotype towards Mφ1 [583, 584]. M-CSF is constitutively expressed by Mφ as well as induced by factors including GM-CSF while the secretion of GM-CSF requires activation with additional stimuli like TNFα or LPS. [109, 582]. Therefore, both cytokines may be involved in an autocrine loop maintaining Mφ viability and polarisation We have shown that short-term (24h) withdrawal of M-CSF or GM-CSF from cultures of differentiated MoMφ did not affect their viability nor phenotype which supports this hypothesis. It is possible that their phenotype is maintained short term via innate memory mechanisms or it may be maintained via autocrine secretion of M-CSF and GM-CSF [332, 586]. Assessment of M-CSF and GM-CSF secretion by MoMφ is needed however for confirmation.

In is important to notice that these studies are limited by the use of a single type of activation and polarisation stimuli and short cell culture following activation (24h). Mφ are long living cells and further work will expand our findings on different types of stimulation and longer culture times as well as will look in more detail on the impact of differentiation method on Mφ response to activation.

Chapter 4 Macrophage plasticity in response to environmental factors

4.1 Introduction

Mφ play a key role in orchestrating immunity and the maintenance of homeostasis including neovascularisation, tissue repair and restoration of pathogen-disturbed homeostasis [98]. In order to perform these functions, Mφ must have the ability to respond efficiently to changes in their microenvironment therefore, extreme plasticity is one of the hallmarks of Mφ family [587]. In many tissues Mφ acquire a long-term tissue-specific phenotype during homeostasis. However, as immune sentinels, Mφ can reprogram shifting their activation states towards the desired point in continuum of Mφ1 or Mφ2 phenotypes in response to environmental signals [588, 589].

A wide network of signalling pathways, transcriptional factors and epigenetic alterations is involved in Mφ plasticity. Transcriptional analysis performed by Martinez *et al*. on MoMφ generated in the presence of M-CSF and polarised with LPS/IFNγ towards Mφ1 or IL-4 towards Mφ2 demonstrated that both types of polarisation had a wide-spread effect on the whole transcriptome [590]. Mφ1 polarisation affected 2053 (5.2%) transcripts in total while Mφ2 polarisation affected 104 (~0.3%) transcripts. Although Martinez *et al*. concluded that Mφ1 polarisation is connected with more dramatic changes in the transcriptome than Mφ2 polarisation it is important to notice that the MoMφ used in this report were initially generated in the presence of M-CSF which already provided some Mφ2 "conditioning". Therefore, the activation

with IL-4 strengthened Mφ2 polarisation while stimulation with LPS/IFNγ could lead to reprogramming of the original pro-Mφ2 state towards Mφ1. It is therefore plausible that the MoMφ generation method used in this research may be responsible for the difference in the number of affected transcripts.

It has been proposed that Mφ are constitutively exposed to steady levels of M-CSF which maintains them in Mφ2-like state [582, 584]. During inflammation, the levels of GM-CSF promote the switch to pro-inflammatory Mφ1 [584]. In many situations Mo may differentiate into Mφ under the influence of one or both cytokines which may result in differential initial phenotypes [584]. For example, both GM-CSF and M-CSF raise in RA [584]. These initial pro-Mφ1/pro-Mφ2 phenotypes may have profound effect on later ability of Mφ to respond to pro- or anti-inflammatory activators. The importance of GM-CSF and M-CSF in the initial Mφ1/Mφ2 polarisation of transcriptome profiles is well demonstrated in a paper by Lacey *et al.* [583]. Go analysis of genes expressed by MoMφ generated in the presence of M-CSF or GM-CSF showed differential bi-polar expression pattern of genes involved not only in immune responses but also in locomotion, receptor activity, adhesion and endocytosis. Interestingly, the polarisation status received by generation of Mφ in M-CSF or GM-CSF could be completely reversed when Mφ were further cultured with opposite cytokines: the M-Mφ in GM-CSF and the GM-Mφ in M-CSF for the same amount of time [591]. These findings were put in a wider perspective by a report by Xue *et al.* [592] in which more diverse Mφ1/Mφ2 stimulation as well as stimuli not linked to Mφ1/Mφ2 polarisation like free fatty acids were used. This research showed that

clustering Mφ1 or Mφ2 stimuli together strengthened the bipolar polarisation of MoMφ but did not change the overall Mφ1/Mφ2 axis. On the other hand, addition of stimuli not linked with Mφ1/Mφ2 polarisation resulted in a spectrum of activation signatures beyond the initial bi-polar axis. These results correspond better to the picture *in vivo* where Mφ represent a continuum of activation states often expressing markers associated with Mφ1 and Mφ2 simultaneously [106].

The expression of both Mφ1 and Mφ2 markers may indicate simultaneous co-activation with Mφ1/Mφ2 factors or shifting from one phenotype to another in changing environment for example during resolution of inflammation or wound healing [588]. Smith *et al*. tried to address both situations by treating BMMφ with simultaneous or consecutive mixture of Mφ1(LPS+IFNγ) and Mφ2 (IL-4, IL-13) stimuli and assessing the expression of Mφ1/Mφ2 markers such as CD86 and CD206 [588]. Their data showed that Mφ exposed to a combination of Mφ1/Mφ2 activating molecules express both Mφ1 and Mφ2 markers however, the expression of Mφ1 markers was lower in mixed conditions then in pure Mφ1 stimulation. In addition, the Mφ1 activation lasted shorter and cells activated with mixed cytokines progressed towards Mφ2-like state. Similarly, consecutive exposure of BMMφ to Mφ1/Mφ2 stimuli did not inhibit the original phenotype but resulted in a mixed activation state with expression of both the original as well as new markers. The initial polarisation towards Mφ1 enhanced the expression of Mφ2 markers upon re-polarisation of Mφ1 towards Mφ2. Interestingly, cells initially polarised towards anti-inflammatory Mφ2 with IL-4/IL-13 required higher levels of

LPS/IFNγ stimulation to express Mφ1 markers, therefore demonstrating certain resistance to reprogramming. Although this report would suggest that Mφ are naturally more prone to anti-inflammatory phenotype, the conditions used for BMMφ generation need to be considered. Similarly, to a report by Martinez *et al*. mentioned earlier [590] this research was performed on Mφ generated for 7 days in the presence of M-CSF possibly skewing their original polarisation status towards Mφ2. Other researchers reported that primary murine Mφ pre-treated with Il-4 or IL-13 enhance their secretion of pro-inflammatory cytokines such as IL-12 and TNFα when subsequently challenged with pro-inflammatory stimuli like LPS or Poly:I C [593-595]. However, there was no effect when Mφ were subsequently activated with a combination of IFNγ and IL-2 [593]. Therefore, the effect of IL-4 on cultured primary murine Mφ is pleiotropic and depends on the initial state of the Mφ as well as the type of subsequent pro-inflammatory stimuli. Mφ primed with IL-4 or IL-13 are not the only type of Mφ2 which can be re-programmed towards pro-inflammatory phenotype. *In vivo* delivery of IL-12 to tumour sites in mice induced pro-inflammatory cytokine secretion like TNFα by TAMφ while at the same time reducing their production of anti-inflammatory IL-10 [596]. Similarly, IFNγ-treated TAMφ from ovarian cancer switched to Mφ1 phenotype [597].

The plasticity of Mφ makes them a very attractive target for therapeutic immunomodulation for treatment of pathologies which involve Mφ dysregulation like cancer, autoimmune disorders and transplant rejection. Recent advances in understanding Mφ plasticity and the molecular

mechanisms governing Mφ polarization provided researchers with tools to potentially repolarise Mφ towards pathology-resolving phenotype. Such strategy uses the cells which are already present at the location in large number to change the environment [598]. This can confer a great advantage over strategies trying to simply eliminate these cells through blocking their recruitment or suppressing their survival. In addition, all three methods can be used in combination to achieve the best results. "Re-education" of TAMφ present in murine ovarian cancer by targeting NFκβ signalling resulted in the induction of tumouricidal and anti-tumour IL-12 dependent activity therefore reversing their tumour-mediated polarisation [599]. Several agents including TLR agonists such as LPS (TLR4), R848 and Imiquimod (TLR7) are known activators of NFkB pathways and can be used in establishment of Mφ1-type tumour-suppressing Mφ. Synthetic LPS-analogues such as 1-8 only 3-O-desacyl-4'-monophosphoryl lipid A (MPL) have been used as cancer vaccine adjuvants [600]. Similarly, topical Imiquimod therapy polarized TAMφ present in cutaneous squamous cell carcinoma towards Mφ1 phenotype [601]. As an example of Mφ2 induction *Schistosoma mansoni*–derived soluble egg Ag (SEA) reprogrammed Mφ1 towards Mφ2 reducing murine atherosclerosis [602].

It is often assumed that Mφ are characterized by infinite plasticity and the ability to freely change between pro-inflammatory and anti-inflammatory states [591]. However, as was suggested before, the initial conditions of Mφ generation and activation can exert a profound and long-term effect on repolarized Mφ. For example, Mφ pre-activated with IL-4 and further

stimulated with LPS/IFNγ can express both Mφ1 and Mφ2 markers. Certain restrictions in the degree of Mφ plasticity were demonstrated by Franklin *et al*. who showed that TAMφ in mammary carcinoma were not generated by conversion of resident mammary tissue Mφ but form a distinctive population discrete from resident mammary tissue Mφ [93, 94]. The lack of Mφ plasticity was reported in COPD where Mφ1-like Mφ persisted in tissue and were unable to switch upon receiving environmental signals [603]. Interestingly gene analysis showed transcriptional changes in Mφ from COPD patients resulting in a loss of inflammatory response consisted with a depletion in the IFNγ linked module [592]. These findings may explain the lack of effectivity of anti-inflammatory treatments in COPD. A better understanding of Mφ plasticity, its degree and mechanisms can provide us not only with improved tools to influence their phenotype but also allow us to choose the most appropriate way for environment immunomodulation through changing Mφ phenotype when it is appropriate, reducing Mφ numbers if it is impossible to sufficiently change their phenotype or combining both strategies together.

In this study, we addressed the hypothesis that Mφ plasticity is restricted by their initial environmental conditioning. We show that Mφ generated in the presence of GM-CSF secrete higher levels of IL-12 and IL-23 and lower IL-10 in response to LPS and IFNγ comparing to Mφ generated with M-CSF. Subsequent repolarisation of GM-Mφ in the presence of M-CSF or M-Mφ in the presence of GM-CSF had little effect on their secretion of IL-23 and IL-10 as well as the expression of HLA-DR and CD86. Furthermore, we also looked at Mφ behaviour in response to reactivation. Primary activation of Mφ with

LPS/ IFNγ influenced their response to secondary activation by reducing IL-23 and IL-10 secretion. Interestingly while treatment of Mφ with IFNγ alone during primary activation increased IL-23 secretion upon secondary activation it also triggered IL-10 secretion. In addition, primary activation with IFNγ resulted in a stable high expression of both CD86 and HLA-DR regardless of further stimulation. However, this effect was abrogated by combining IFNγ with LPS during primary activation. Finally, we addressed the effect of TLR stimulation on Mφ polarisation. We show that LPS often portrayed as pro-Mφ1 stimulus [109, 604] acts as a universal activation factor co-operating with pro-Mφ1(IFNγ) and pro-Mφ1 (IL-4) inducers to stimulate cytokine secretion by Mφ. In contrast, triggering of TLR7/8 with R848 resulted in a mixed Mφ phenotype characterised by high secretion of IL-12, IL-23 and IL-10. Furthermore, the effect of R848 was more pronounced in M- Mφ comparing to GM-Mφ. Importantly, although combination of R848 with IFNγ increased IL-12 and decreased IL-10 secretion, there was no effect on IL-23. Overall our results provide better understanding of Mφ polarisation in response to their environment and may provide new ways to manipulate Mφ behaviour for novel immunotherapeutic strategies for cancer, autoimmune disorders and transplant rejection.

4.2 Results

4.2.1 Effect of LPS and IFNγ on GM-Mφ and M-Mφ

Signalling through IFNγ receptor provides a very strong pro-Mφ1 signal able to repolarise many features of anti-inflammatory Mφ to a more pro-inflammatory phenotype [597]. Moreover, M-Mφ activated with LPS and

IFNγ are often used as a representative for Mφ1 [597]. However, a body of evidence suggests that original programming of Mφ caused by M-CSF or GM-CSF may influence the final phenotype of Mφ after activation. Therefore, we hypothesized that Mφ generated in the presence of GM-CSF or M-CSF will display differential pro-inflammatory and regulatory properties when activated in the presence of IFNγ. We focused on the secretion of pro-inflammatory IL-12 and IL-23, and regulatory IL-10 to differentiate inflammatory Mφ1 and immuno-regulatory Mφ2. IFNγ was necessary for the secretion of IL-12 and IL-23 by GM-Mφ and M-Mφ activated with LPS and there was no secretion in the presence of LPS alone (Figure 4.1, Supplementary material Figure S1A-B). Both activating factors cooperated with each other and the production of both IL-12 and IL-23 increased proportionally to increased dose of LPS and IFNγ. Secretion of IL-12 and IL-23 increased proportionally to the increase in LPS dose reaching maximum levels at 5μg and could only be increased by increase in IFNγ dose. IL-12 was secreted in a dose-dependent manner whilst IL-23 reached a maximum level at 1,000IU of IFNγ for GM-Mφ and 10,000IU for M-Mφ.

There was variability between GM-Mφ and M-Mφ with regards to their IFNγ response. Overall, there was 21.1% reduction in IL-12 secretion by M-Mφ as compared to GM-Mφ at the standard activation dose (Table 4.1). However, there was donor to donor variability. While one donor (A) showed a doubling in IL-12, three retained between 70-85%, and in the remaining two donors IL-12 was reduced to 34 and 15% (Figure 4.1, Table 4.1, Supplementary material Figure S1A-B). IL-12 secretion was mostly affected at higher doses of IFNγ

and LPS and was similar in GM-Mφ and M-Mφ stimulated with lower. Even more pronounced differences were observed in IL-23 secretion from GM-Mφ and M-Mφ. All donors exhibited reduced IL-23 responses to LPS/IFNγ by M-Mφ regardless of the dose of IFNγ or LPS used for activation. While one donor retained 60% of IL-23 secretion (compared to GM-Mφ), three retained 23-39 % while two failed to secrete IL-23.

The secretion of IL-10 differed between MoMφ generated in the presence of GM- or M-CSF (Figure 4.1, Supplementary material Figure S1C, Table 4.1). IL-10 secretion by M-Mφ was proportional to LPS doses up to 5μg after which it remained the same or was reduced. As expected it was inversely proportional to IFNγ dose. GM-Mφ did not secrete IL-10 upon stimulation with LPS alone or with the addition of 10 000IU/mL IFNγ. Surprisingly two donors readily secreted IL-10 when LPS was combined with intermediate doses of IFNγ. IL-10 secretion reached maximum at 5μg/mL of LPS. In summary, although IFNγ provided a strong pro-inflammatory signal there were significant differences between GM-Mφ and M-Mφ with regards to cytokine production.

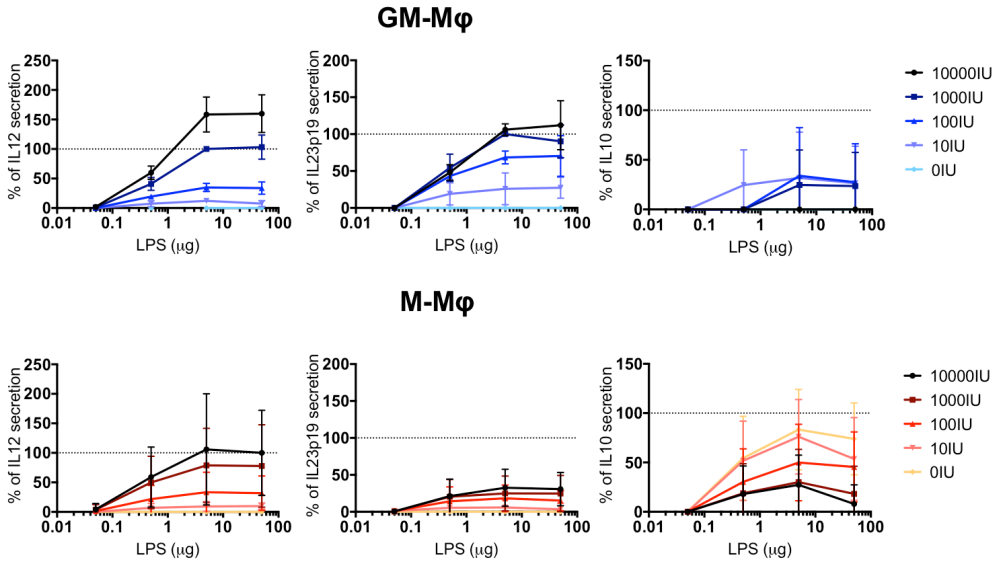


Figure 4.1 Effect of LPS and IFNγ dose on cytokine secretion by MoMφ. MoMφ from 6 donors were activated with increasing doses of LPS and IFNγ. The secretion of IL-12p70, IL-23 and IL-10 were measured at 24h by ELISA and is expressed as a percentage of cytokine secretion as comparing to secretion of GM-Mφ activated with 5μg LPS/1000IU IFNγ (100%-dotted line) for IL-12 and IL-23 and M-Mφ activated with 5μg LPS (100% - dotted line) for IL-10. Shown is mean + SD of singlicate stimulation of 6 donors for each condition.

Donor	IL-12	IL-23	IL-10
A	195.34	60.93	0
B	15.67	0	0
C	75.81	39.96	0
D	70.98	25.48	0
E	34.017	23.39	0
F	81.61	0	0
Overall	**78.90**	**24.96**	**0**

Table 4.1 Effect of Mφ type on the cytokine secretion in response to LPS and IFNγ. Summary table showing percentage secretion of IL-12p70 and IL-23 by M-Mφ activated with standard dose of LPS/IFNγ (5μg LPS/1,000IU IFNγ) as comparing to GM-Mφ and IL-10 secretion by GM-Mφ activated with LPS only as comparing to M-Mφ (5μg LPS) with same dose of LPS.

4.2.2 Effect of IFNγ vs IL4 on MoMφ

After establishing the impact of IFNγ on MoMφ we assessed whether other T-cell cytokines also impacted on Mφ polarity. The major Th2 cytokine, IL-4, polarises Mφ towards Mφ2a type characterised by high IL-10 and low IL-12 and IL-23. Therefore, we compared the effect of IFNγ and IL-4 on MoMφ. Mφ generated in the presence of GM-CSF or M-CSF were activated with LPS combined with IFNγ or IL-4 and the secretion of IL-12, IL-23 and IL-10 determined. In accordance with previous results LPS alone did not elicit the secretion of IL-12, IL-23 and IL-10 from GM-Mφ and IL-12 and IL-23 from M-Mφ while it stimulated IL-10 secretion by M-Mφ (Figure 4.2). Addition of IFNγ induced IL-12 and IL-23 by both GM-Mφ and to a lesser degree M-Mφ while it also induced IL-10 secretion in GM-Mφ from 2/5 donors. On the other hand, IFNγ reduced slightly although not significantly IL-10 secretion by M-Mφ comparing to activation with LPS alone. Activation of Mϕ with IL-4 alone did not elicit cytokine secretion (data not shown). The addition of IL-4 to LPS did not elicit IL-12 or IL-23 from GM-Mφ and M-Mφ. IL-4 together with LPS induced the secretion of IL-10 by GM-Mφ (3/5 donors) at levels comparable to activation with LPS / IFNγ ($p>0.05$). On the other hand, the combination of IL-4 and LPS in M-Mφ resulted in a high IL-10 which was significantly higher than from activation with LPS alone or LPS/IFNγ ($p<0.05$).

In summary LPS presented a dichotomy in its properties; exacerbating pro-inflammatory effects of IFNγ and anti-inflammatory effects of IL-4 and M-CSF while remaining silent in combination with pro-Mφ1 signal, GM-CSF. IFNγ acted as pro-Mφ1 signal and partially repolarised M-Mφ to pro-

inflammatory phenotype. IL-4 cooperated with two other pro-Mφ2 signals (LPS and M-CSF) to promote IL-10. Its effect in the presence of GM-CSF was less pronounced however considering the induction of IL-10 with lack of secretion of IL-12 or IL-23 it showed certain ability to repolarise GM-Mφ towards Mφ2.

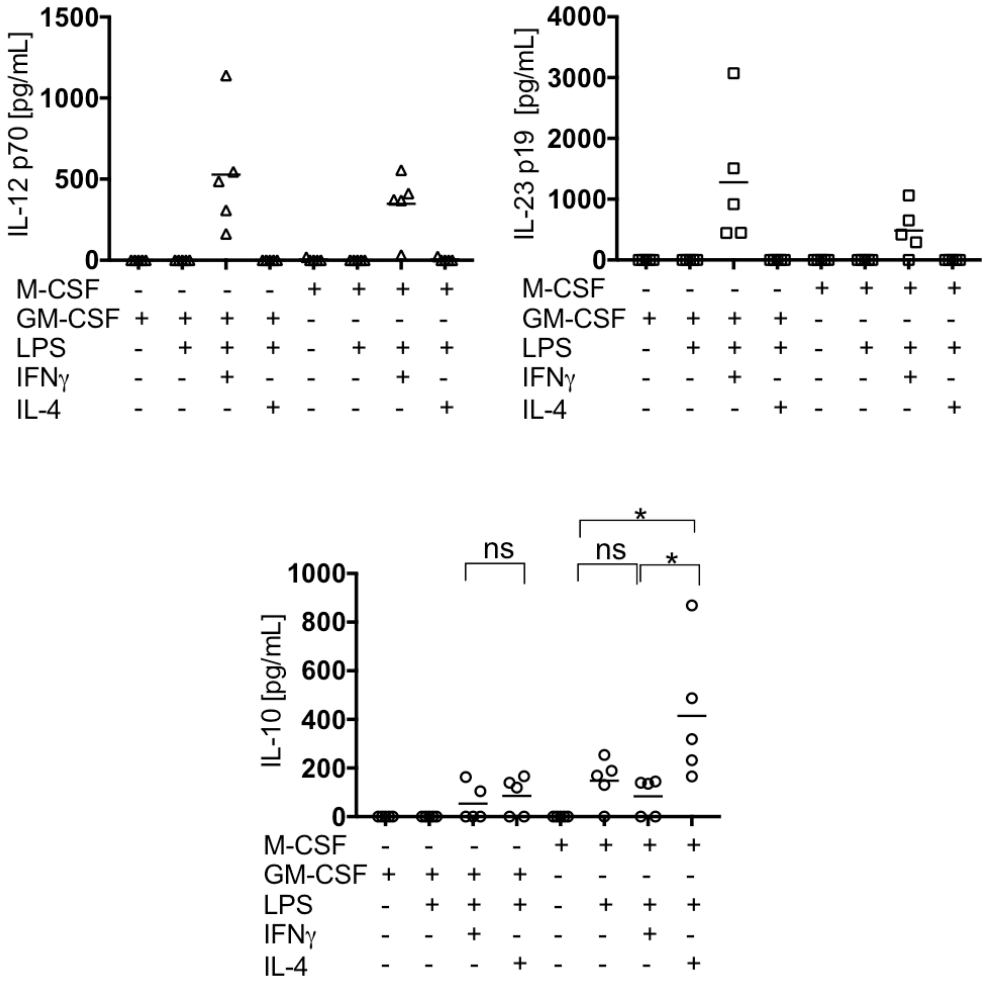


Figure 4.2 Effect of IFNγ and IL-4 on cytokine secretion by MoMφ. MoMφ were activated with LPS in the presence or absence of IFNγ and IL-4 and secretion of IL-12p70, IL-23 and IL-10 was measured at 24h by ELISA. Graphs show collective data from 5 tested donors. Shown is mean of triplicate stimulation for each donor for each condition with a line at mean for a group. Statistically significant differences were determined by the unpaired student's t-test.

We evaluated the expression of co-stimulatory molecules by Mo-Mφ. As described before unstimulated GM-Mφ displayed higher expression of HLA-DR and CD86 comparing with M-Mφ. CD86 expression, although low on both types of resting Mφ was nevertheless slightly higher on M-Mφ. CD83 remained negative and CD40 was low (2-fold increase over isotype control) on both M-Mφ and GM-Mφ.

Activation of Mφ with LPS and IFNγ resulted in enhanced expression of all tested markers (Figure 4.3A-C). IFNγ alone lead to a similar increase of the expression of CD86 by GM-Mφ and CD40 by both GM-Mφ and M-Mφ. The expression of HLA-DR and CD80, by both types of Mφ as well as CD86 by M-Mφ stimulated with IFNγ alone was lower than in combination with LPS. Furthermore, LPS alone induced higher expression of CD86 comparing to IFNγ alone.

Treatment of Mφ with IL-4 resulted in increased levels of all markers tested. IL-4 alone and in combination with LPS stimulated similar expression of HLA-DR by GM-Mφ as IFNγ alone. Interestingly, while IL-4 alone resulted in HLA-DR expression by M- Mφ similar to IFNγ together with LPS and higher than IFNγ alone, the addition of LPS to IL-4 reduced HLA-DR expression (~14-fold MFI for IL-4 alone versus ~ 8-fold MFI for IL-4 with LPS). Furthermore IL-4 induced the highest expression of CD86 by both GM-Mφ and M-Mφ. The expression of CD80 was higher in cells treated with IL-4 in the presence of LPS than with IL-4 alone however lower than in cells induced with IFNγ with LPS.

Interestingly cells expressed low level of CD83 under the influence of IL-4 suggesting the possibility of differentiation towards DC. In conclusion, both T-cell cytokines promote the expression of co-stimulatory molecules and therefore the Mφ-T-cell cross-talk at the same time directing specificity in Mφ responses. This effect is further reinforced by LPS.

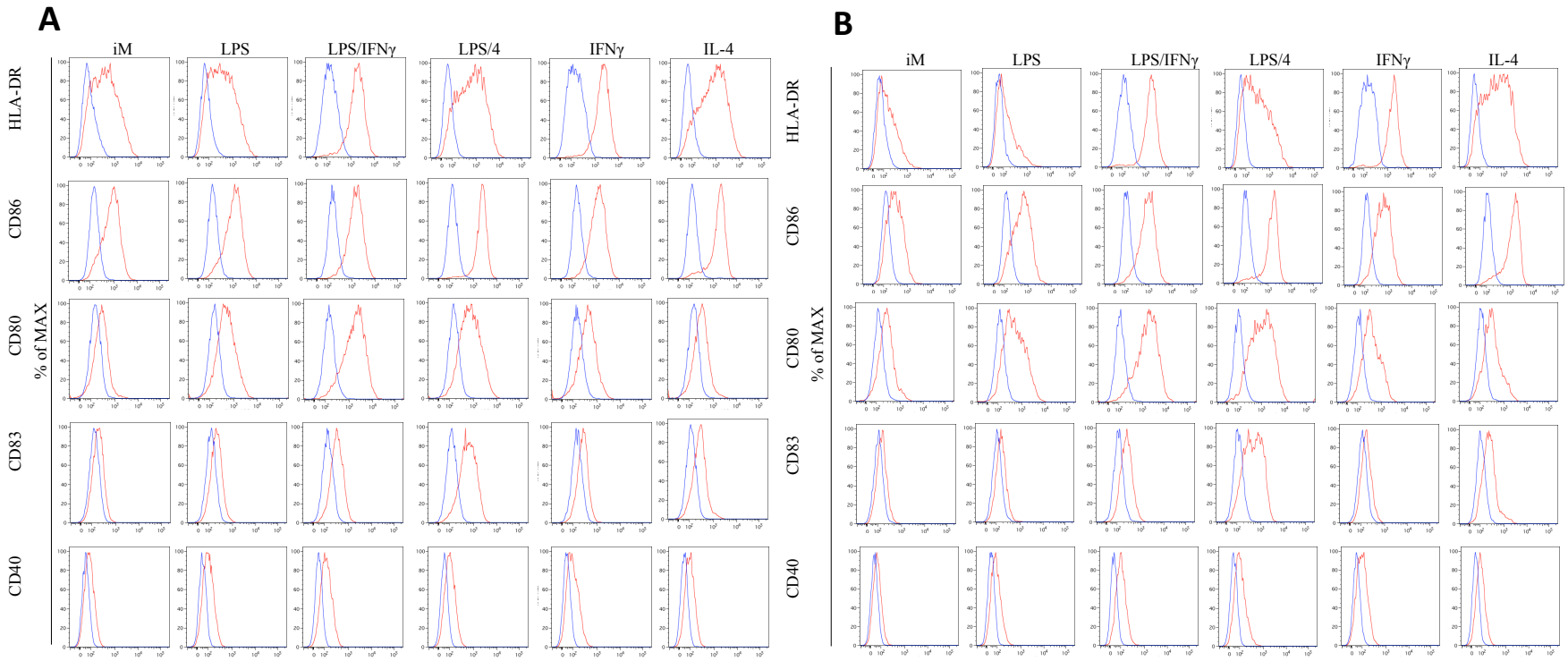


Figure 4.3A, B Effect of IFNγ and IL-4 on the expression of co-stimulatory molecules by MoMφ. MoMφ were activated with LPS in the presence or absence of IFNγ and IL-4. After 24h cells were collected by trypsinisation and stained with HLA-DR, CD86, CD80, CD83 and CD40 antibodies and matching isotype controls for flow cytometry. **A, B**- Histograms show the expression of co-stimulatory molecules by GM-Mφ (**A**), and M-Mφ (**B**) of one of 4 tested donors. **C**-Summary figure showing normalised MFI fold expression of co-stimulatory molecules in 4 donors. Shown is mean + SD of singlicate stimulation of 4 donors for each condition.

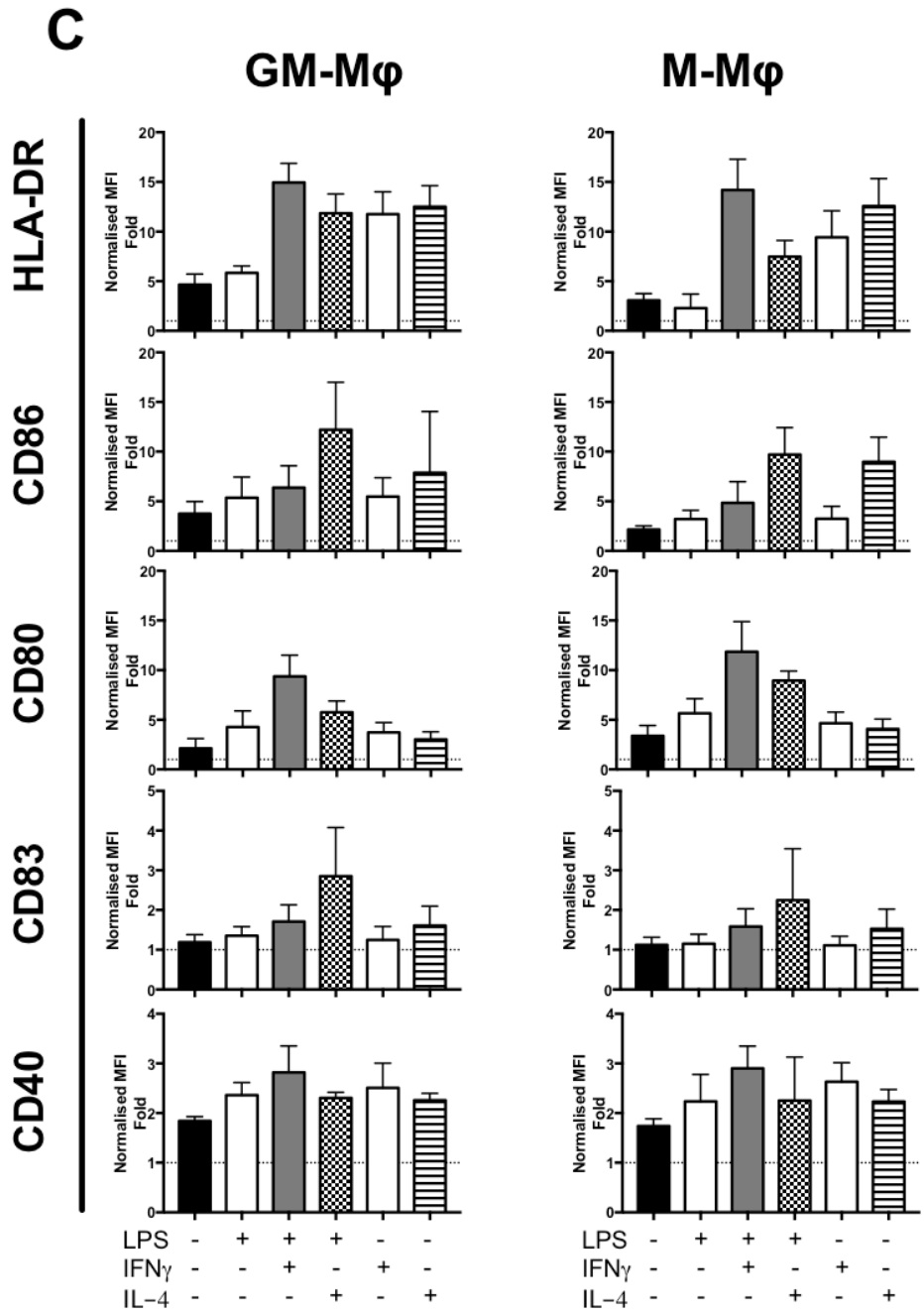


Figure 4.3C Effect of IFNγ and IL-4 on the expression of co-stimulatory molecules by MoMφ. MoMφ were activated with LPS in the presence or absence of IFNγ and IL-4. After 24h cells were collected by trypsinisation and stained with HLA-DR, CD86, CD80, CD83 and CD40 antibodies and matching isotype controls for flow cytometry. **A, B-** Histograms show the expression of co-stimulatory molecules by GM-Mφ (**A**), and M-Mφ (**B**) of one of 4 tested donors. **C-**Summary figure showing normalised MFI fold expression of co-stimulatory molecules in 4 donors. Shown is mean + SD of singlicate stimulation of 4 donors for each condition.

4.2.3 Effect of switching polarisation signals on MoMφ behaviour

Previous experiments established the importance of initial GM-/ M-CSF polarisation on the final phenotype of activated Mφ. Our next question was whether repolarising of Mφ with opposite cytokine –culture of M-Mφ in GM-CSF and GM-Mφ in M-CSF - could reverse their initial polarisation and therefore influence the final activated phenotype (Figure 4.4) as reported earlier by Xu *et al.* with regards to IL-6 and TNFα secretion [591].

Repolarisation of GM-Mφ with M-CSF had little effect on the secretion of IL-23 and IL-10 regardless of stimulation protocol (Figure 4.5 A, B). Similarly, GM-CSF had little effect on IL-23 secretion by M-Mφ but decreased IL-10 levels. Pre-stimulation with LPS, alone or in combination with IFNγ, attenuated IL-23 secretion and significantly decreased IL-10 during secondary activation. IFNγ increased IL-23 levels in both GM-Mφ and M-Mφ. However, it also stimulated IL-10 release from both GM-Mφ and M-Mφ with the exception of GM-Mφ cultured further with GM-CSF during secondary polarisation.

The expression of CD86 and HLA-DR by Mφ did not differ significantly regardless of whether their phenotype was reinforced by secondary polarisation with the same cytokine or they were repolarised (Figure 4.6A-F). Pre-stimulation with IFNγ resulted in a stable high expression of both CD86 and HLA-DR regardless of further stimulation. This effect was abrogated when IFNγ was combined with LPS and there was a requirement for the presence of IFNγ during final stimulation for enhanced expression of both

markers. Furthermore, pre-stimulation of Mφ with LPS alone resulted in increased expression of HLA-DR and again IFNγ was required during final stimulation for the increase in CD86 expression. These results demonstrate that, in contrast to established dogma, there are certain limitations on the plasticity of Mφ.

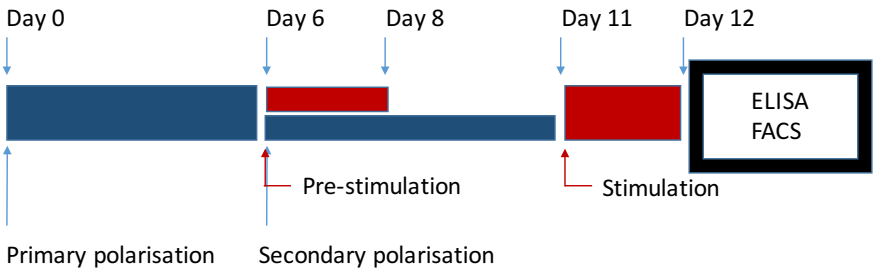


Figure 4.4 Repolarisation protocol. GM-Mφ and M-Mφ were cultured in medium alone or M-CSF or GM-CSF to examine their functional commitment towards inflammation (IL-23) or regulation (IL-10). At the same time cells were pre-stimulated with LPS, IFNγ or LPS/IFNγ or medium alone to assess the importance and hierarchy of polarising stimuli. After 48h cells were washed to remove the pre-stimulation and new medium alone or containing GM-CSF or M-CSF was added to continue secondary polarisation. Finally, after 4 days cells were washed and activated with LPS, IFNγ or LPS/IFNγ or left resting. Supernatants were examined after 24h by ELISA and cells were collected for FACS.

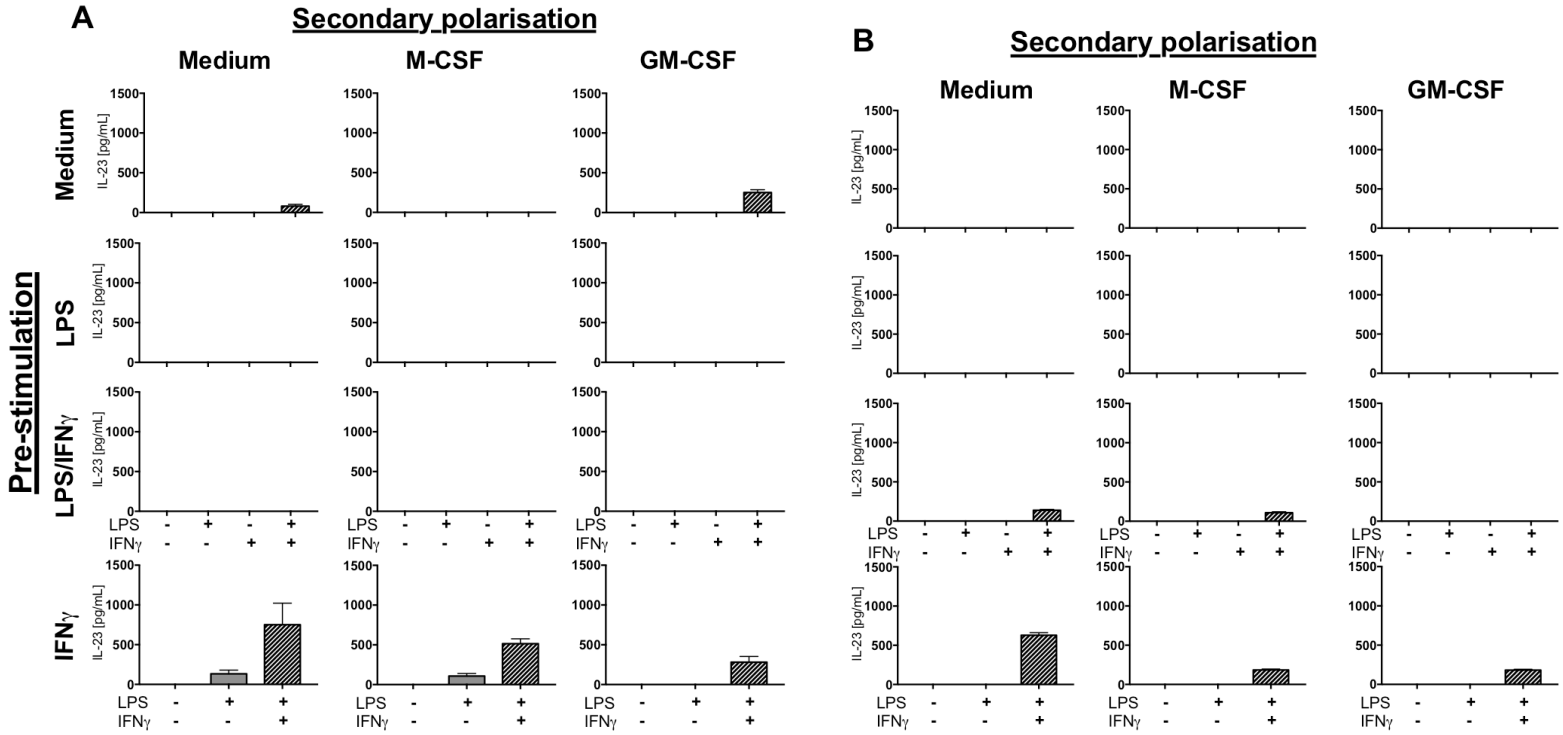


Figure 4.5 A,B. Effect of Mφ repolarisation on cytokine secretion. Mφ were generated for 6 days in the presence of GM-CSF for GM-Mφ **(A, C)** or M-CSF for M-Mφ **(B, D)** during primary polarisation. Next cells were collected, washed 1x with PBS and seeded in the presence or absence of M-CSF or GM-CSF (secondary polarisation) and activated with LPS, IFNγ or LPS/IFNγ or left in medium (Pre-stimulation). After 2 days' cells were washed and new medium alone or containing GM-CSF or M-CSF was added. After 4 days' cells were washed and activated with LPS, IFNγ, LPS/IFNγ or medium alone (stimulation). Supernatants were collected after 24h and assayed by ELISA for IL-23 **(A, B)** and IL-10 **(C, D)**. Graphs showing results from 1 donor.

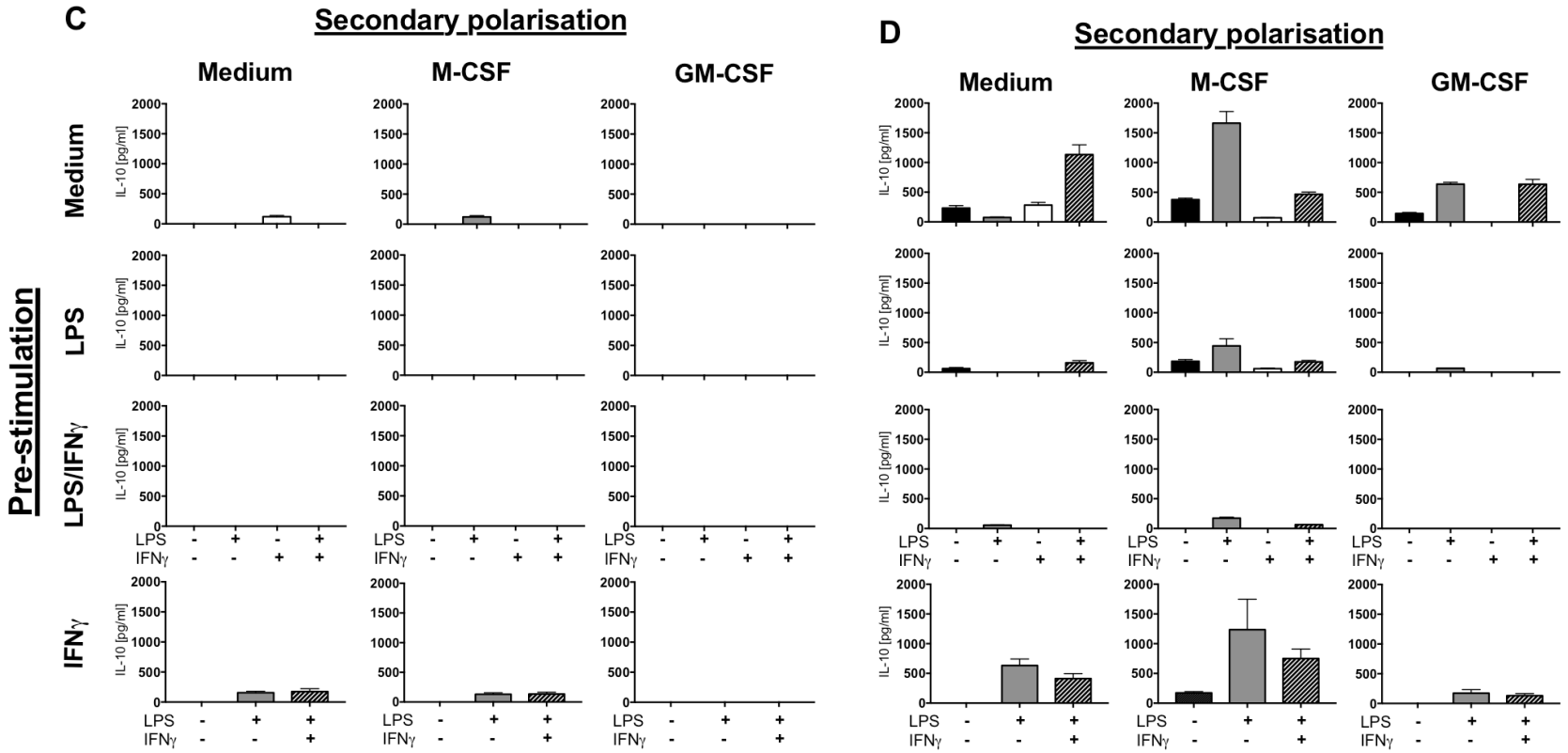


Figure 4.5 C,D. Effect of Mφ repolarisation on cytokine secretion. Mφ were generated for 6 days in the presence of GM-CSF for GM-Mφ **(A, C)** or M-CSF for M-Mφ **(B, D)** during primary polarisation. Next cells were collected, washed 1x with PBS and seeded in the presence or absence of M-CSF or GM-CSF (secondary polarisation) and activated with LPS, IFNγ or LPS/IFNγ or left in medium (Pre-stimulation). After 2 days' cells were washed and new medium alone or containing GM-CSF or M-CSF was added. After 4 days' cells were washed and activated with LPS, IFNγ, LPS/IFNγ or medium alone (stimulation). Supernatants were collected after 24h and assayed by ELISA for IL-23 **(A, B)** and IL-10 **(C, D)**. Results from 1 donor are shown.

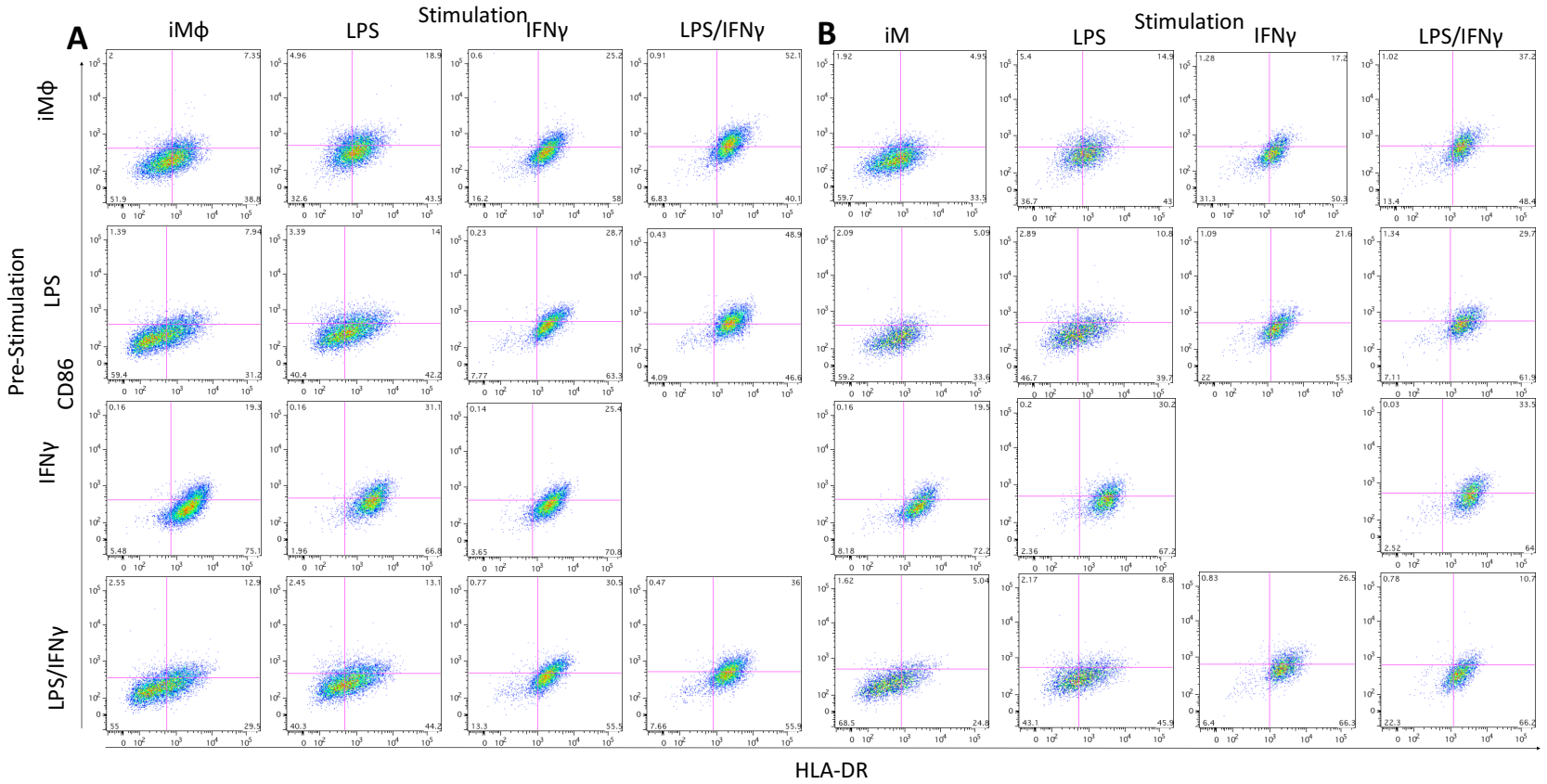


Figure 4.6A,B Effect of repolarisation on MoMφ phenotype. Mø were generated for 6 days in the presence of GM-CSF for GM-Mφ **(A, B, C)** or M-CSF for M-Mφ **(D, E, F)** during primary polarisation. Next cells were collected, washed 1x with PBS and seeded in medium alone **(A, D)** or with M-CSF **(B, E)** or GM-CSF **(C, F)** and pre-stimulated as indicated. After 2 days' cells were washed and new medium alone or containing GM-CSF or M-CSF was added. After 4 days' cells were washed and activated with LPS, IFNγ, LPS/IFNγ or medium alone (stimulation). Expression of HLA-DR and CD86 was assessed by FACS 24h after stimulation. Results show one of three donors tested.

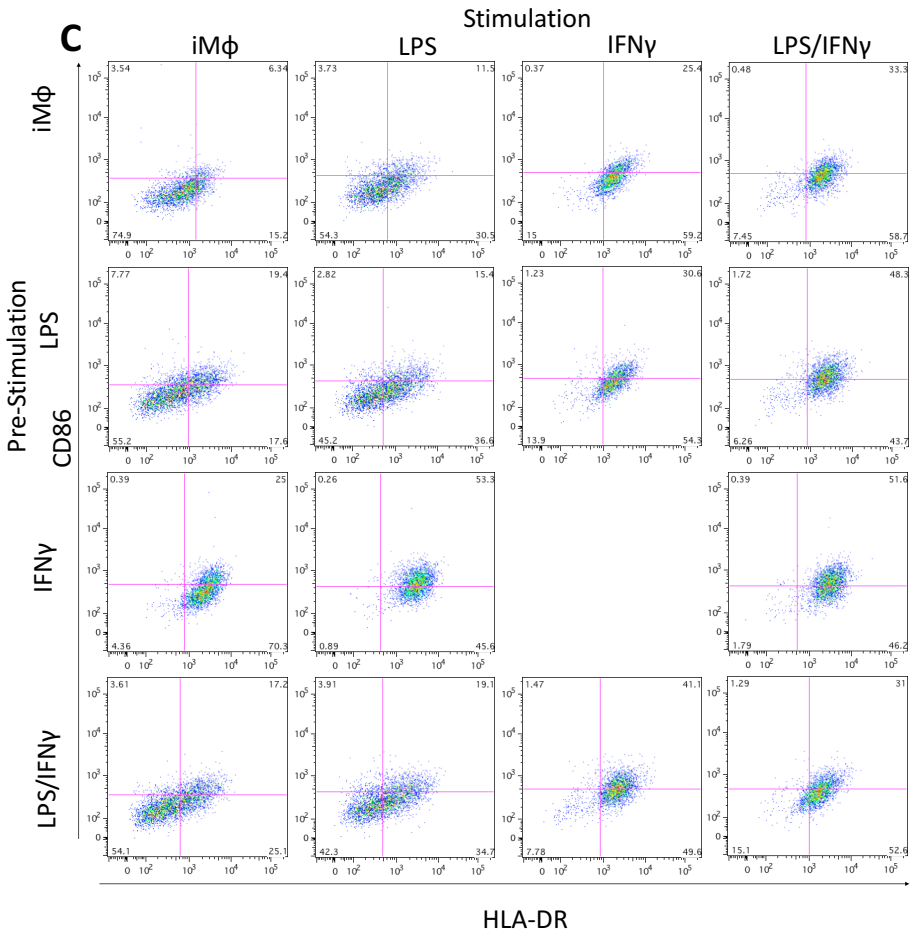


Figure 4.6C Effect of repolarisation on MoMφ phenotype. Mø were generated for 6 days in the presence of GM-CSF for GM-Mφ **(A, B, C)** or M-CSF for M-Mφ **(D, E, F)** during primary polarisation. Next cells were collected, washed 1x with PBS and seeded in medium alone **(A, D)** or with M-CSF **(B, E)** or GM-CSF **(C, F)** and pre-stimulated as indicated. After 2 days' cells were washed 1x with PBS and new medium alone or containing GM-CSF or M-CSF was added. After 4 days' cells were washed and activated with LPS, IFNg, LPS/IFNg or medium alone (stimulation). Expression of HLA-DR and CD86 was assessed by FACS 24h after stimulation. Results show one of three donors tested

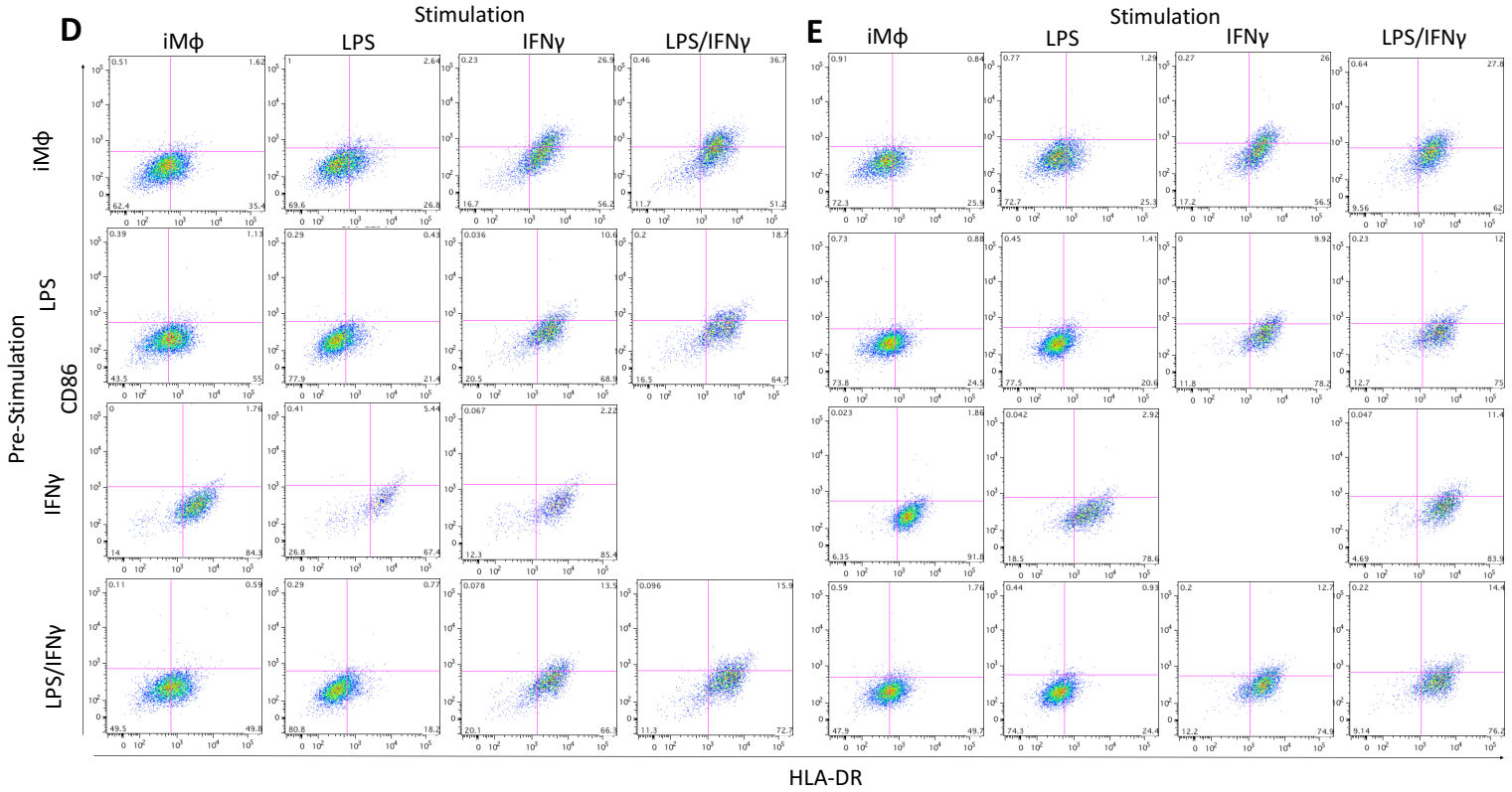


Figure 4.6.D,E Effect of repolarisation on MoMφ phenotype. Mφ were generated for 6 days in the presence of GM-CSF for GM-Mφ **(A, B, C)** or M-CSF for M-Mφ **(D, E, F)** during primary polarisation. Next cells were collected, washed 1x with PBS and seeded in medium alone **(A, D)** or with M-CSF **(B, E)** or GM-CSF **(C, F)** and pre-stimulated as indicated. After 2 days' cells were washed 1x with PBS and new medium alone or containing GM-CSF or M-CSF was added. After 4 days' cells were washed and activated with LPS, IFNγ, LPS/IFNγ or medium alone (stimulation). Expression of HLA-DR and CD86 was assessed by flow cytometry after 24h. Results show one of three donors tested.

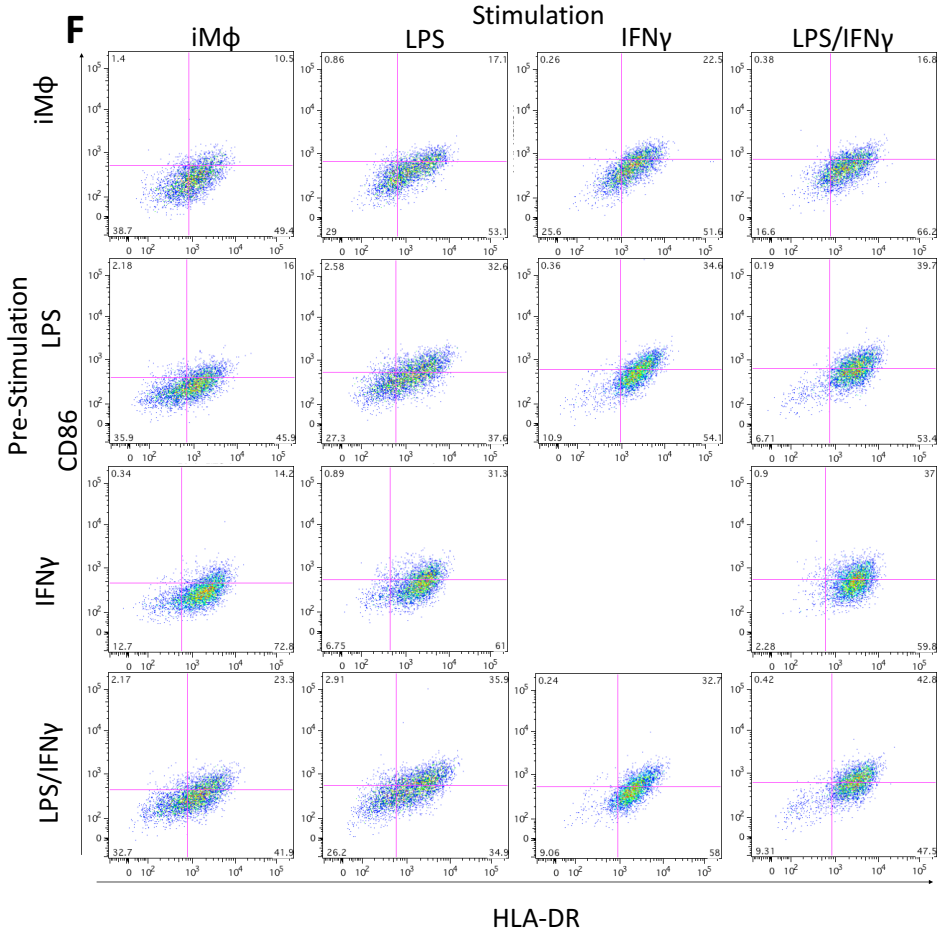


Figure 4.6.F Effect of repolarisation on MoMφ phenotype. Mø were generated for 6 days in the presence of GM-CSF for GM-Mφ **(A, B, C)** or M-CSF for M-Mφ **(D, E, F)** during primary polarisation. Next cells were collected, washed 1x with PBS and seeded in medium alone **(A, D)** or with M-CSF **(B, E)** or GM-CSF **(C, F)** and pre-stimulated as indicated. After 2 days' cells were washed 1x with PBS and new medium alone or containing GM-CSF or M-CSF was added. After 4 days' cells were washed and activated with LPS, IFNγ, LPS/IFNγ or medium alone (stimulation). Expression of HLA-DR and CD86 was assessed by FACS 24h after stimulation. Results show one of three donors tested.

4.2.4 Modulation of the effect of IFNγ and IL-4 on MoMφ by TLR activation

Activation of Mφ with a TLR4 agonist in conjunction with T-cell cytokine played a key role in establishing Mφ polarisation profiles. Our results confirmed the importance of cooperation between TLR4-signalling and factors including M-CSF or IFNγ. We hypothesised that similar cooperation may exists with different TLR. Several groups investigated other TLR including the TLR7/8 by employing the R848 agonist [605, 606]. At doses between 0.01μg-10μg R848 is understood to stimulate TLR7 while at doses between 0.1μg-10μg it also activates TLR8 [214]. Therefore, a dose titration was carried out in the presence or absence of IFNγ or IL-4.

At the lowest dose of 0.04μg/mL, R848 induced IL-12 secretion by M-Mφ but not by GM-Mφ (Figure 4.7). Furthermore, there was no secretion of IL-23 or IL-10. At higher levels (0.4μg/mL), R848 induced IL-12 secretion by GM-Mφ (1/3 donors) and IL-10 and IL-12 by M-Mφ (2/3 donors). At 4μg/mL R848, all donors secreted IL-10 and IL-23 with the exception of IL-12 by GM-Mφ (1 donor). Therefore, further experiments were undertaken with this dose of R848. It is worth noting that the overall levels of cytokine secretion triggered by R848 from GM-Mφ were lower than those from M-Mφ.

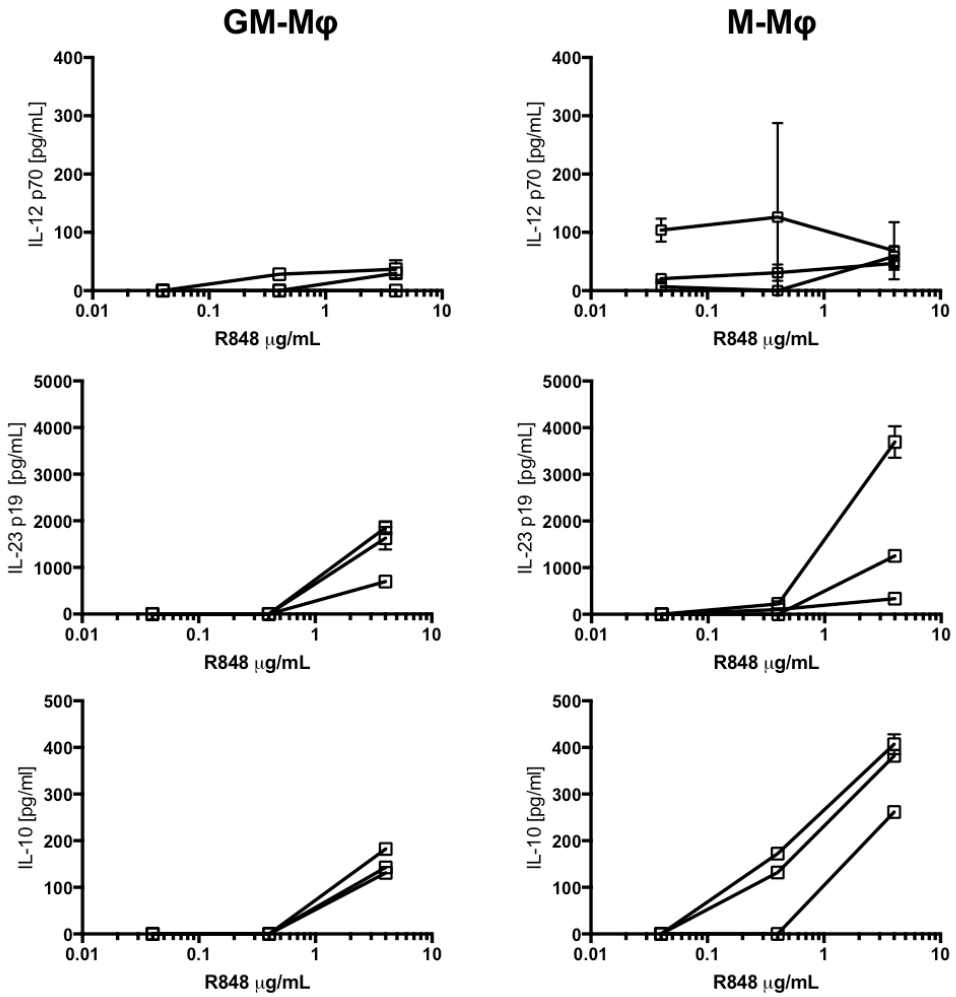


Figure 4.7 Titration of R848 on MoMφ. GM-Mφ and M-Mφ from 3 donors were stimulated with 0-4μg/mL R848. IL-12p70, IL-23 and IL-10 secretion was measured at 24h by ELISA. Shown is mean +/- SD of triplicate stimulation.

After establishing the optimal dose of R848 for Mφ stimulation we examined whether IFNγ and IL-4 exerted a similar polarising effect on Mϕ activated with R848 as observed with LPS. As expected, LPS required IFNγ for IL-12 and IL-23 secretion by Mφ (Figure 4.8, Supplementary material Figure S2A-E). Furthermore, the secretion of pro-inflammatory cytokines was higher in LPS/IFNγ activated GM-Mφ than M-Mφ. Mφ activated with LPS alone or LPS/IL-4 did not secrete IL-12 or IL-23. In contrast, R848 resulted in higher secretion of pro-inflammatory cytokines by M-Mφ in comparison to GM-Mφ.

Furthermore, while the combination of R848 and IFNγ was required for IL-12 secretion by both GM-Mφ and M-Mφ, it did not affect IL-23. IL-4 decreased secretion of both IL-12 and IL-23 by Mφ activated with R848.

Treatment of M-Mφ with LPS alone induced IL-10 which was decreased by addition of IFNγ or increased by IL-4. R848 triggered IL-10 secretion by both M-Mφ and GM-Mφ and, similarly to LPS, its effect was decreased by IFNγ and up-regulated by IL-4. Secretion of all three cytokines was higher in cells activated with R848 versus LPS (IL-12 6-fold higher, IL-23 and IL-10 3-fold higher). Furthermore, R848 triggered higher secretion of IL-12 and IL-10 by M-Mφ comparing to GM-Mφ, while the levels of IL-23 were similar in both types of Mφ. This contrasts with LPS which co-operated with GM-CSF to induce IL-12 and IL-23.

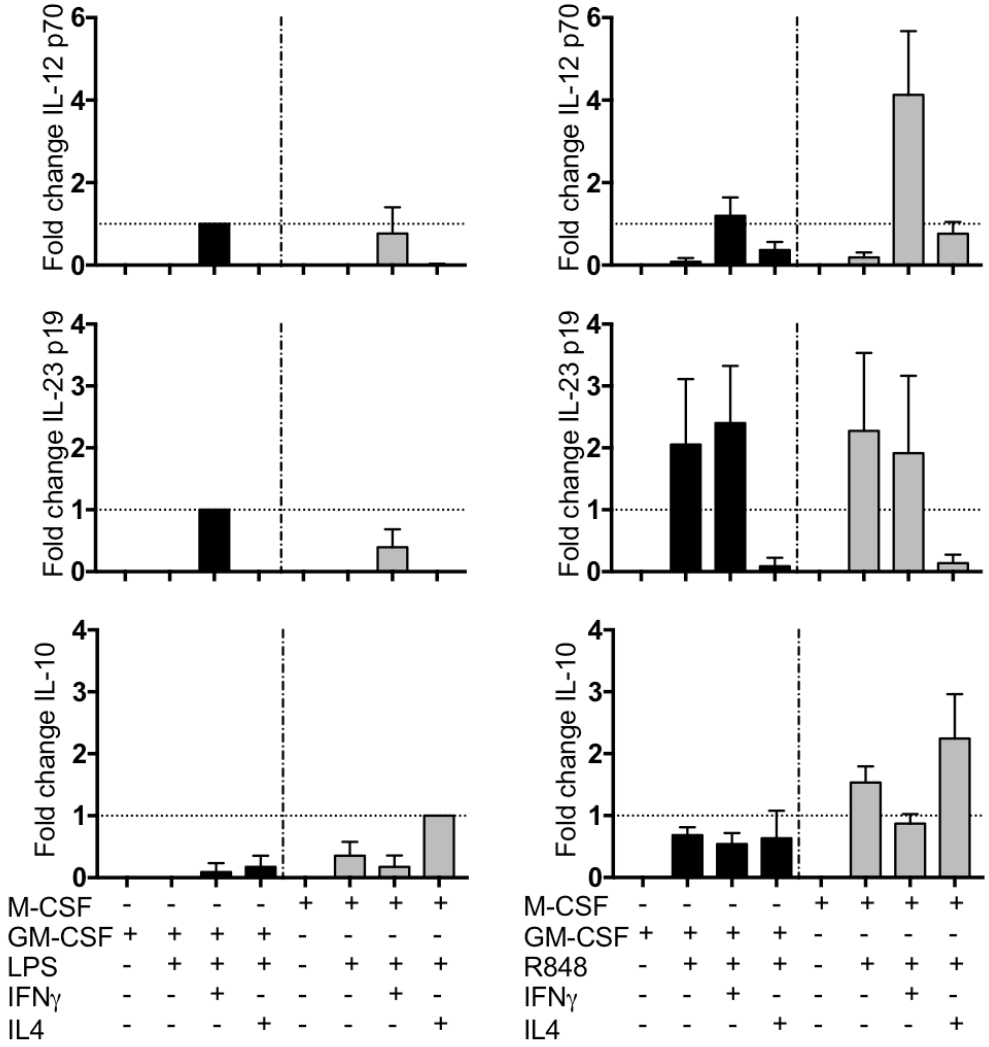


Figure 4.8 Comparison of the effect of LPS and R848 stimulation on MoMφ in the presence or absence of IFNγ and IL-4. M-Mφ and GM-Mφ from 5 donors were stimulated with LPS (5μg/mL) or R848 (40μg/mL) in the presence or absence of IFNγ and IL-4. Graphs present a fold change of IL-12p70 and IL-23 secretion by MoMφ as compared to GM-Mφ stimulated with 5μg/ml LPS and 1000IU IFNγ (dotted line) and s fold change of IL-10 secretion by MoMφ as compared to M-Mφ stimulated with 5μg/mL LPS and 20ng/mL IL-4 (dotted line). Shown is mean + SD of triplicate stimulation of 5 donors for each condition.

4.3 Discussion

In this chapter we demonstrate the restrictions in Mφ plasticity and the importance of microenvironmental conditions during the early process of differentiation of Mo into Mφ for final Mφ polarisation status. Furthermore,

we show the importance of initial activation on Mφ response to further stimulation as a result of innate memory.

As mentioned earlier GM-CSF and M-CSF are often used in MoMφ generation protocols to provide Mφ1 and Mφ2 respectively. As was shown by Lacey *et al*. [583] M-CSF directs the phenotype of MoMφ towards Mφ2 while GM-CSF towards Mφ1 phenotype. The importance of these initial phenotypes is often disregarded due to high plasticity of Mφ. Treatment of TAMφ with IFNγ or IL-12 was shown to switch their phenotype to Mφ1-like with high secretion of pro-inflammatory cytokines such as TNFα while reducing their secretion of IL-10 [596, 597]. On the other hand, Smith *et al*. showed that consecutive or simultaneous treatment of MoMφ with Mφ1/Mφ2 stimuli results in mixed phenotypes where cells express both Mφ1 and Mφ2 markers to some degree [588]. Similarly, in the tissues Mφ often display the characteristic of both pro and anti-inflammatory Mφ [588, 607]. We hypothesized that environmental conditions during differentiation of Mo towards Mφ may leave a specific imprint on Mφ which affects further response to pro- and anti-inflammatory stimuli and results in varied activation phenotypes. The combination of LPS and IFNγ for activation is often used to trigger Mφ1 polarisation however a comparison between Mφ activated with this combination but generated with GM-CSF or M-CSF was never performed. We found that Mφ generated in the presence of GM-CSF were characterised by higher production of pro-inflammatory cytokines (IL-12 and IL-23) and lower secretion of IL-10 than Mφ generated with M-CSF. These findings were especially striking in regards to IL-23 which secretion was

completely abrogated in M-Mφ from some donors. These results agree with findings by Xue *et al*. [592] who demonstrated strengthening of Mφ polarisation when Mφ1 or Mφ2 stimuli where combined in our case GM-CSF used for Mφ generation followed by pro-inflammatory LPS and IFNγ. Furthermore our results highlight the independence of regulatory mechanisms responsible for secretion of these two cytokines in Mφ similar to DC [289, 572].

After establishing the importance of initial GM-CSF/ M-CSF polarisation on the phenotype of activated Mφ we evaluated the sensitivity of Mφ to re-polarising signals . Report by Xu *et al*. demonstrated that consequent culture of GM-Mφ and M-Mφ with M-CSF or GM-CSF respectively was able to completely reverse their initial polarisation with regards to IL-6 and TNFα secretion and partially with regards to IL-10 secretion [591]. In contrast, our results suggest certain stability of the initial Mφ phenotype. Repolarisation of Mφ with GM-Mφ with M-CSF alone had little effect on the secretion of IL-23 and IL-10 as well as expression of HLA-DR and CD86 regardless of stimulation protocol used. These results support findings by Franklin *et al*. in breast cancer showing that the TAMφ population differentiate *de novo* from circulating Mo rather than from local Mφ [93, 94].

Our findings regarding the importance of the differentiation signals on Mφ phenotype are reflected in the profound effect of the initial activation of Mφ on the result of their subsequent activation profile. Primary activation of Mφ

with LPS, alone or in combination with IFNγ decreased both IL-23 and IL-10 secretion during secondary activation. Interestingly, although IFNγ up-regulated IL-23 levels in both GM-Mφ and M-Mφ, it also stimulated IL-10 release. These results demonstrate an activating role of IFNγ for Mφ. Furthermore, it may be tempting to suggest the ability of Mφ to develop certain degree of immunologic memory which allow for stronger response upon reactivation by microbial infection or stimulation by adaptive immune responses. Such mechanism may play a role in the exacerbation of autoimmune disorders. In this respect, it would be interesting to expand our findings on other kinds of stimulation by a wide array of TLR in combination with signalling from other types of Th-cells to assess for any specificity in secondary response. Furthermore, the effect of re-activation on other cytokines especially IL-12 would provide a valuable insight. The definition of innate memory has been postulated by Natea *et al*. in 2011 [332]. However, first reports of prolonged activation states of innate immune cells were published more than half a century ago. In 1969, Mackaness *et al*. described temporary activation of Mφ in response to IFNγ secreted by lymphocytes [608]. As a result, a state of temporary heightened innate immunity against a secondary infection was generated which decreased rapidly after elimination of the primary pathogen. Furthermore, Mφ have been shown to be able to develop different kinds of memory characterised by lesser or higher reactivity to secondary challenge depending on the type of priming [609]. Epigenetic reprogramming was postulated as the possible mechanism responsible for the development of innate memory [610-612].

We also examined the effect of different TLR on cytokine secretion by Mφ in the presence or absence of additional influence from adaptive immune responses (IFNγ and IL-4). LPS, which is often treated as a pro- Mφ1 stimulus, co-operated with both pro- and anti-inflammatory stimuli (with the excepion of GM-CSF) promoting the secretion of respective pro- or anti-inflammatory cytokines. On the other hand, R848, acting on TLR7/8, induced strong, mixed phenotype characterised by secretion of IL-12, IL-23 as well as IL-10. Furthermore, cells activated with R848 demonstrated resistance to enhancing effects of IFNγ with regards to IL-23 secretion. Interestingly R848 induced significantly stronger secretion of cytokines by M-Mφ in comparison to GM-Mφ suggesting co-operation between these two stimuli. TLR7/8 agonists including R848 have been used as vaccine adjuvants due to their ability to promote inflammatory responses via NFκB activation [613-615]. Furtermore it has been shown that Mφ induced with R848 become resistant to activation by other TLR agnists. Our findings that IFNγ added at the time of R848 activation promote IL-12 while not affecting IL-23 and reducing IL-10 secretion may be helpful in designing therapeutic strategies aimed at stimulating specifically Th1 responses such as cancer.

In summary our results suggest the existance of innate memory in Mφ which may be resposible for excacerbation of immune pathologies. Furthermore, we demonstrate differential regulation of IL-12 and IL-23 by M-Mφ and GM-Mφ activated by the same stimuli. Although both IL-12 and IL-23 have pro-inflammatory functions, IL-12 drives anti-tumour Th1 responses and promotes resolving of inflammation while IL-23 drives Th17 which promote

chronic inflammation and are involved in the development and maintenance of many autoimmune and cancerous diseases [243, 616]. The ability of selective down-regulation of IL-23 by Mφ may have implications for the utilisation of therapeutic interventions on Mφ in autoimmunity and cancer. Our further work will focus on the regulation of IL-23 by macrophages in response to RT.

Chapter 5. Ionising radiation down-regulates macrophage IL-23 preserving their pro-inflammatory phenotype

5.1 Introduction

Radiotherapy (RT) provides an important, cost-effective and widely used option for the treatment and palliation of many types of cancer including Squamous Cell Carcinoma (SCC) [617], breast [618] and metastatic brain cancer [619]. The main purpose of RT is to damage transformed tumour cells and initiate their demise by generating free radicals which induce stress response and consequently cell death if the damage cannot be repaired [620-622]. However, over recent years reports demonstrated that some of the effects of IR are mediated through immune responses. Irradiated tumour cells up-regulate MHCI expression and secrete chemokines and cytokines such as CXCL16, TNFα, IL-1β, IL-6 which stimulate infiltration and activation of immune cells [623]. In addition, irradiated tumour cells can serve as a source of TAA (tumour associated antigens) which allow for tumour specific T-cell responses when processed and presented by APC [623]. Furthermore, in several cancer models, irradiated tumour cells demonstrated enhanced expression of death receptors [624, 625], costimulatory molecules [626], adhesion molecules, and stress-induced ligands which increased their recognition and killing by T-cells [627-629]. By coupling the release of new Ag with immune adjuvant-like effect, IR engages both the innate and adaptive arms of immune responses. Finally, although infrequent, abscopal effects, characterised by a tumour response in a metastasis after local RT of different tumour site have been seen in several types of cancer [630-635]. These

findings led to suggestions of utilising RT, in addition to its palliative role, as an adjuvant for immunotherapy. Such strategies have been tested in preclinical models with a degree of success. Injection of exogenous syngeneic DC following irradiation promoted development of tumour-specific T-cells and tumour regression in murine melanoma, sarcoma and fibrosarcoma models [636, 637]. In another approach injection of CpG resulted in a strong DC activation [638-640]. In these models the result of combination of RT and immunotherapy was seen more effective than any of these therapies used alone. Adoptive transfer of T-cells resulted in improved killing of colon carcinoma cells if vaccination was given before radiation, with boosts following radiation [624, 641]. This effect was mediated through radiation-induced up-regulation of Fas/CD95 in tumour cells. Combination of RT with anti-CTLA-4 resulted in successful T-cell–mediated antitumor responses in tumours resistant to anti-CTLA-4 [642, 643]. The effect was due to irradiated tumour acting as an in-situ vaccine and was mediated through CXCL16 and the NKG2D ligand retinoic acid early inducible 1 (RAE-1) induced on irradiated tumour cells which interacted with effector CD8 T-cells.

However, whilst irradiation can promote immune responses, it also exerts powerful immunosuppressive effect through activation of TGFβ and accumulation of regulatory T-cells (T-reg) and Mφ2 [643, 644]. Furthermore, whilst tumour cells are the main target for RT, immune and stromal cells residing in the tumour microenvironment (and in non tumour regions that nevertheless lie in the beam path) are also irradiated. In addition, alterations in the behaviour of stromal cells following RT can lead to severe side effects

including fibrosis [645]. Therefore, a better understanding of the effect of RT on tumour stroma is needed in aim to improve the outcomes while reducing detrimental side effects of exposure to IR [646, 647].

Mφ constitute a major component of inflammatory tumour stroma and can contribute up to 50% of tumour mass [648]. Tumours contain highly heterogeneous population of TAMφ (tumour associated macrophages) which can display characteristics of Mφ1- and Mφ2-like cells. TAMφ of the Mφ1-like phenotype are often found in normoxic areas and during early stages of tumour development. It is believed that Mφ1-like cells convert to Mφ2-like cells during progression of cancer and in hypoxic areas [649-651]. Furthermore, stromal Mφ can sensitize some tumour cells to irradiation while exhibiting protective behaviour in others [652, 653].

Many effects of Mφ on tumours are exerted through released cytokines and growth factors. Mφ2-like TAMφ impair anti-tumour immunity through the secretion of TGFβ and IL-10 which promote the recruitment of T-reg and inhibit Th1 activity [654-657]. Growth factors, such as VEGF and Platelet-derived growth factor (PDGF), stimulate angiogenesis and lymphangiogenesis therefore facilitating tumour metastasis and growth [648, 655, 658]. In contrast, Mφ1-like TAMφ secrete pro-inflammatory IL-12 and IFNγ which promote anti-tumour adaptive responses [659, 660]. Secretion of the chemokines Chemokine (C-X-C motif) ligand 9 (CXCL9) and CXCL10 stimulates the influx of Th1 [659]. Due to their ability to stimulate anti-tumour responses the presence of Mφ1-like TAMφ in tumours often correlate with

more favourable prognosis comparing to Mφ2 [661]. However, many factors released by Mφ1 TAMφ also support tumour growth [662].

In much the same way as with IL-12, secretion of IL-23 is regarded as an archetypal Mφ1-like cytokine [663]. Sharing a common p40 subunit with IL-12, IL-23 promotes inflammation and angiogenesis [309, 310]. Importantly, it has been shown that certain TAMφ subpopulations downregulate IL-12 while at the same time up-regulate both IL-23 and IL-10 therefore presenting a mixed phenotype [664]. These findings are in agreement with current view that the Mφ1-Mφ2 classification represents extremes of Mφ polarisation and, depending on unique environmental signalling cues, TAMφ acquire phenotypical and functional properties from the whole polarisation range [109, 665]. Importantly, although both IL-12 and IL-23 have pro-inflammatory functions, they play opposing roles in maintaining tumour in equilibrium with host immunity [660]. Whilst inhibition of IL-23 with antibodies prevents metastatic potential of methycholanthrene (MCA)-induced murine cancers, blocking IL-12 stimulates tumour outgrowth [660]. Whereas IL-12 induction leads to the development of Th1 secreting IFNγ and promotes cytotoxic and anti-tumour responses, IL-23 stimulates Th17 recruitment and maintenance thereby prolonging and exacerbating inflammation, angiogenesis and matrix degradation [143, 145, 150, 153, 309, 310, 666]. These processes are vital for tumour growth, invasion and metastasis. IL-23 also directly promotes the proliferation of some tumours [307]. Several studies demonstrated up-regulation of IL-23 in various cancers including ovarian, colon, lung, gastric

and breast cancer [310, 667] with TAMφ pointed as the main source of IL-23 in tumours [310, 668].

Although the importance of TAMφ in tumour progression is well recognized, the effect of RT on TAMφ and their cytokine secretion including IL-23 requires further investigation. Many studies have been performed in murine models using low or moderate doses of IR (0.1-1.5 Gy). Those are not relevant in context of tumour RT for humans which use higher doses of IR per fraction (2-6Gy). In addition, using different strains of mice often leads to contrary results. Exposure of radio-sensitive Balb-c mice to doses of 0.5-0.7Gy resulted in a decrease in IL-1β secretion by Mφ and concomitant increase of TGFβ while TNFα was affected only at higher (2Gy) doses [503, 669]. On the other hand, the inhibition of TNFα secretion was seen at lower doses (0.5Gy) in RAW264.7 mouse Mφ cell line [670]. However, no effect on IL-1β or TNFα was observed in less radiosensitive but radiation fibrosis-prone C57BL/6 mice [669, 671]. Therefore, it was suggested that IR has anti-inflammatory effect on stromal Mφ and polarizes them towards Mφ2 [503, 647, 653, 672]. Some findings though indicated the possibility of initial Mφ1 polarisation following RT which over time switched towards Mφ2 [647]. On the other hand, an increase of inflammatory markers such as CD80, CD86 and HLA-DR with concomitant decrease of CD163 and IL-10 was reported in human MoMφ treated with cumulative dose of 10Gy [646].

Our present study aimed to test the hypothesis that IR regulates cytokine secretion by human Mφ. We used MoMφ generated in the presence of GM-

CSF or M-CSF to ensure that the effect of IR is not an artefact of a particular generation method. We show that IR selectively downregulates IL-23 by GM-Mφ and M-Mφ and IL-6 secretion by GM-Mφ while not affecting their expression of antigen-presentation markers or secretion of IL-12, IL-1β and TNFα. The selective down-regulation of IL-23 without effect on IL-12 agrees with our previous findings showing a degree of independent regulation of the pathways responsible for production of these two cytokines in Mφ. Moreover, we show that the effect of IR on IL-23 secretion is mediated by ATM kinase and further executed by CREB-CBP interaction. As described earlier ATM is one of the key regulatory kinases activated in response to IR. In addition to its role in DDR pathway its also exerts immunoregulatory functions through its target proteins such as CREB transcription factor. Irradiation of MoMφ resulted in activation of ATM and this in effect supressed the production of IL-23. Inhibiting ATM with highly selective inhibitor KU55933 [673], resulted in a significant increase in IL-23 secretion from both irradiated but also non-irradiated MoMφ. This effect was abrogated by CREB-CBP inhibitor. IR led to the phosphorylation of inhibitory S121 residue on CREB transcription factor. The activation of S21 was completely blocked by ATM inhibitor and led to the recovery of IL-23 secretion. ATM-dependent increase in IL-23 secretion was abrogated by inhibiting CREB-CBP interactions with C-Ci (2-Naphthol-AS-E phosphate), confirming the involvement of CREB transcription factor in ATM-dependent regulation of IL-23 production by MoMφ. Overall our data demonstrates important effects of IR on Mφ functions and identifies novel pathway involved in the regulation of MoMφ IL-23 production. These findings may be utilised in the development of new

strategies for combining immunotherapy with RT through targeting of TAMφ polarisation.

5.2 Results

5.2.1 IR inhibits IL-23 secretion by MoMφ

We hypothesised that IR was likely to affect the function of Mφ and therefore initially determined the effect of IR on Mφ IL-23 secretion. IR significantly ($p<0.01$) downregulated IL-23 secretion by GM-Mφ activated with LPS/IFNγ in all donors tested. Radiation-induced suppression was dose dependent and maximal at 6Gy (Figure 5.1). There was no secretion of IL-23 by M-Mφ activated with LPS regardless of their irradiation status (Table 5.1). Assessment of MoMφ viability by PI staining at the time of collecting supernatants (24h post activation) was undertaken in order to account for the impact of radiation-induced cell death. Although M-Mφ had slightly lower viability comparing to GM-Mφ (~80% and ~90% respectively) there was no significant difference in death rate between irradiated and non-irradiated cells (Figure 5.2). Therefore, the cell death could not account for changes in cytokine secretion.

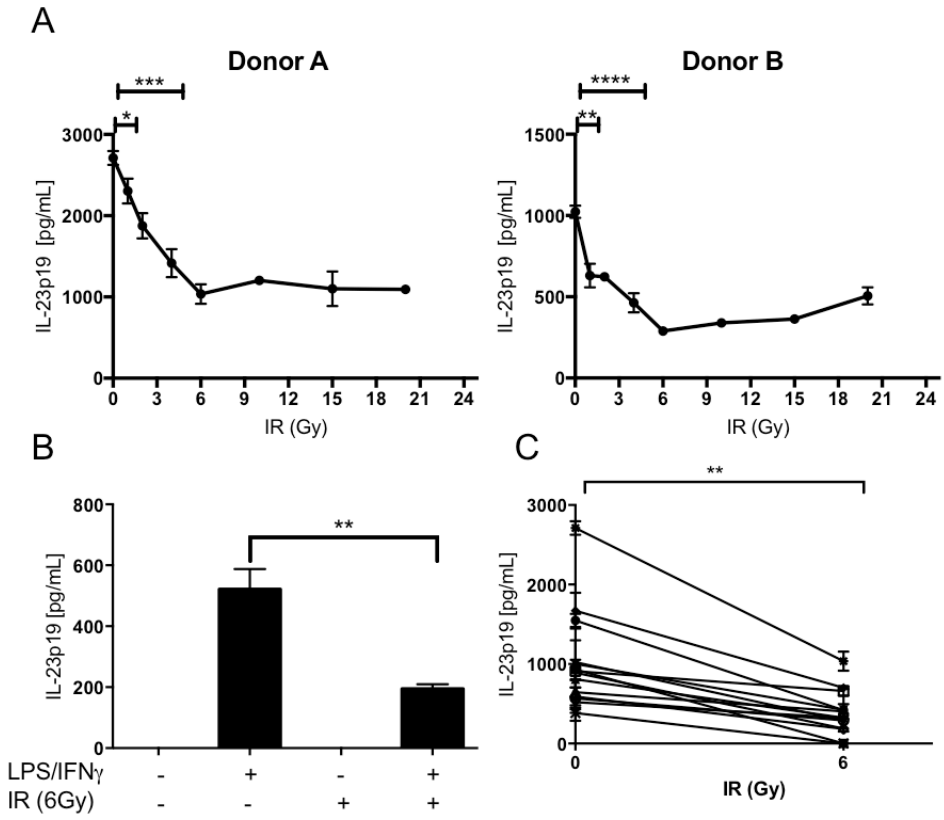


Figure 5.1 Effect of IR on IL-23 secretion by GM-Mφ (LPS/IFNγ). **A**-Mφ were irradiated with increasing doses of IR (0-20Gy) 1h prior to stimulation with LPS/IFNg for 24h and cytokine secretion was measured by ELISA. Shown are 2 out of 4 donors tested. **B, C**-Mφ were irradiated (6Gy) or left non-irradiated 1h prior to addition of LPS/IFNγ. Shown 1 donor (B) and a summary of 14 donors tested (C). Shown are means + SD of triplicate stimulation for each donor. Statistical analysis by t-test.

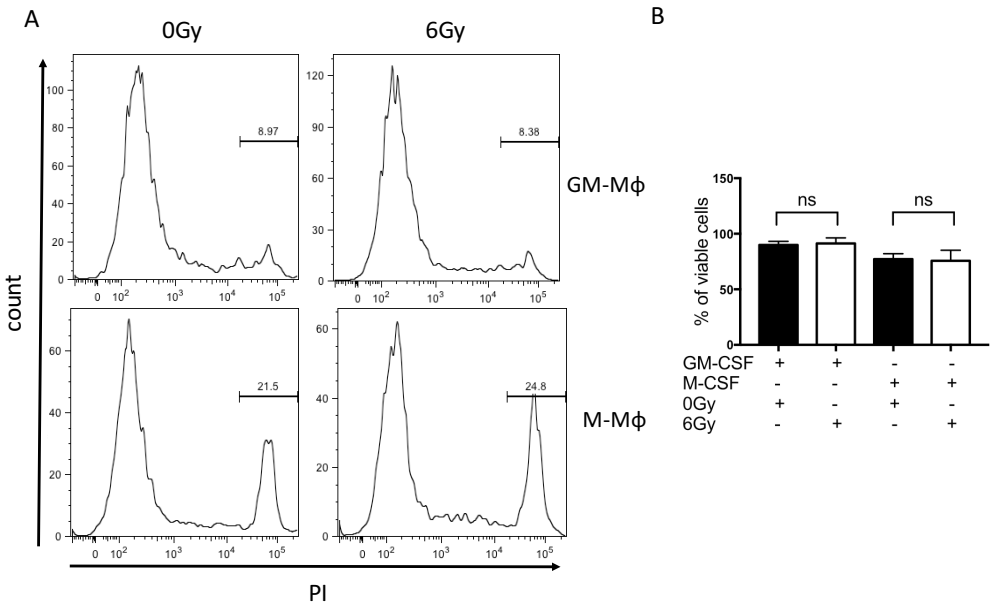


Figure 5.2 Effect of IR on MoMφ viability. MoMφ generated in the presence of GM-CSF or M-CSF were washed and rested for 2h prior to irradiation. After 24h of incubation MoMφ were detached by trypsinisation, stained with PI and assessed by FACS. (A) Shown is a representative of 3 tested donors. The histograms show the percentage of positive cells stained with PI compared to positive control treated with 25% DMSO. (B) A summary figure of 3 donors. Shown is mean + SD for each condition. The significance between conditions was measured by paired t-test.

5.2.2 Effect of IR is independent of MoMφ generation method

Our previous results showed that M-Mφ activated with LPS/IFNγ are deficient in IL-23 secretion in comparison to GM-Mφ. Therefore, we sought to assess whether the effect of IR depended on MoMφ generation conditions. In agreement with our previous results, M-Mφ were deficient in IL-23 compared to GM-Mφ and only 3/7 donors studied secreted IL-23 at 0Gy following activation with LPS/IFNγ (Figure 5.3). In all three donors secretion of IL-23 was down-regulated by IR. These findings suggest that the inhibitory effect of IR on IL-23 secretion is independent of MoMφ generation conditions.

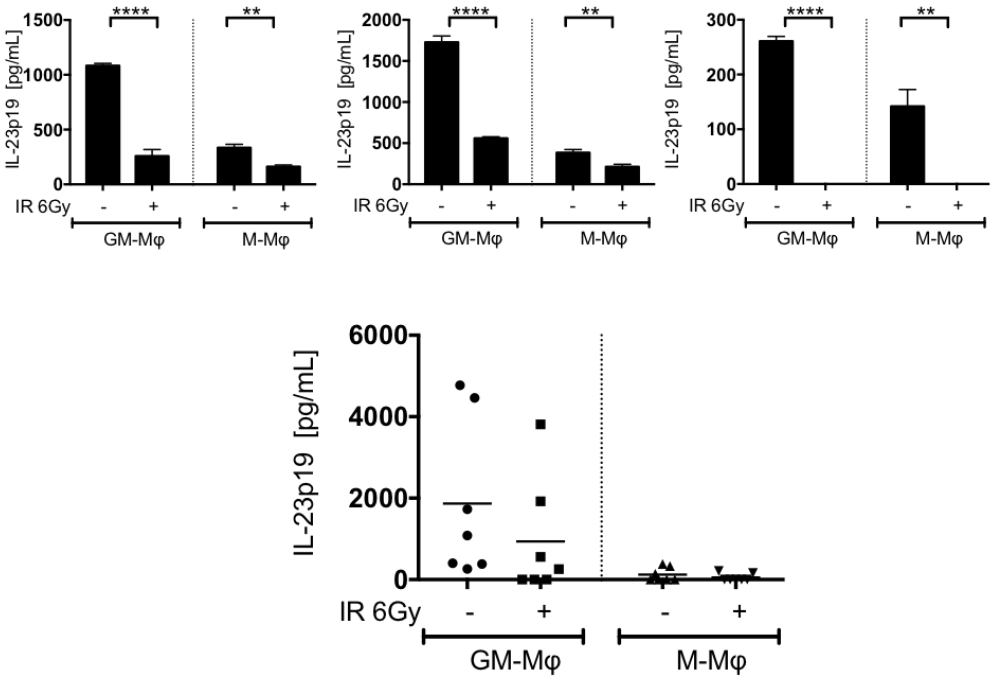


Figure 5.3 Comparison of the effect of IR on GM-Mφ and Mφ-M. MoMφ were generated in the presence of GM-CSF or M-CSF and activated with LPS/IFNγ 1h after treatment with 6Gy of IR. 24h later supernatants were collected and IL-23 secretion was analysed by ELISA. Shown are 3 individual donors and a summary graph of all 7 donors tested. Shown are means+/-SD. Significance was assessed by t-test.

5.2.3 Effect of IR on cytokine secretion and expression of HLA-DR and CD86 by MoMφ.

Having established the role of IR in regulating IL-23 secretion, we assessed its effect on other cytokines. In previous chapter, we showed that it is possible to affect the IL-23 pathway separately from IL-12. In agreement with those results despite IL-23 being down-regulated by IR, IL-12 secretion remained unaffected or was increased in some donors (Figure 5.4, Table 5.1). Similarly, IL-10, IL-1β and TNFα secretion by Mφ1 and Mφ2 were not significantly affected by IR. In contrast IL-6 was significantly down-regulated ($p<0.05$) in all for 4 donors tested in GM-Mφ but not in Mφ-M.

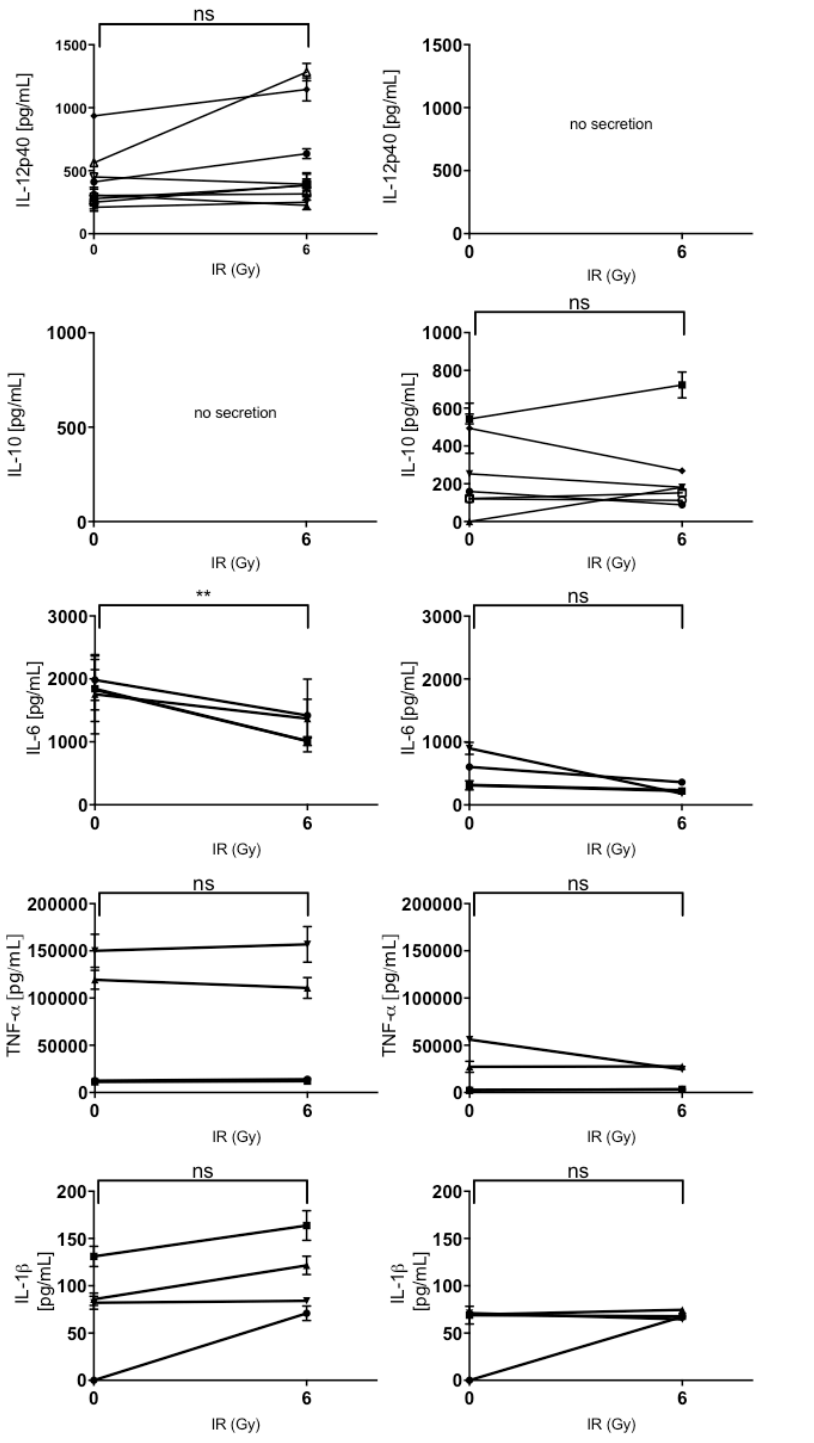


Figure 5.4 Effect of IR on cytokine secretion by GM-Mφ(LPS/IFNγ) and M-M(LPS). MoMφ were harvested, rested for 2h and irradiated with 6Gy or left non-irradiated for 1h prior to stimulation with LPS/IFNγ or LPS only respectively. 24h after stimulation supernatants were collected and cytokine secretion assessed by ELISA. Experiments were undertaken in triplicates in a minimum of 4 donors. Shown is mean + SD for each donor. The significance between conditions was measured by paired t-test.

cytokine	MoMφ type	0Gy		6Gy		p value
		Average pg/mL	St.dev.	Average pg/mL	St.dev.	
IL-23	Mφ1	1045.3	675.8	463.6	259.5	<0.01
	Mφ2	-		-		-
IL-12	Mφ1	412.3	225.1	557.9	391.8	ns
	Mφ2	-	-	-	-	-
IL-10	Mφ1	-	-	-	-	-
	Mφ2	241.6	203.6	243.9	219.1	ns
IL-6	Mφ1	1852.5	95.4	1203.5	221.1	<0.05
	Mφ2	532.9	279.3	248.4	78.6	ns
IL-1β	Mφ1	74.7	54.5	110.0	41.7	ns
	Mφ2	52.3	34.9	68.5	4.2	ns
TNFα	Mφ1	73385.3	72018.6	73498.8	73498.8	ns
	Mφ2	22040.0	25457.4	14731.1	14731.1	ns

Table 5.1 Effect of IR on cytokine secretion by MoMφ. MoMφ were irradiated with 6Gy or left non-irradiated for 1h prior to activation. Cytokine secretion was assessed by ELISA in supernatants collected 24h following stimulation. All experiments were repeated at least 4 times in triplicate and significance was assessed with by paired t-test.

To evaluate whether the effect of IR is selective to indicated cytokines and does not affect MoMφ polarisation status we assessed the expression of HLA-DR and CD86 by irradiated Mφ. In agreement with Mφ1/Mφ2 phenotypic profile irradiated GM-Mφ displayed higher levels of HLA-DR and CD86 expression comparing to M-Mφ (Figure 5.5). Stimulation with LPS/IFNγ further increased the expression up to 9-fold for HLA-DR and up to 3.6-fold for CD86. In contrast activation of M-Mφ with LPS only modestly increased the expression of HLA-DR and CD86 to levels similar to those expressed by immature GM-Mφ.

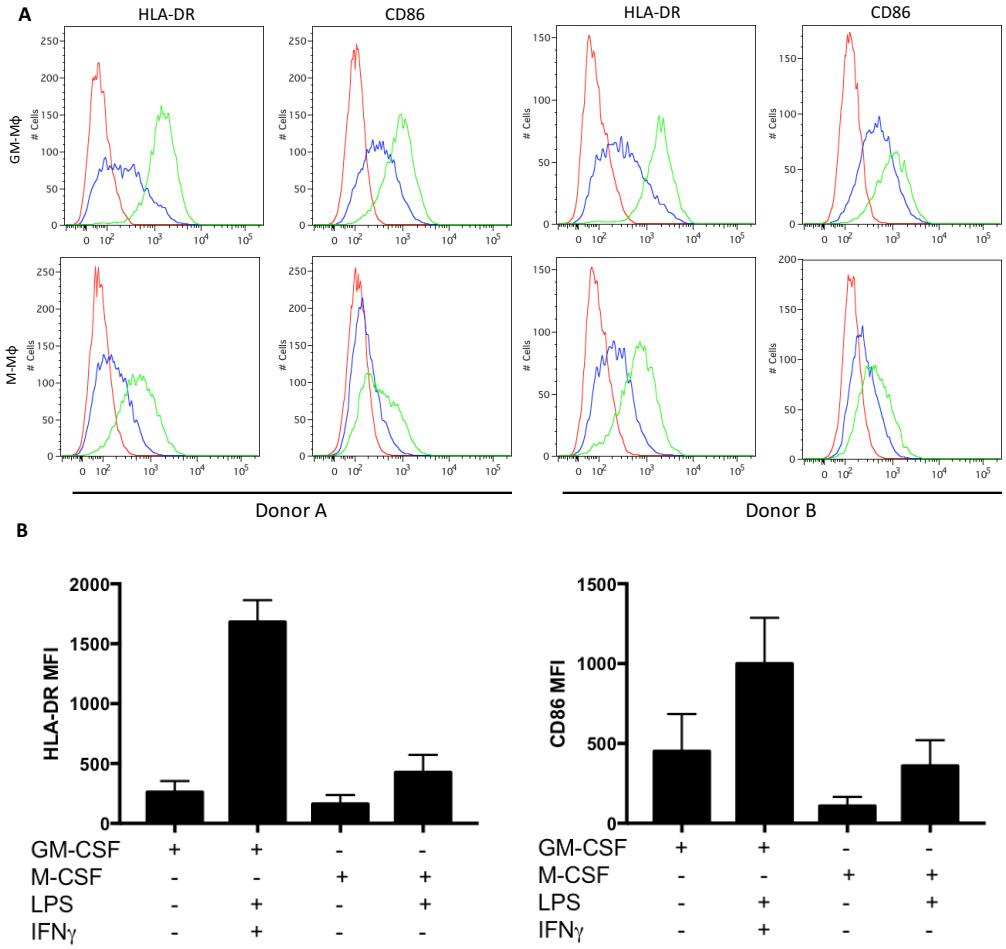


Figure 5.5 Expression of HLA-DR and CD86 by irradiated MoMφ. MoMφ were rested for 2h and irradiated with 6Gy or left non-irradiated for 1h prior to stimulation with LPS/IFNγ or LPS only. 24h after stimulation supernatants cells were detached by scraping and gentle pipetting and expression of HLA-DR and CD86 was assessed by flow cytometry. (A) Shown are 2 out of 4 donors tested. Red line-isotype, blue-non-activated MoMφ, green-activated MoMφ. (B) Summary graph showing normalised MFI of HLA-DR and CD86 expression in all 4 donors. Shown are means +SD for each condition.

5.2.4 Regulation of ATM activation by IR

Irradiation of cells results in DNA damage leading to double strand breaks which are sensed by ATM kinase [620]. Therefore, we tested whether irradiation of MoMφ with 6Gy results in the activation of ATM. In agreement

with previous reports raised levels of pATM were consistently observed in all donors tested in cells irradiated with 6Gy as compared to non-irradiated MoMφ (Figure 5.6). This was seen regardless of whether MoMφ were generated in the presence of GM-CSF or M-CSF.

Having shown that IR activates ATM kinase, we wanted to assess whether the effect of IR on ATM is dose-dependent as we could see with regards to IL-23. Irradiation of MoMφ with dose as low as 1Gy already resulted with activation of ATM kinase which increased proportional to the IR dose up till 6Gy (Figure 5.7). Further increase in the dose of IR up to 20Gy only minimally increased p-ATM levels. These results suggest maximal activation of ATM at the dose of 6Gy.

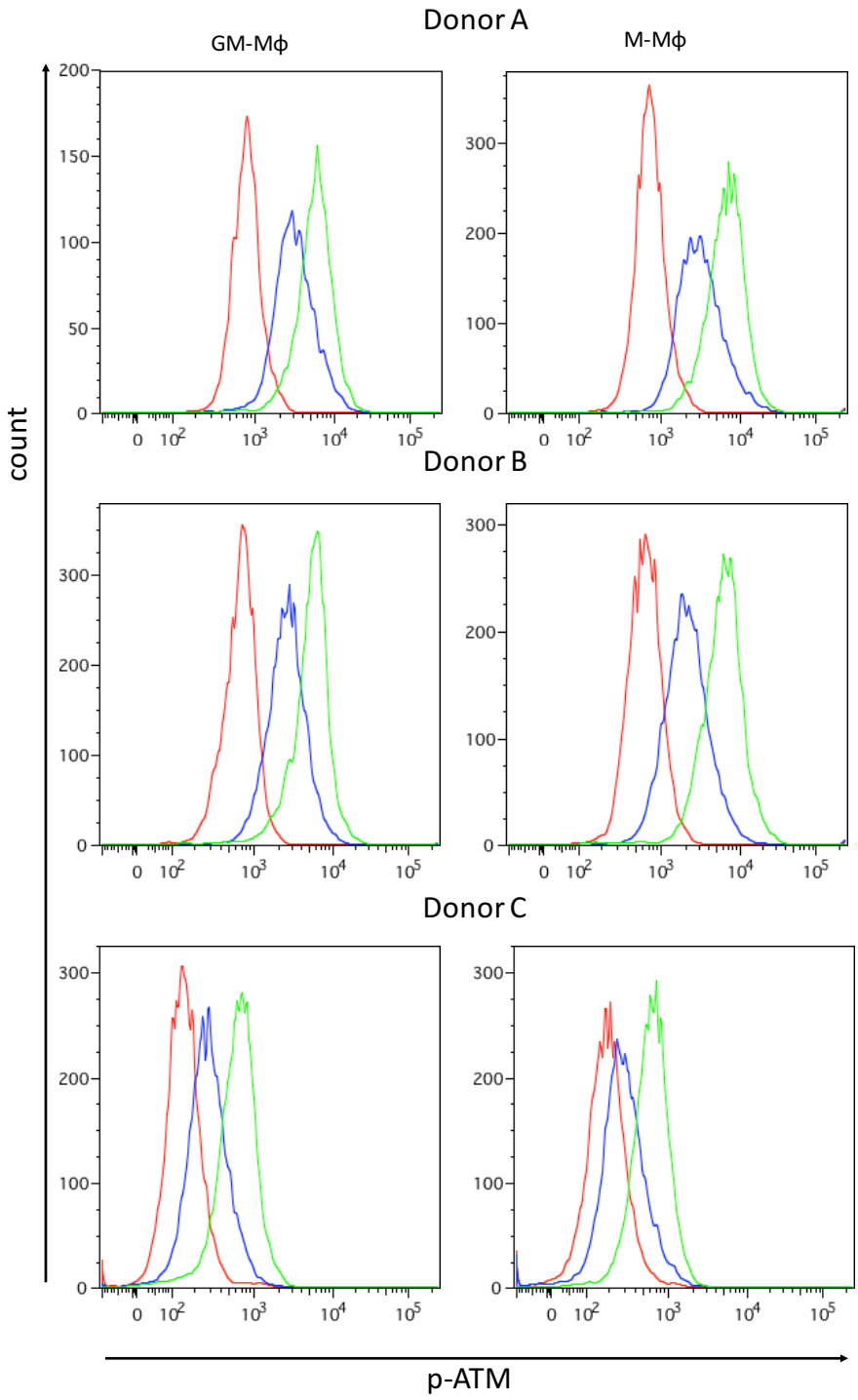


Figure 5.6 IR activates ATM kinase in MoMφ. MoMφ were rested for 2h and irradiated with 6Gy or left non-irradiated for 1h prior collection for assessment of ATM phosphorylation by flow cytometry. Cells were collected by scraping and p-ATM levels were assessed by indirect intracellular staining. Red line-secondary antibody only, blue line- MoMφ exposed to 0Gy, green line- MoMφ exposed to 6Gy. Shown 3 out of 4 donors tested.

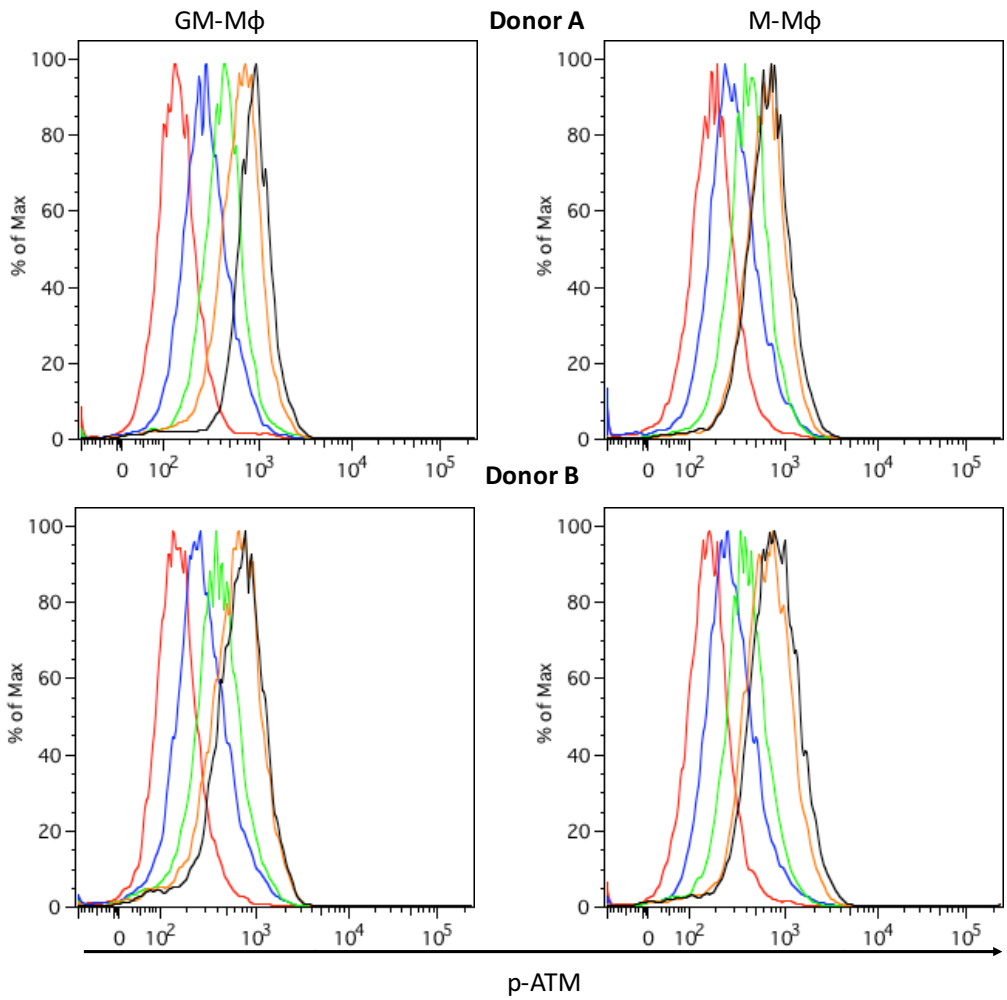


Figure 5.7 Effect of IR on ATM kinase is dose dependent. MoMφ were rested for 2h prior to treatment with 0-20Gy of IR and levels of ATM phosphorylation were confirmed by intracellular indirect staining for p-ATM. Red line-secondary Ab only, blue- 0Gy, green-1Gy, orange-6Gy, black-20Gy. Shown are 2 donors tested.

5.2.5 Effect of IR on cytokine secretion is propagated by ATM kinase

Recently ATM was reported to be involved in regulation of immune functions in addition to its role in check-point blockade [563, 564]. Therefore, we evaluated whether the inhibitory effect of IR on IL-23 secretion by MoMφ was propagated through an ATM-dependent signalling pathway. To achieve this, we utilised KU55933 which is a highly specific inhibitor of ATM kinase [673]. Firstly, we demonstrated by western blot the effectiveness of KU55933

in prevention of ATM phosphorylation. In agreement with our previous results, irradiation with 6Gy resulted in increased phsophorylation of ATM. The level of ATM phosphorylation was reduced by pre-treatment of cells with KU55933 (Figure 5.8).

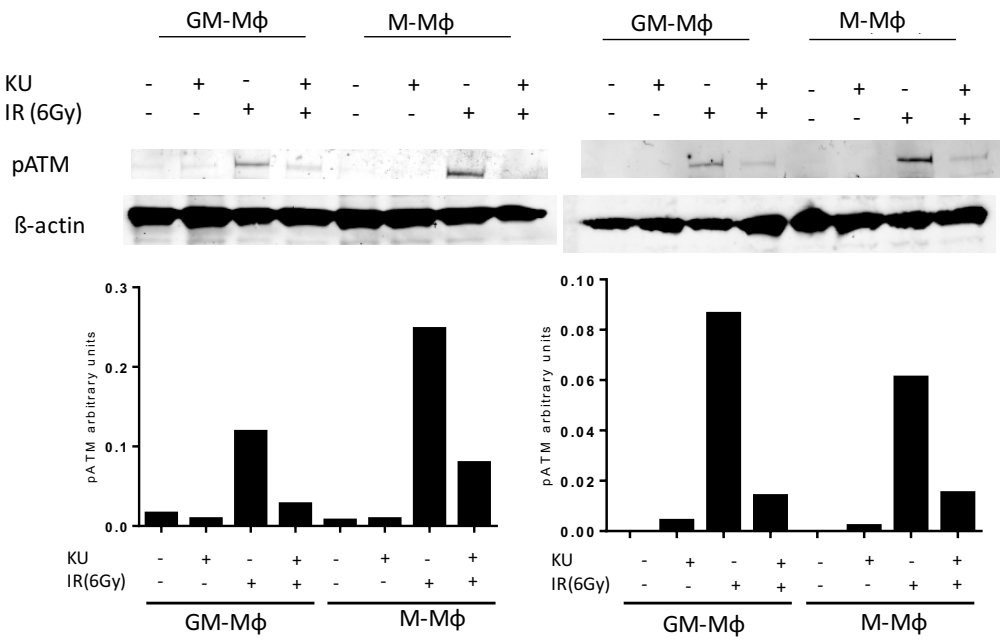


Fig5.8 KU55933 effectively inhibits ATM kinase. Western blot analysis of phosphorylated ATM expression of non-irradiated or irradiated (6Gy) MoMφ with or without pre-treatment with KU55933 (10μM). An example of 2 out of 6 donors tested. Graphs represent quantification of p-ATM levels normalised to b-actin.

Having shown the efficacy of KU55933 in inhibiting ATM we sought to assess the effect of ATM on IL-23 secretion by MoMφ. Interestingly inhibition of ATM resulted in a significant up-regulation of IL-23 by both non-irradiated and irradiated MoMφ activated with LPS/IFNγ in all donors tested (Figure 5.9). The increase of IL-23 in irradiated MoMφ was equal or higher than non-irradiated non-treated cells. Up-regulated IL-23 secretion was seen in MoM generated in the presence of GM-Mφ as well as M-Mφ

demonstrating independence of ATM regulation mechanism from specific culture conditions.

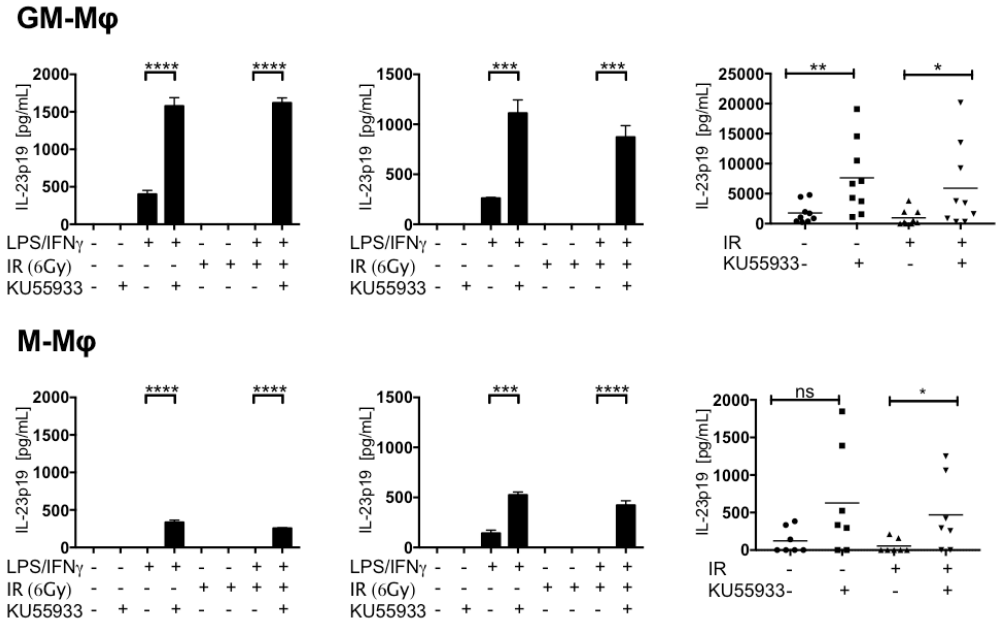


Figure 5.9 Effect of ATM inhibition on IL-23 secretion by MoMφ. Cells were pre-treated with ATM inhibitor (KU55933 10μM) for 1h prior to irradiation with 6Gy and activation with LPS/IFNg. IL-23 secretion was assessed 24h post activation by ELISA. Shown are 2 individual donors and summary graphs of all 9 donors tested. Data is shown as mean+SD for individual donors for each condition. Significance is assessed by t-test. For summary graphs data is shown as mean for each condition for all the donors and significance determined by 2-way Anova.

Since ATM kinase plays crucial role in cellular responses to DNA damage we evaluated whether the effect of ATM inhibition on IL-23 secretion was not connected with altered cells survival upon irradiation. MoMφ were treated with KU55933 1h prior to irradiation and viability was assessed 24h after irradiation by flow cytometric analysis of PI staining. Pre-treatment of MoMφ with ATMi did not affect their viability within 24h after irradiation (Figure 5.10).

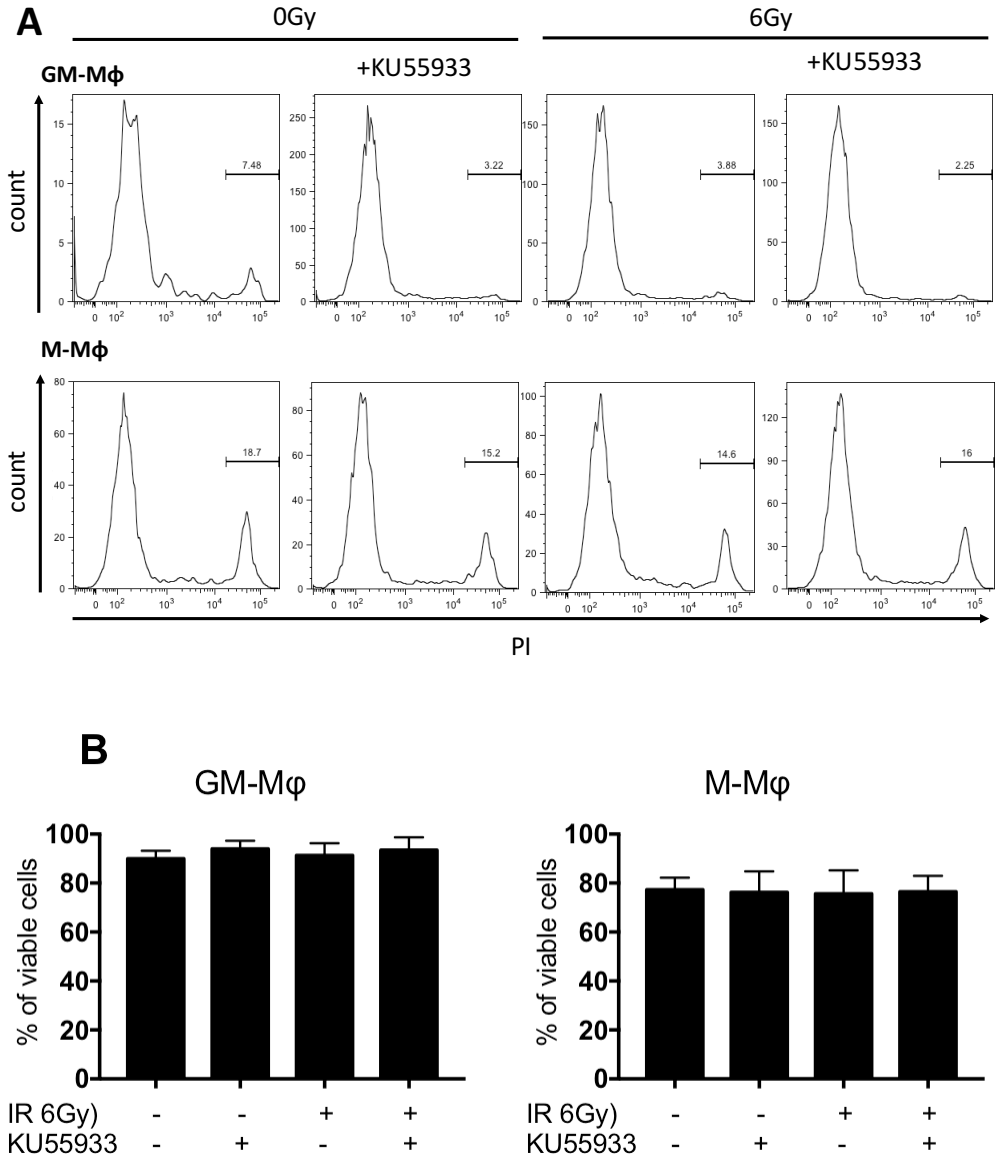


Figure 5.10 Effect of ATM inhibition on viability of irradiated MoMφ. MoMφ generated in the presence of GM-CSF or M-CSF were rested for 2h and pre-treated with KU55933 1h prior to irradiation. After 24h of incubation MoMφ viability was assessed by flow cytometric analysis of cells stained with PI. **A**-Shown is a representative of 3 donors tested. The histograms show the percentage of positive cells stained with PI compared to positive control treated with 25% DMSO. **B**- A summary figure of 3 donors. Shown is mean + SD for each condition.

5.2.6 ATM-dependent IL-23 secretion by MoMφ is TLR4-dependent

The triggering of IL-23 secretion requires activation of MoMφ with dual stimulus by LPS and IFNγ. Since inhibition of ATM kinase in combination with dual stimulation resulted in a significant up-regulation of IL-23 we sought to assess if the pathway triggered by LPS or IFNγ is affected by ATM. There was no secretion of IL-23 when ATM inhibitor was used alone or in conjunction with IFNγ (Figure 5.11). In 5 out of 8 donors there was significant up-regulation of IL-23 when cells activated with LPS were pre-treated with KU55933 suggesting that the effect of ATM inhibition on IL-23 secretion is at least partially TLR-dependent. However, the levels of IL-23 remained below of the levels secreted upon activation with combination of LPS/IFNγ.

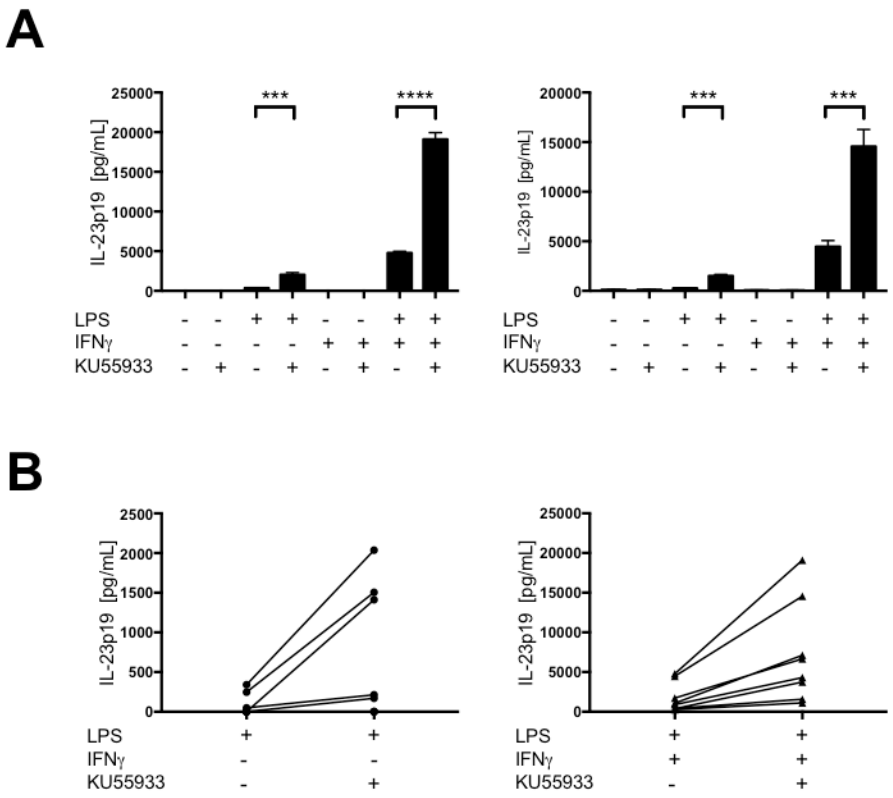


Figure 5.11 Inhibitory effect of ATM on IL-23 is TLR-dependent. GM-Mφ were pre-treated with ATM inhibitor for 1h or left untreated and activated with LPS and IFNg separately or in combination for 24h. IL-23 secretion was measured in collected supernatants by ELISA. **A**- 2 individual donors, **B**- summary graphs showing all 8 donors tested. Data is shown as mean+/-SD. Significance was assessed by t-test.

5.2.7 Inhibition of ATM triggers IL-23 secretion from Mφ2-like MoMφ

Next, we assessed whether ATM inhibition will be able to trigger IL-23 secretion from Mφ2-like Mφ. MoMφ were directed towards Mφ1 or Mφ2 by generation in the presence of GM-CSF or M-CSF respectively. Activation of GM-Mφ with LPS/IFNγ and M-Mφ with LPS resulted in the expected Mφ1/Mφ2 secretory profiles as shown in Fig 5.4 and Table 5.1. As expected pre-treatment of cells with ATM inhibitor 1h before activation resulted in significant increase of IL-23 secretion by GM-Mφ (Figure 5.12). Interestingly M-Mφ directed towards Mφ2 also secreted IL-23 upon pre-treatment with

KU55933 in 9 of 12 donors tested although at levels below those secreted by activated Mφ1 with or without treatment with KU55933.

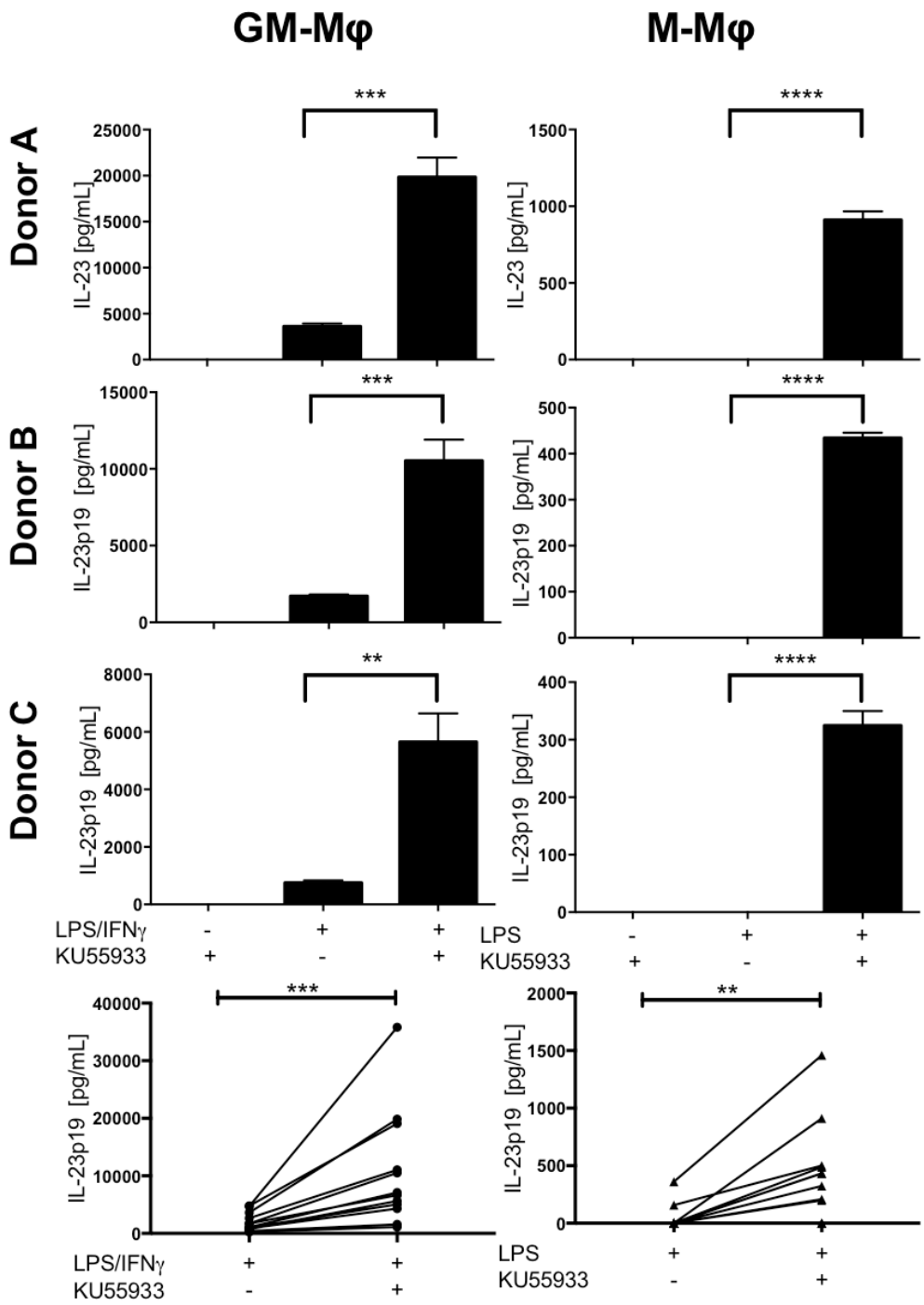


Figure 5.12 Inhibition of ATM triggers IL-23 secretion by Mφ2 MoMφ. MoMφ were generated in the presence of GM-CSF and activated with LPS/IFNg towards M1 phenotype or were generated with M-CSF and activated with LPS only towards Mφ2 phenotype. 1h prior to activation cells were treated with KU55933 (10μM) or left untreated. IL-23 secretion was measured in supernatants 24h post-activation by ELISA. Graphs represent 3 individual donors and summary graphs for all 12 donors tested. Data is shown as mean+/- SD. Statistical significance is assessed by student t-test for individual donors and 2-way Anova for grouped graphs.

5.2.8 Effect of IR-induced ATM is mediated by CREB/CBP interactions.

ATM was reported to regulate the activity of CREB transcription factor which is involved in IL-23 secretion mediated by PGE2 [289]. Therefore, we hypothesised that the inhibitory effect of ATM on IL-23 secretion by irradiated MoMφ is mediated by CREB. S121 is one of few residues on CREB associated with diminished function of this transcription factor. Radiation-induced phosphorylation of ATM coincided with phosphorylation of CREB at S121 (Figure 5.13). Pre-treatment of cells with KU55933 decreased the phosphorylation of ATM and resulted in reduced phosphorylation of CREB at S121. The changes in phosphorylation of ATM and CREB were closely followed by changes in IL-23 secretion by irradiated MoMφ. To confirm the role of CREB we utilised C-Ci, a small molecule inhibitor which blocks CREB interaction with CBP [674]. Pre-treatment of MoMφ with C-Ci resulted in the significant reduction of ATMi-enhanced production of IL-23 in all the donors tested (Figure 5.14).

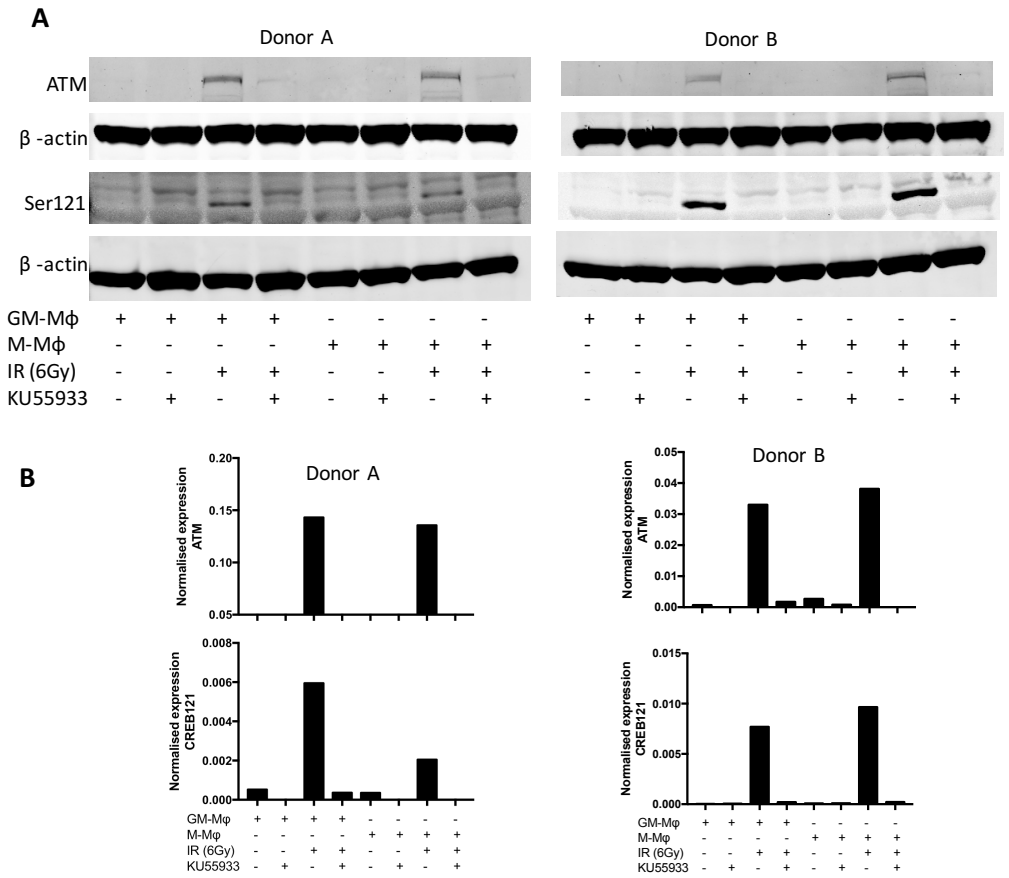


Figure 5.13 Regulation of CREB activity by IR-induced ATM. Cells were pretreated with KU55933 1h prior to irradiation with 6Gy or left unterated. Phospho-ATM and CREB phospho-S121 were examined at 1h following IR. **A-** shows western blots of 2 representative donors out of 3 donors tested. **B-** shows phospho-ATM and CREB phospho-S121 levels normalised by using the ratio of reading of the protein bands to the corresponding β-actin bands.

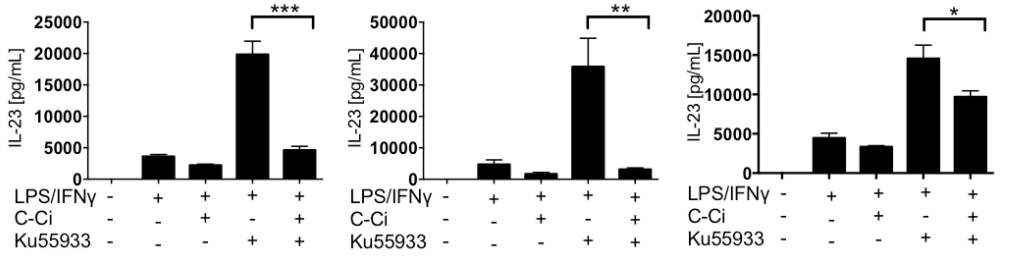


Figure 5.14 Effect of the inhibition of CREB-CBP interaction on ATM-dependent IL-23 secretion. GM-Mφ were pre-treated with 40μM C-Ci 1h prior to treatment with KU55933. 1h later cells were activated with LPS/IFNγ. IL-23 secretion was checked 24h after activation by ELISA. Graphs represent 3 individual donors. Shown are mean+SD. Significance was assessed by t-test.

5.3 Discussion

The preponderance of Mφ in the microenvironment of solid tumours has potential implications for local RT as both tumour and stromal cells are irradiated. However, the effect of therapeutic interventions on IL-23 secretion by Mφ has not been studied and the knowledge of IL-23 regulation and signalling pathways involved, remains poorly understood. We demonstrate for the first time that IR, at doses routinely used for cancer RT, selectively down-regulates IL-23 secretion by human Mφ. Importantly, the effect of IR and ATM on IL-23 secretion occurred regardless whether Mφ were generated in the presence of GM-CSF or M-CSF. Moreover, we describe a novel mechanism of IL-23 regulation in human Mφ where IR-induced phosphorylation of ATM, a key regulator of DDR response to DNA DSB, down-regulates IL-23 secretion through inhibition of CREB transcriptional activity.

The opposing roles served by IL-12 and IL-23 in anti-tumour immunity (namely Th1 vs Th17 induction respectively) [675] makes their study of particular importance for developing therapies against cancers well as autoimmune disorders. Our results demonstrate that IR down-regulates IL-23. Importantly there was no effect of IR on IL-12, IL-1β and IL-10 secretion as well as the ability of Mφ to present Ag to T-cells. IL-6 was also affected by IR, however, in contrast to IL-23, which was down-regulated regardless of the presence of GM-CSF or M-CSF during Mφ generation, IL-6 was consistently decreased only in GM-Mφ. M-CSF-generated Mφ are commonly used as a model of tissue Mφ [108]. However, our previous results demonstrated

significant difference between M-CSF- and GM-CSF-generated Mφ in the ability to secrete IL-23 upon activation with various stimuli (Chapter 4). Importantly, both M-CSF and GM-CSF are commonly up-regulated in human cancers, while GM-CSF have also been used as cancer vaccine adjuvant and to correct neutropenia following chemotherapy and radiation [676-680]. Our findings suggest that the regulation of IL-23 secretion by IR is independent on Mφ generation method and represent a real biological effect rather than an artefact of generation protocol.

Selective down-regulation of IL-23 secretion by IR without impacting the general inflammatory phenotype are surprising given the previously suggested role of IR in driving Mφ2 responses [681]. Those conclusions were however based mainly on murine models in contrast to our findings in human Mφ. More recently, Pinto *et al*. [646] showed that RT may drive Mφ towards pro-inflammatory phenotype through down-regulation of IL-10 production with concomitant up-regulation of HLA-DR and CD86 expression on human M-Mφ following multiple doses of IR. In contrast to this study we assessed Mφ phenotype after a single radiation dose and it is possible that repeating doses of IR are required to influence these markers.

IR stimulates activation of DDR responses which are governed by the serine/threonine kinase protein, ATM [682]. Although key function of ATM is to coordinate responses to DSB, several lines of evidence suggest its involvement in immune-regulatory function [564, 683, 684]. Deficiency in ATM expression has been implicated in autoimmune diseases such as MS and

RA [564, 683, 684]. Inhibition of ATM increases expression of innate immune response genes in drosophila while ATM deficiency in mice mounts a strong immune response [684, 685]. In addition, ATM is involved in IL-6 secretion by senescent FB in response to IR and oncogenic stimuli [686]. All together this evidence suggest that lack of ATM signalling leads to increased and sustained inflammatory responses. Our results showing the down-regulation of IL-23 secretion by irradiated Mφ occurs as an effect of ATM activation agree with this data. Limiting chronic inflammatory responses promoted by IL-23 without affecting acute inflammation driven by IL-12 in response to cell damage may act to limit pathology and facilitate tissue repair.

Inhibiting the function of ATM with a small molecule antagonist markedly increased IL-23 from both irradiated and non-irradiated Mφ. Due to its high selectivity and potency KU55933 is the inhibitor of choice for ATM studies [673]. It is a competetive inhibitor with an IC_{50} for ATM of 13nM while other targets require substantially higher concentrations to inhibit function e.g. DNA-PK, mTOR, PI3K, PI4K and ATR are inhibited with IC_{50} of 2500, 9300, 16000, >100000 and >100000nM respectively [673]. The 200-fold difference in IC_{50} between ATM and the next target makes KU55933 ideal for studies on DDR pathways. However, possible off-target effects should be considered while using inhibitors. Alternatively, a second ATM inhibitor could be used to confirm the phenotype observed with KU55399. There are several ATM inhibitors that would be suitable for this, such as, KU60019 (analogue of KU55399), CP466722 and KU59403. Another approach is a use of small interfering RNA. Our group made attempts to knock-down ATM expression

using a panel of SiRNA. However, we have not been able to achieve complete knock-down of ATM in human cells. In addition to incomplete knock-down one of the key issues we encounter is the susceptibility of APC to activation by manipulation. As described earlier ATM is a massive molecule with only 10% of its function mapped. Therefore, an ATM knock-down may result in an unintended inhibition of other functions of ATM.

The increase in IL-23 secretion by non-irradiated cells treated with KU55933 may suggest certain basal level ATM activity in Mφ. This hypothesis is supported by recent findings showing increased levels of NBS1, a component of MRN complex responsible for activating ATM, in Mφ activated with LPS and IFNγ [687]. Since activated Mφ produce large amounts of reactive oxygen species which cause DNA lesions, activation of DDR pathway is important to protect them from self-inflicted damage [687]. ROS are also induced by certain microbes which result in triggering inflammation through generating DSB [688]. This process in negatively regulated by DDR [688]. Overall this data suggests the involvement of DDR pathway and ATM in limiting immunopathology while promoting resolving of inflammation and tissue repair.

Finally, we demonstrated that the effect of ATM on IL-23 secretion is mediated via CREB and its interaction with CBP. CREB is a basic leucine zipper family transcription factor involved in the regulation of approximately 4,000 genes [689]. Activation of CREB via binding of its kinase-inducible domain (KID) to the transcriptional coactivator CREB-binding protein (CBP)

is required for cell survival [690]. In response to DBS ATM blocks this interaction by phosphorylation of inhibitory residues on CREB including S121 [691]. In addition to its function in DDR response, CREB has been involved in Mφ polarization promoting Mφ2 phenotype [569, 692]. While CREB-mediated induction of CBP was required for induction of anti-inflammatory genes such as *Arg-1, Il-10, Il-13ra* and *Msr1* in murine models, it seem to be dispensable for Mφ1 genes including *IL-1β, IL-12* and *Tnfα* [569]. However, the interaction between CREB and CBP induced by PGE2 or CD40 stimulated the production of IL-23 by DC through involvement of *IL23a* promoter activation [289, 693]. Our results support these findings and present IL-23 as a novel target for CREB/CBP in Mφ. Interestingly these results indicate that despite being one of the most important Mφ1 cytokines, IL-23 regulation in contrast to IL-12 is governed by mechanisms involved in Mφ2 polarisation. The involvement of CREB as a downstream effector of IR-induced ATM-dependent modulation of cytokine secretion supports the lack of effect of IR on other pro-inflammatory cytokines. Intriguingly, although CREB activation was reported to stimulate *IL-10* gene activation in Mφ, we did not see significant down-regulation of IL-10 secretion following irradiation. This may suggest the specificity of ATM effect on CREB/CBP. It is also possible that further translational or posttranslational mechanisms are needed for enhanced secretion of IL-10 in addition to the activated gene. The specific and independent regulation of individual cytokine secretion by Mφ is required to allow them to precisely respond to discrete signals from their environment. Interestingly Ruffell *et al.* denstrated that CREB/CBP is dispensable for IL-6 expression by murine BMMφ cultured in the presence of M-CSF. [569]. On

the other hand, NFκB has been implicated in induction of IL-6 gene expression of human Mφ generated from U937 cell line [694]. We may speculate that in humans CREB transcription factor is involved in IL-6 regulation by human GM- Mφ while NFκB promotes IL-6 in M- Mφ.

Overall our results on the effect of IR on Mφ may have important meaning for combinatorial RT and Immunotherapy protocols for cancer treatment. In addition, confirmation of a new regulatory mechanism for IL-23 secretion in Mφ can provide new targets for modulation of immune responses in cancer and auto-immune diseases. Although in our research we utilised a range of IR doses so far, we focused only on a short-time effects after a single dose of IR. It would be interesting to expand the time-scale of our experiments to include multiple doses of IR distributed over several days to observe the timeline of changes in Mφ functions in aim to identify the window for most efficient immunomodulation. So far, we focused on only one type of immune cell present in tumour stroma. Expanding our findings on other types of cells present in tumour microenvironment will provide a more complete view of the effect of IR tumour stroma. Therefore, in the next chapter, we will look at the effect of IR on another type of APC, DC which are commonly used for cancer therapy and their interactions with T-cells.

Chapter 6. Effect of IR on cytokine secretion by MoDC

6.1 Introduction

Tumour-infiltrating DC (TIDC) are a common feature of many solid tumours including ovarian, breast, colorectal, lung and renal [695]. However, they often exhibit an immature phenotype characterised by low co-stimulatory molecule expression [696, 697], poor Ag cross-presentation [696], and elevated expression of regulatory molecules and receptors [697, 698], factors that facilitate immune escape. Tumour cells inhibit or reverse DC maturation through a range of secreted mediators such as VEGF, TGFβ and IL-10, as well as display of inhibitory molecules such as PD-L1 and PD-L2 [697, 699, 700]. Studies show that the phenotype and number of TIDC is dynamic, changing with disease progression. In murine ovarian cancer models, PD1 expression on DC was minimal during early stages of tumour development but significantly increased during tumour progression [697]. Scarlett *et al.* demonstrated, in a spontaneous murine model of ovarian cancer, that disease progression associated with an increase in immune infiltrate while aggressive growth correlated with a switch from immune-stimulatory to immune-suppressive DC [701]. In agreement with these findings, depletion of DC early during disease promoted tumour expansion and aggression, whereas depletion later led to abrogation of tumour growth [702]. Kocián *et al*. also demonstrated correlation between maturation state of TIDC and disease recurrence in human colorectal cancer [703].

Numerous approaches have been developed to restore the function of DC in cancer patients. These include targeting the PD-1/PD-L1 or intra-tumoural injections of TLR agonists (CpG or Poly I:C) [704, 705]. Furthermore, the use

of DC in a form of cancer vaccines continues to be an active area of research. In this regard combining DC vaccine with RT resulted in induction of anti-tumour T-cell responses [636, 637, 706].

It is important to understand the functional consequences of irradiating DC in the context of tumour immunotherapy. DC are resistant to radiation induced apoptosis and do not show reduced viability even at high doses (up to 30Gy) [707]. The mechanism of such resistance is expression and up-regulation of DNA repair proteins [620]. However, a body of evidence suggests that direct exposure to radiation suppresses key functions. CCR7 expression is down-regulated on irradiated DC impairing their ability to migrate from tissues to draining lymph nodes [708]. In addition, irradiated DC are less effective in priming naive T-cells [707] or stimulating T-cell proliferation [709]. Importantly, irradiation of DC down-regulates the production of IL-12 without affecting IL-10 thus changing the balance in pro and anti-inflammatory stimuli towards tolerance [707].

Considering our findings on the regulation of Mφ cytokine secretion by IR we hypothesised that IR exerts similar effect on human DC. We have demonstrated that IR down-regulates IL-23 secretion by MoDC through reduction of transcription of the IL-23p19 gene and this resulted in reduced capacity of irradiated DC to stimulate Th17 responses. The effect of IR was mediated by ATM and inhibiting ATM activation with a highly selective ATM antagonist, KU55399 [673] resulted in increased IL-23 secretion by both irradiated and non-irradiated DC. Our data showed that an ATM regulated

pathway cooperates with TLR4 signalling for enhanced IL-23 production, but not with IFNγ. Moreover, we demonstrated that the action of ATM is mediated via CREB-CBP interactions. The inhibitory residue, S121, on CREB was phosphorylated when DC were exposed to IR. The expression of phospho-CREB-S121 was blocked by KU55933 which was associated with recovery of IL-23 production. Importantly, the increased level of TLR4 dependent IL-23 caused by ATM inhibition in DC was blocked by a specific CREB-CBP interaction inhibitor, C-Ci. This study describes a novel effect of IR on DC and has important implications for designing DC-based immunotherapy.

6.2 Results

6.2.1 DC characterisation and phenotype

The quality of MoDC preparations and their maturation status were confirmed by morphologic examination and assessment of their phenotypic and secretory profile. In microscopic examination MoDC were recognised as un-attached, small, rounded cells, which did not display dendrites or form groups (not shown). By 10h after stimulation with LPS/IFNγ DC acquired elongated shape suggesting migration and by 24h DC displayed dendrites and tightly clumped together. Phenotypic maturation was determined by examination of HLA-DR, CD80, CD86, CD83 and CD40 24 hours post-activation with LPS/IFNγ (Figure 6.1). Levels of HLA-DR, CD80 and CD86 were low on immature DC and increased upon maturation. CD83 was negative on immature DC and triggered by maturation while CD40 was similar on both immature and mature DC.

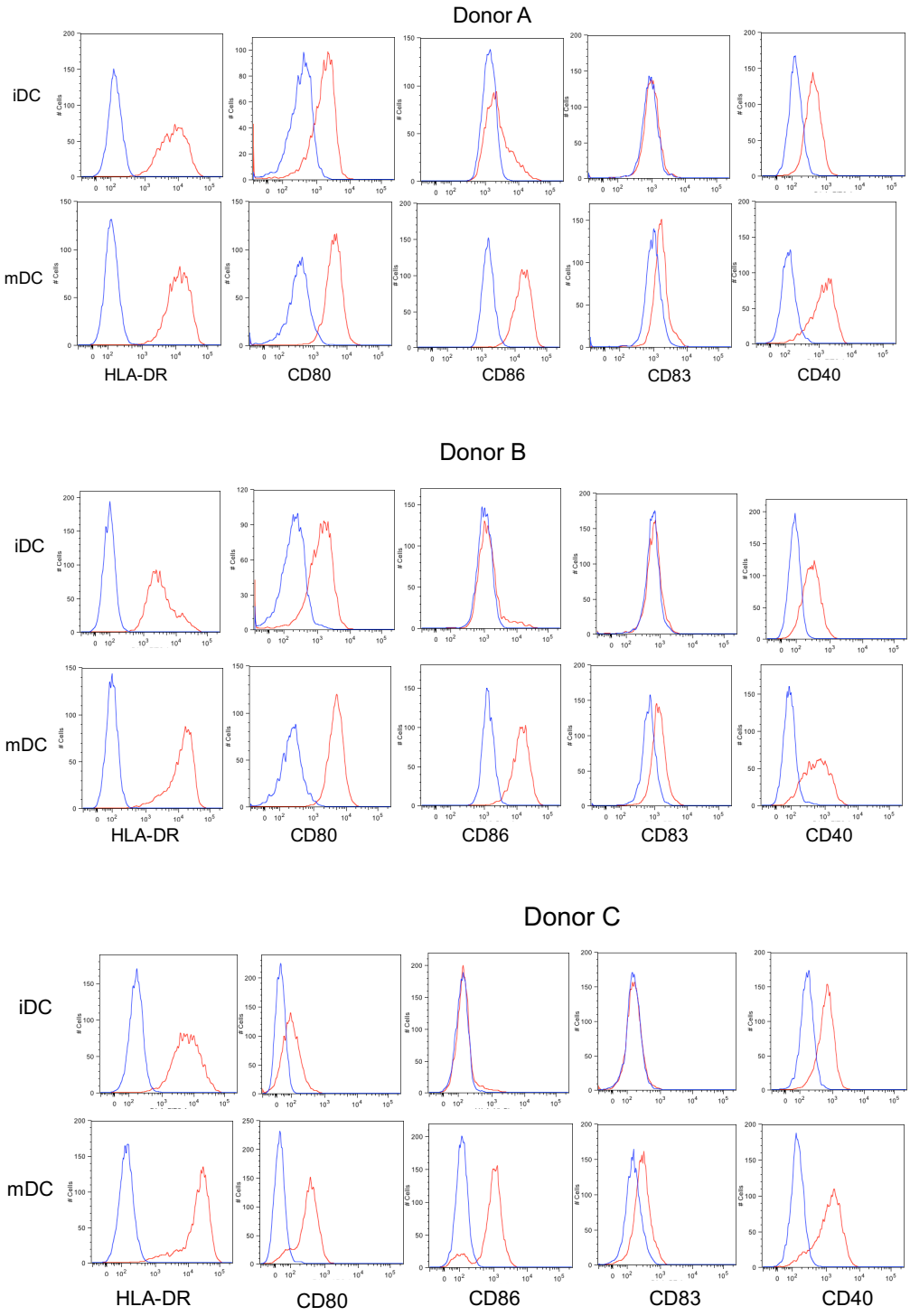


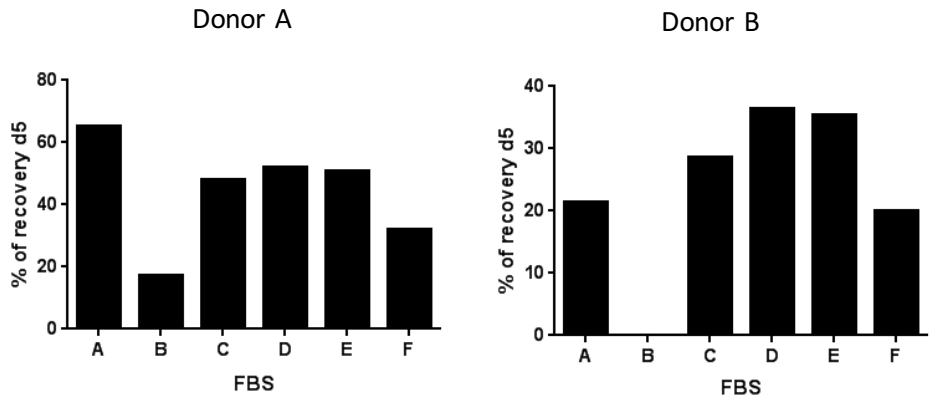
Figure 6.1 Phenotype of immature and mature MoDC. MoDC were activated with LPS/IFNγ or left untreated. After 24h their maturation status has been assessed by examining HLA-DR, CD80, CD86, CD83 and CD40 by flow cytometry. Cells were stained with antibodies (red lines) or matched isotype controls (blue lines) and acquired by flow cytometry. Shown are three representative donors.

As described earlier, FBS used in cell culture influences the quality as well as functional properties of immune cells [573]. Therefore, different batches and types of FBS were tested before moving to a new batch. Six FBS types were used for testing in a blind experiment. Important differences were found in both the numbers of generated DC as well as their cytokine secretion (Figure 6.2). FBS B did not sustain generation of DC from Mo with only 0-10% recovery of cells on day 5 and was dropped from further testing (Figure 6.2 A). FBS A produced highest number of DC in Donor A (60% of recovery) however, the effect was donor dependent and resulted in only 20% recovery in Donor B. Regardless of the high cell recovery in one of the donors, DC generated and activated in FBS A secreted low levels of IL-12 and IL-23 comparing to other batches used (Figure 6.2 B). Batches C, D and E produced similar numbers of DC (~45%) in Donor A and 30-40% in Donor B. Cells generated in FBS C secreted highest levels of IL-12, IL-23 and IL-10 while batch D generated DC with lowest cytokine secretion among the three batches. Culturing cells in medium with FBS F generated slightly less DC (30-37% recovery depending on donor) with secretion of cytokines falling within the ranges of DC generated with other batches.

Finally, we assessed whether different batches of FBS affected the response to ATM inhibition (Figure 6.2 C). KU55933 increased IL-23 secretion in both FBS batches tested as expected. Interestingly, the strength of these responses depended on the donor-FBS combination with donor C secreting higher IL-23 levels with FBS C while donor D secreted higher IL-23 levels with FBS F. In

summary, the effect of FBS depends both on the FBS batch as well as the donor used.

A



B

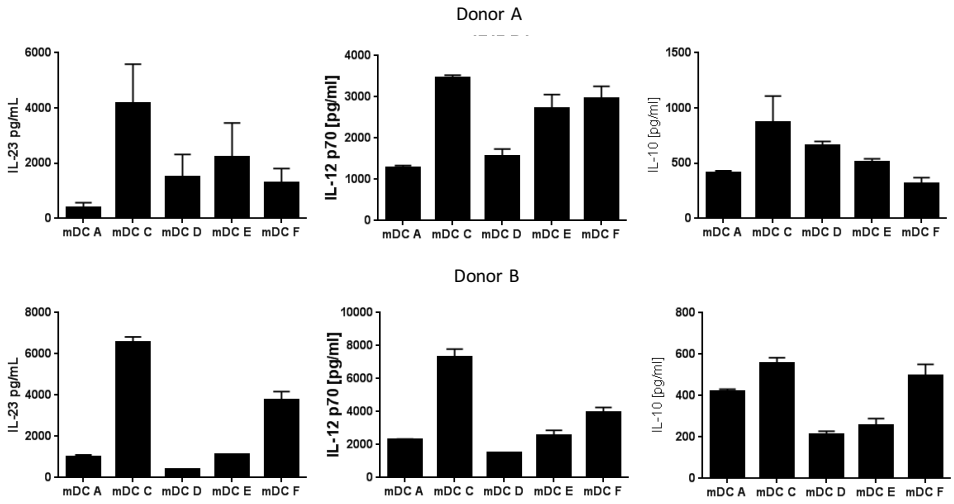


Figure 6.2 A,B. Effect of FBS in medium on DC generation. MoDC were generated by culture with IL-4/GM-CSF in RPMI in the presence of five different FBS and assessed upon collection. **A**- % of MoDC collected on day 5 comparing to the number of seeded Mo in two tested donors. **B**- comparison of the secretion of IL-23, IL-12 and IL-10 by MoDC generated and activated in medium containing different FBS in two tested donors. MoDC generated in DC medium containing different FBS were activated with LPS/IFNγ and cultured for further 24h in indicated DC medium containing indicated FBS. Cytokines levels were measured in supernatants by ELISA. **C**-Effect of FBS on MoDC response to KU55933. DC from two donors were pre-treated with KU55933 before activation and cultured in DC medium containing indicated FBS. IL-23 secretion was measured 24h post activation by ELISA. Blue bar - FBS C, red-FBS F.

C

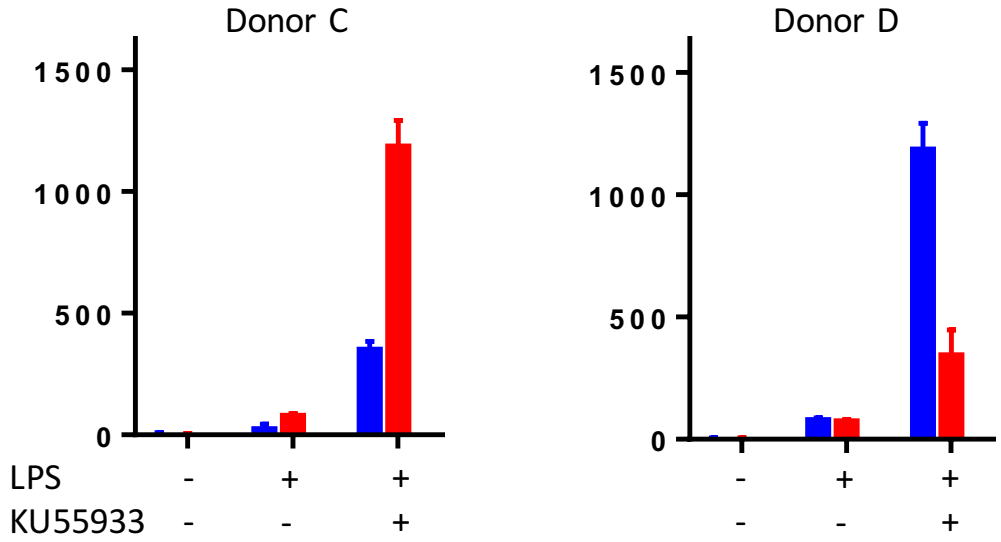


Figure 6.2C. Effect of FBS in medium on DC generation. MoDC were generated by culture with IL-4/GM-CSF in RPMI in the presence of five different FBS and assessed upon collection. **A**- % of MoDC collected on day 5 comparing to the number of seeded Mo in two tested donors. **B**- comparison of the secretion of IL-23, IL-12 and IL-10 by MoDC generated and activated in medium containing different FBS in two tested donors. MoDC generated in DC medium containing different FBS were activated with LPS/IFNγ and cultured for further 24h in indicated DC medium containing indicated FBS. Cytokines levels were measured in supernatants by ELISA. **C**-Effect of FBS on MoDC response to KU55933. DC from two donors were pre-treated with KU55933 before activation and cultured in DC medium containing indicated FBS. IL-23 secretion was measured 24h post activation by ELISA. Blue bar - FBS C, red-FBS F.

6.2.2 IR does not affect DC viability

Mφ are relatively radio-resistant and the effect of IR on IL-23 secretion does not result from decreased viability. Both DC and Mφ were found radio-

resistant in previous reports in contrast to Mo which are defective in DNA-repair mechanism [620, 646, 707]. Although for 2/3 donors survival decreased with time (~64% survival at 72h compared to ~83% at 24h) there was no significant reduction in viability of irradiated DC comparing to un-irradiated control group (Figure 6.3A,B).

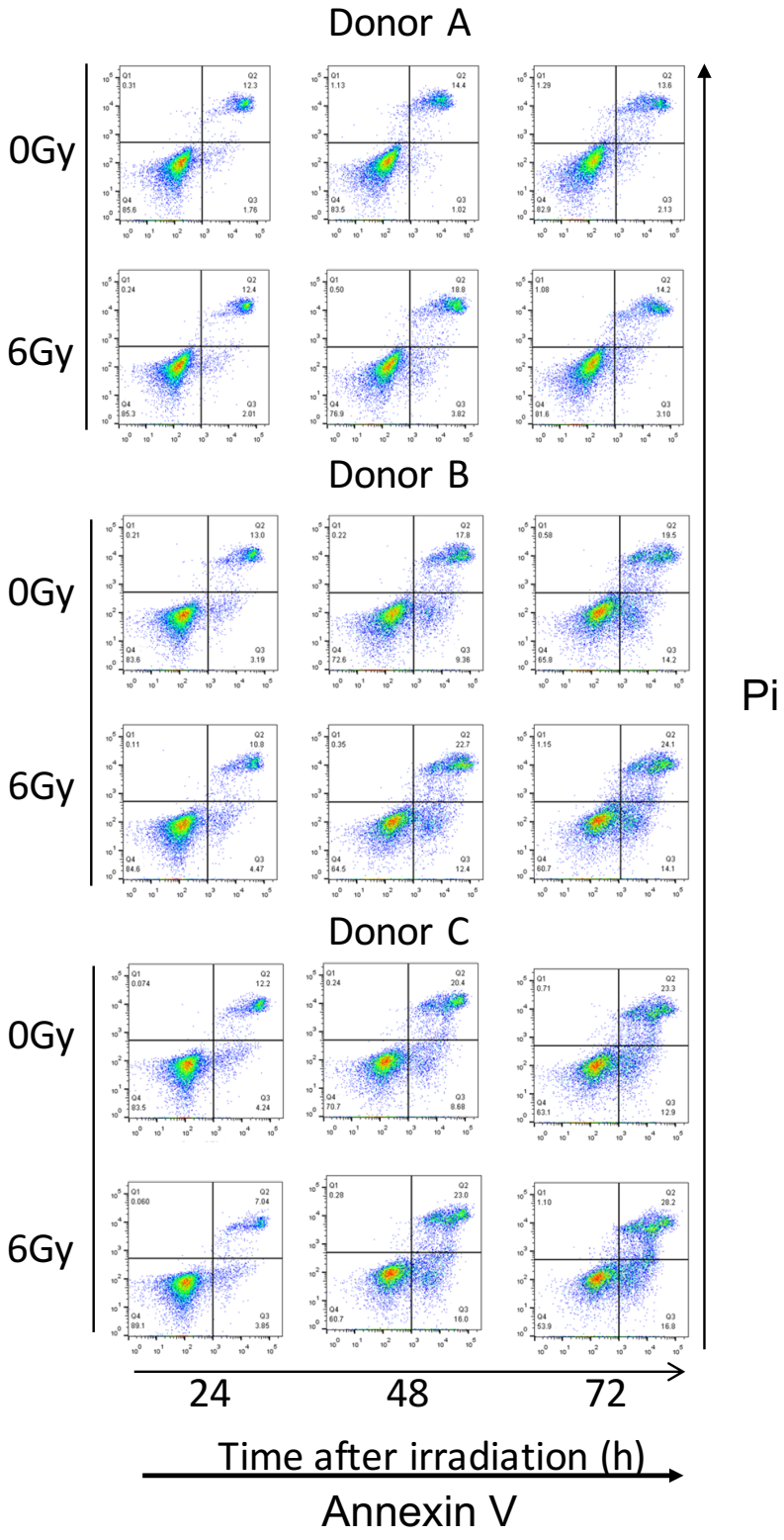


Fig 6.3A Determination of DC viability following exposure to IR. DC were irradiated with 6Gy or left un-irradiated and their viability was assessed 24,48 and 72h following irradiation by annexin V/PI double staining. Shown are scatter plots for 3 donors tested and a summary figure representing the frequency of cell survival (double negative cells) induced by IR in all 3 donors collectively.

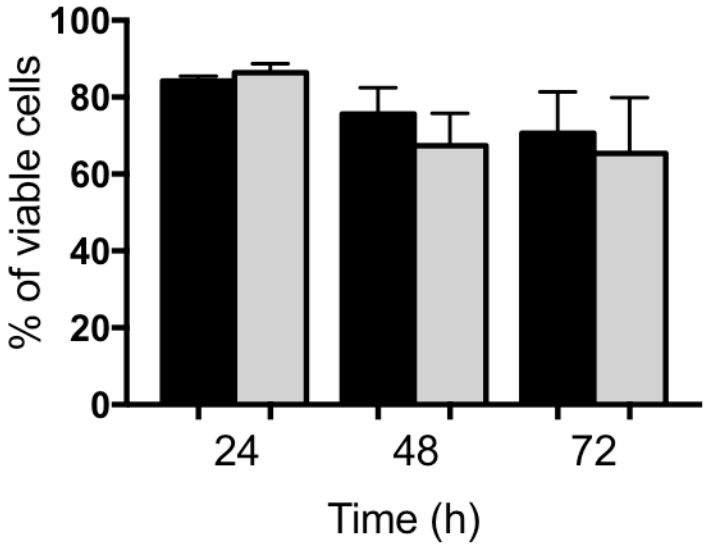


Fig 6.3B Determination of DC viability following exposure to IR. DC were irradiated with 6Gy or left un-irradiated and their viability was assessed 24,48 and 72h following irradiation by annexin V/PI double staining. Shown are scatter plots for 3 donors tested and a summary figure representing the frequency of cell survival (double negative cells) induced by IR in all 3 donors collectively.

6.2.3 IR modulates cytokine secretion by MoDC

Stimulation of MoDC with LPS/IFNγ induced IL-23 production which was significantly lowered ($p<0.05$) by pre-treatment with IR and occurred in all donors tested (Figure 6.4 A, B). Unstimulated cells did not secrete IL-23 regardless of irradiation status (Figure6.4 A). The maximal suppression of IL-23 by IR occurred at 6Gy (($p<0.001$ Figure 6.4 C).

To determine which stage of cytokine production is affected by IR transcription levels of the IL-23-specific *IL-23A* gene or the common *IL-12B* gene that encodes the p40 chain were evaluated. Irradiation of DC (6Gy) significantly suppressed transcription of *IL-23A* when compared with non-

irradiated DC. Although the effect of IR on *IL-12B* was also inhibitory it was nevertheless less marked than on *IL-23A* (Figure 6.4 D).

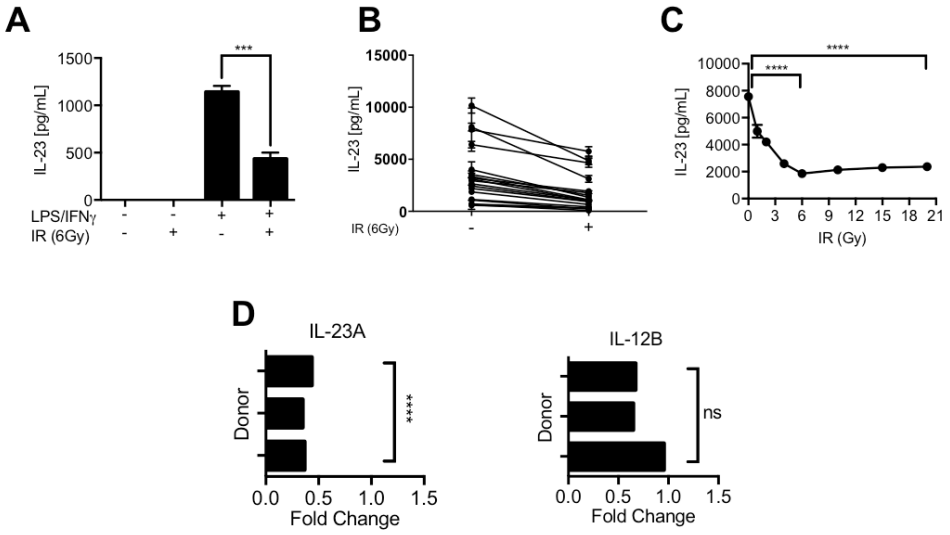


Figure 6.4 Effect of IR on IL-23 secretion by MoDC. A, B-IR downregulates IL-23 by MoDC. MoDC were activated with LPS/IFNγ one hour post irradiation with 6Gy or left untreated. IL-23 secretion was assessed by ELISA 24h after activation. **A**- representative donor, **B**-all 18 donors tested, **C**-Effect of IR on IL-23 secretion is dose dependent. DC were treated with various dose of IR (0-20Gy) before activation. IL-23 secretion was measured 24h after activation by ELISA. Shown is a representative donor out of 3. **D**- Changes in IL-23 secretion were associated with decreased transcription of the IL-23A gene and IL-12B gene as shown using Q-RT-PCR in 3 donors. Statistical significance was measured by the student t-test.

Previous results showed that IR differentially affects cytokine secretion by Mφ. Furthermore, earlier reports suggested that IR skewed cytokine profile towards tolerance [707]. Therefore, we assessed the effect of IR on a panel of cytokines (Figure 6.5, Table 6.1). The inhibitory effect of IR was restricted to IL-12 and IL-23. IR potently down-regulated IL-23 ($p<0.0001$) while its effect on IL-12, although significant, was less pronounced ($p<0.0005$). In contrast, IL-27, IL-6, TNFα and IL-1β and IL-10 were unaffected.

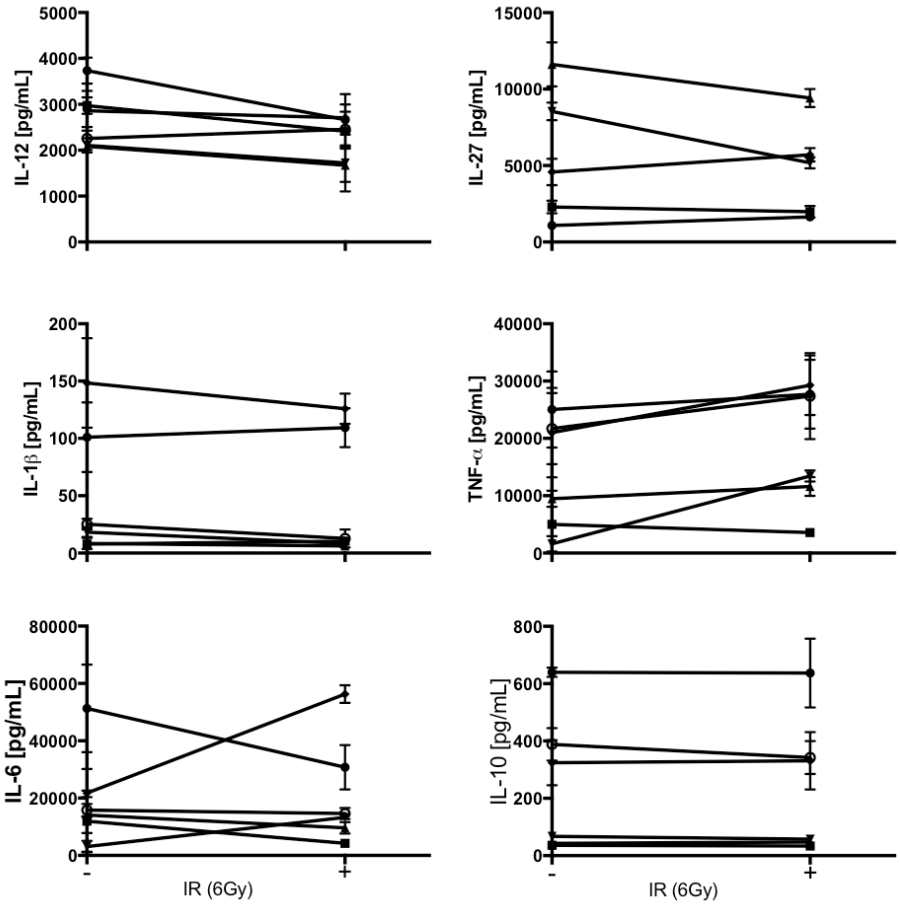


Figure 6.5. Effect of IR on cytokine secretion by mDC. DC were irradiated (6Gy) or non-irradiated before activation with LPS/IFNγ. Supernatants were collected following 24h incubation and secreted IL-23, IL-12, IL-27, IL-1β, TNFα, IL-6 and IL-10 were measured by ELISA. All experiments were repeated at least 5 times in triplicate.

Cytokine	% remaining secretion after IR	% of SD	P value
IL-23	43	11	<0.0001
IL-12	89	16.3	0.0005
IL-27	100.9	36.6	ns
IL-1β	93.5	34.4	ns
TNFα	111.8	29.6	ns
IL-6	174.1	114.4	ns
IL-10	95.9	6.2	ns

Table 6.1 Effect of IR on cytokine secretion by mDC. DC were irradiated with 6 Gy or left non-irradiated before activation with LPS/IFNγ. Supernatants were collected following 24h incubation and secreted cytokines were measured by ELISA. All experiments were repeated at least 5 times in triplicate and significance was assessed with 2-way ANOVA.

6.2.4 IL-23 secretion by MoDC is regulated by ATM

To evaluate the involvement of ATM kinase in the regulation of IL-23 secretion by MoDC we exposed DC to 6Gy to activate ATM. As expected the level of phospho-ATM (S1981) increased in moDC 15 min after irradiation (4/4 donors). Treatment with ATMi (KU55933 10μM) prior to radiation prevented ATM activation (Figure 6.6). These results were further confirmed by intracellular flow cytometry (Figure 6.7).

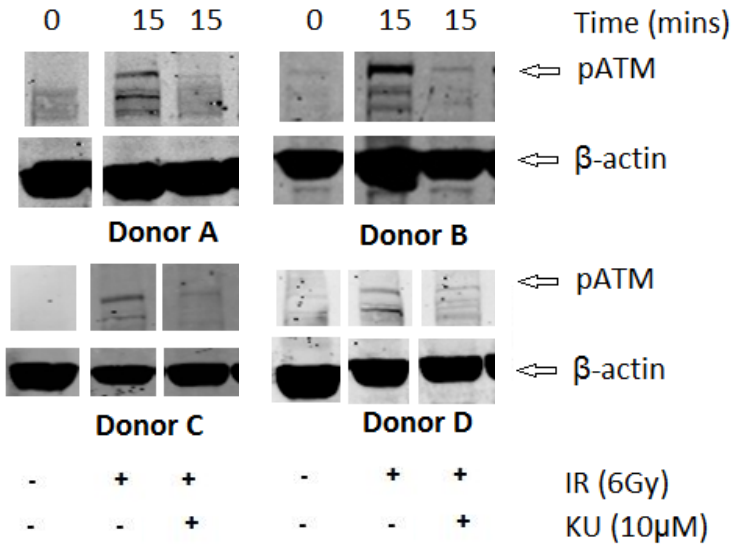


Figure 6.6 Activation of ATM following IR. Cells were pretreated with KU55933 1h prior to irradiation with 6Gy or left untreated. Phospho-ATM was examined at 1h following IR. Shown are WB for 4 tested donors.

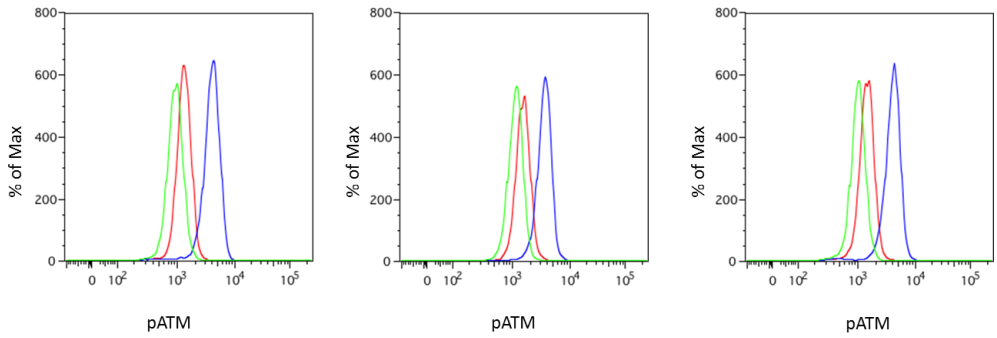


Figure 6.7 Phosphorylation of ATM following irradiation and treatment with KU55933. DC were exposed to 6Gy of IR with or without pre-treatment with KU55933 for 1h. The phosphorylation of ATM was examined 1h after irradiation by indirect intracellular staining with a phospho-ATM-specific antibody, green line: immature DC, blue line: 6Gy X-ray exposed DC, red line: KU55933 pretreated DC after 6Gy X-ray exposure, three plots represent 3 donors.

Since irradiation suppressed IL-23 secretion, we proposed that inhibition of ATM phosphorylation would reverse this effect. Indeed, blocking ATM in TLR4 stimulated MoDC prior to IR abolished the effect of IR and consequently IL-23 secretion was increased in 11/11 donors (Figure 6.8). Pre-treatment of TLR4 stimulated MoDC with KU55933 significantly increased

IL-23 levels in non-irradiated cells which were equal to those from irradiated cells. The effect of KU55933 was TLR4-dependent as there was no IL-23 secretion by DC treated with KU55933 alone or in conjunction with IFNγ (Figure 6.8).

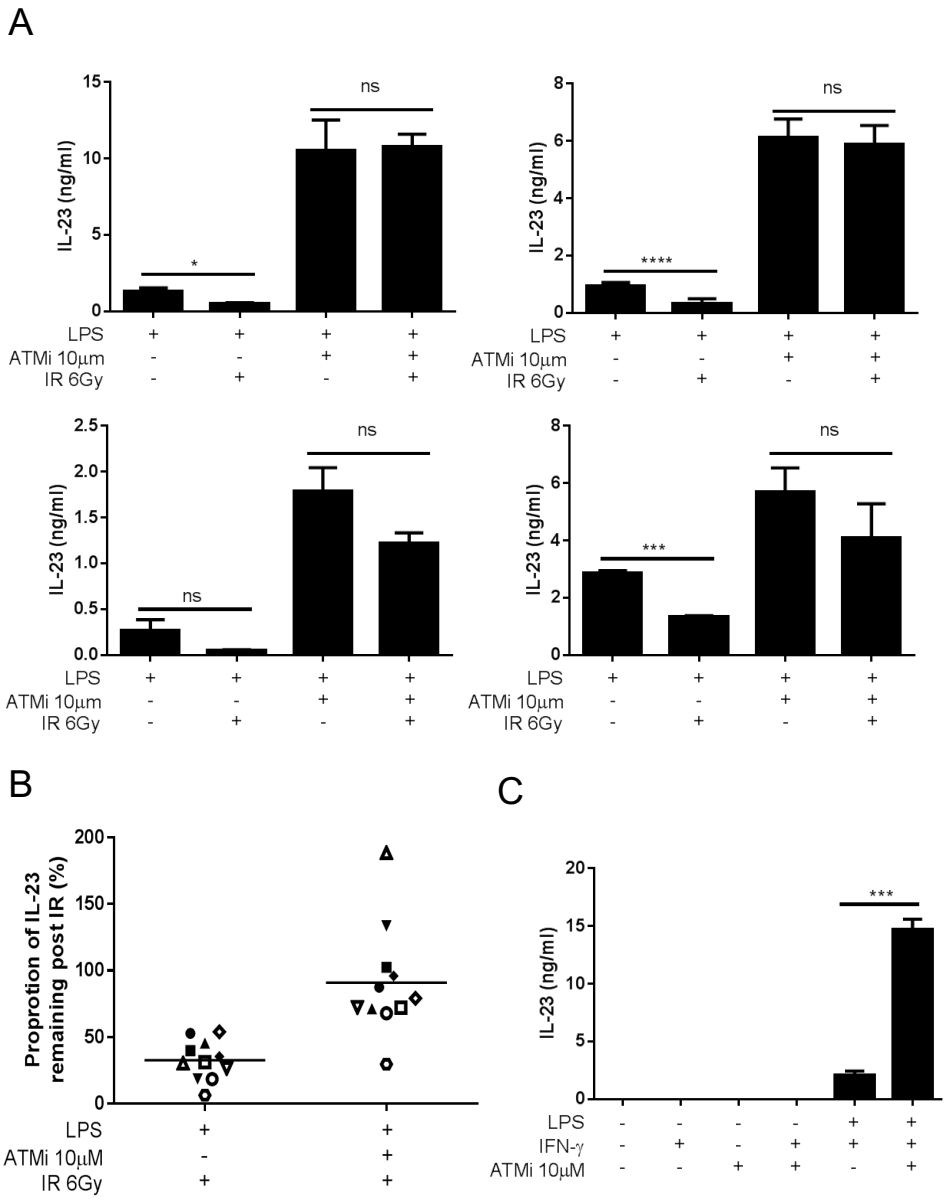


Figure 6.8 Effect of ATM inhibition on IL-23 secretion by MoDC. **A-** ATM inhibition up-regulates IL-23 secretion by MoDC. MoDC were exposed to ATMi, KU55933 (10μM) for 1 hr or left untreated prior to 6Gy IR and then immediately activated with LPS. Data from 4 donors out of 11 are presented, **B-** shows the percentage change in IL-23 production following IR in each indicated treatment group for 11 donors where individual donor is symbolized by a discrete shape. **C**-ATM effect is TLR4 dependent. MoDC were treated with KU55933 (10μM) 1h prior to stimulation with LPS and IFNγ and IL-23 were measured in supernatants 24h after stimulation. Shown a representative donor of 5 donors tested. Statistically significant differences were determined by the unpaired student's t-test.

Importantly, ATM inhibition in MoDC did not impact significantly on their survival following exposure to X-rays. The viability of irradiated or untreated DC pre-treated or not with KU55933 was assessed. There was no significant increase in cell death up to 72h after irradiation (Figure 6.9). Furthermore, viability was not adversely affected by KU55933 regardless of irradiation in any of the donors tested.

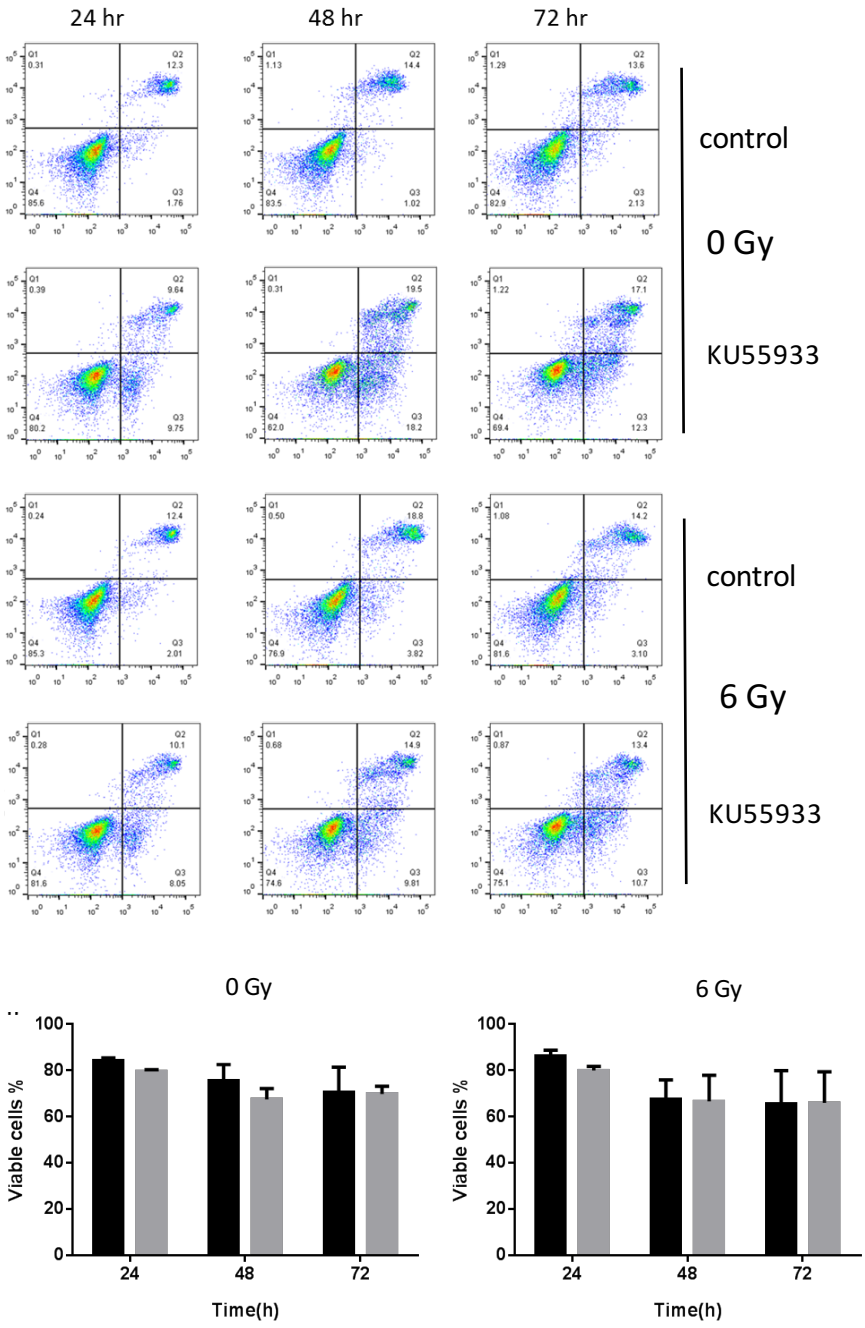


Figure 6.9 Effect of ATM inhibition on MoDC survival. MoDC were treated with KU55933 10μM and 1h later irradiated or left untreated. Cell death was assessed by annexin V and PI staining in flow cytometry at 24, 48 and 72h after irradiation. **A**-scatter plots of one out of 3 donors tested. **B**-summary of 3 donors.

6.2.5 The effect of ATM on IL-23 is mediated through CREB/CBP interactions

CREB is an important transcription factor under the influence of ATM as described earlier. It is also essential for PGE2 mediated IL-23 expression [710]. Therefore, we hypothesised that CREB was involved in mediating the effects of ATM. Irradiation of DC resulted in phosphorylation of ATM (S1981) and at the inhibitory S121 residue on CREB (Figure 6.10 A). Inhibition of ATM with KU55933 resulted in reduced phosphorylation of CREB at S121.

To further evaluate the role of CREB, C-Ci, an inhibitor of CREB-CBP interactions [674], was used. C-Ci did not affect LPS-dependent IL-23 at any of the concentrations used (Figure 6.10 B). However, ATMi-dependent IL-23 release was diminished to the control level by 10μM C-Ci in all the donors tested, suggesting the ATM effect on IL-23 is mediated by CREB-CBP (Figure 6.10 C).

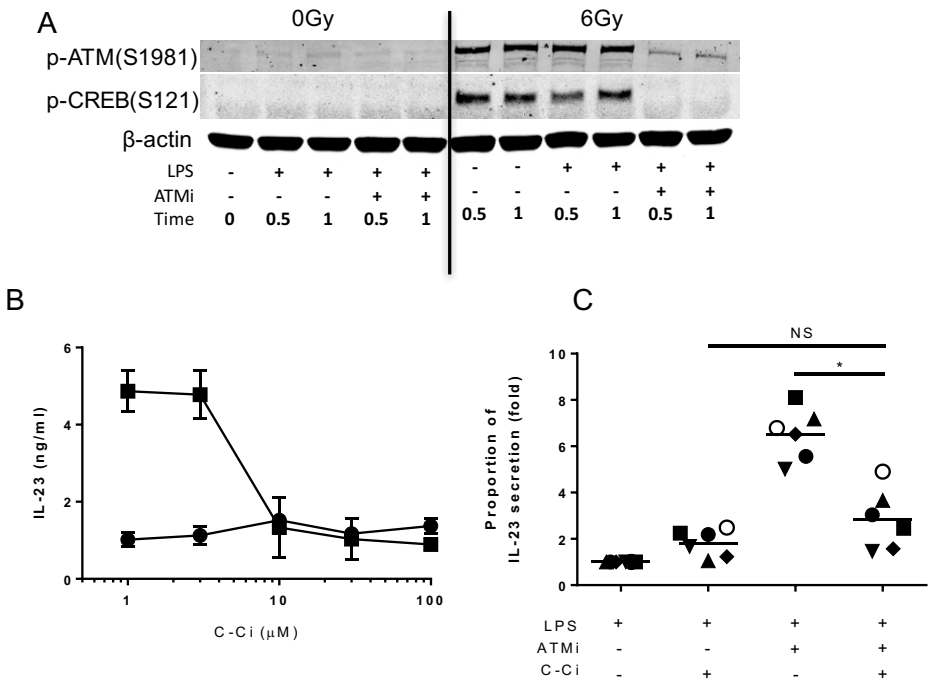


Figure 6.10 ATM-dependent inhibition of IL-23 is mediated through CREB/CBP interactions. A-Western blot showing phosphorylation of ATM and CREB121 of MoDC irradiated with 6Gy or left untreated following 1h pre-treatment with KU55933 10μM. Cells for WB were collected 1h after exposure to IR. Shown a representative donor out of 3 donors tested. **B**-Effect of C-Ci on ATM-dependent regulation of IL-23 is dose-dependent. MoDC received different concentrations of C-Ci (1μM, 3μM, 10μM, 30μM, 100μM) 1h before treatment with KU55933 10μM 1h followed by LPS 1h later (squares) or LPS only (circles). IL-23 levels were measured by ELISA from supernatants collected 24h after stimulation with LPS. **C-** Summary graph of six donors for a single dose of C-Ci (10μM). Statistical significance was measured by one-way anova.

6.2.6 Effect of IR-mediated IL-23 down-regulation on Th-17.

Finally, we examined the capacity of irradiated MoDC to polarise naïve CD4 T-cells to Th17 cells. Activation of Naïve T cells through anti-CD3/anti-CD28 alone resulted in a small number of IL-17A production by T-cells which was significantly increased in the presence of IL-1β and IL-6 and further enhanced by addition of IL-23 (Figure 6.11). Replacing cytokine mixture with a supernatant from LPS/IFNγ activated DC primed prominent Th17 responses in

all donors tested. In contrast, irradiated DC primed significantly lower Th17 responses in two out of three donors tested which correlated with the decreased secretion of IL-23. In one donor irradiation of DC (6Gy) did not significantly reduce their ability to stimulateIL-17 secretion by T-cells.

A

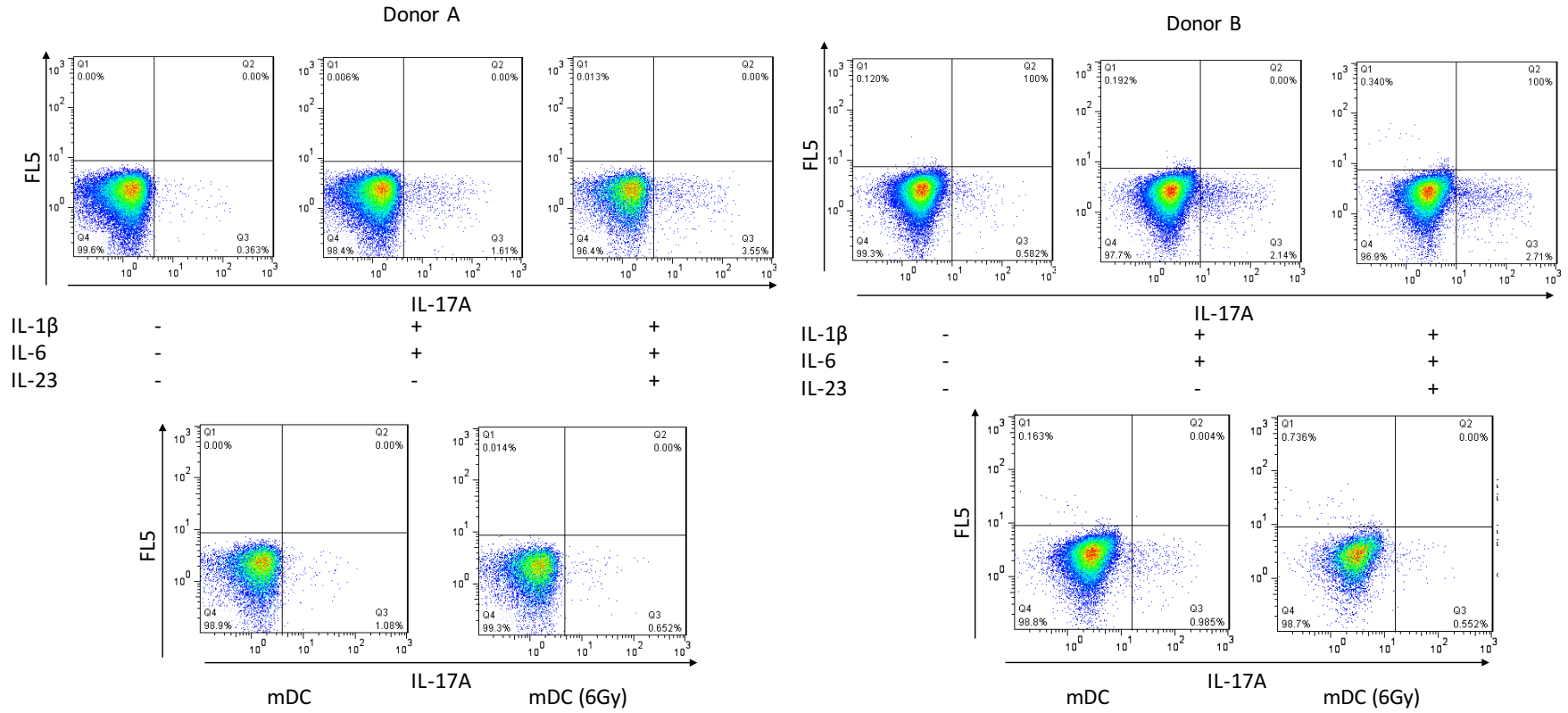


Figure 6.11A Effect of IR on DC ability to stimulate Th17 responses. Naive CD4+CD45RA+T cells were co-stimulated for 5 d with anti-CD28/CD3 in the presence of recombinant cytokines or MoDC supernatants. After resting for 2 d, cells were re-stimulated with anti-CD3/anti-CD28, treated with Brefeldin A to inhibit cytokine secretion and IL-17A release was determined by FACS. Data are 2 representatives of 3 donors.

B

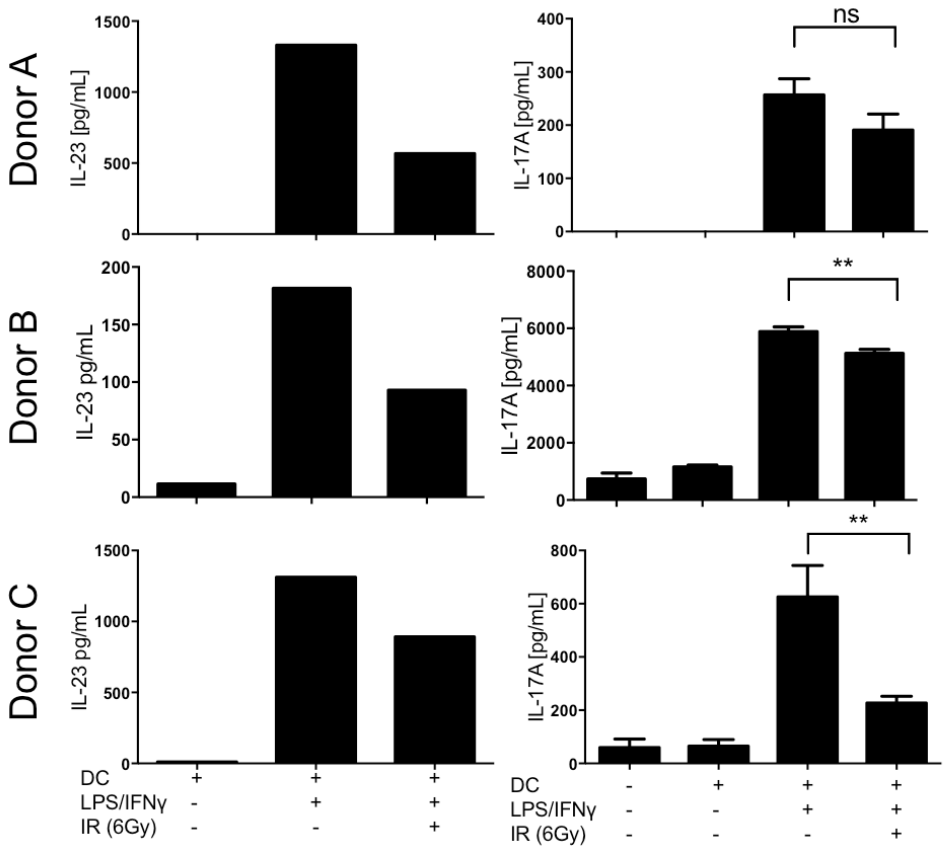


Figure 6.11B Effect of IR on DC ability to stimulate Th17 responses. Naive CD4+CD45RA+T cells were co-stimulated for 5 d with anti-CD28/CD3 in the presence of recombinant cytokines or MoDC supernatants. Cytokine secretion was measured 48 hours after re-stimulation with anti-CD3/anti-CD28 by ELISA. Shown are 3 tested donors. The level of IL-23 was measured in singlicate. IL-17 levels were measured in triplicates and statistical significance of IL-17 changes were measured by unpaired student's t-test.

6.3 Discussion

The importance of DC in tumour immunotherapy is well acknowledged and preclinical studies and early patient trials continue to be developed [711]. Both *in vitro* applications of DC as well as *in vivo* immunotherapy strategies often involve irradiation of DC be it incidental during RT or part of combined modality treatment [636, 707].

We demonstrate that IR down-regulates IL-23 and to a lesser degree IL-12 secretion by human DC whilst the secretion of IL-27, IL-6, IL-1β, TNFα and IL-10 remained unaffected. Importantly the effect of IR on DC inadvertently affected their ability to prime Th17 responses. IL-23 and IL-12 belong to the same family and share a common subunit p40 encoded by *IL12B* gene. In agreement with these results, our data shows that IR reduce the production of both cytokines at transcriptional level mainly affecting *IL23A* gene and to a lesser extend the *IL12B* gene. Interestingly, another cytokine from the IL-12 cytokine family, IL-27, has not been significantly affected by IR. Although IL-27 belongs to the same family it does not share the p40 subunit but instead it comprises of the EBI3 which is homologous to IL-12p40 subunit [712]. It suggests therefore that the effect of IR is specific mainly to the p19 and to a lesser extend to p40 subunits.

Previous studies describe conflicting findings on cytokine secretion by irradiated DC. Merrick *et al*. (2005) observed down-regulation of IL-12 secretion at doses of IR as low as 2Gy [707] while Shigematsu *et al*. (2007) reported increased expression of IL-12 at 0.05Gy and this effect was reversed to the level of non-irradiated DC at 1Gy [713]. The disparity between these findings may be due to different models (human vs murine DC) and irradiation protocols (low vs high IR dose) used. It is plausible that various doses of IR exert differential effect on IL-12 secretion where lower doses promote while higher doses inhibit it. In addition, our results suggest differential donor sensitivity to IR regarding IL-12 secretion. IL-12 was down-regulated in four out of six donors while in two it remained unaffected when DC were irradiated with 6Gy. It is possible that by further increasing the dose of IR we may see inhibition of

IL-12 in all the donors. However, it is important to note that the maximum impact of IR on IL-23 secretion occurred at 6Gy and did not increase with increasing radiation dose. Whether similar dose-dependency occurs for IL-12 needs to be further elucidated. It is also plausible that with the decrease of IL23p19 subunit there is less competition between IL-23 and IL-12 for the shared IL-12p40 subunit, therefore making more p40 available for IL-12 generation.

Importantly, although IL-23 was consistently down-regulated at 6Gy in all donors, IL-10 was unaffected agreeing with previous reports on MoDC [707]. This supports the notion postulated by Merrick and colleagues that IR at therapeutic doses directs immune responses towards tolerance due to changing the balance between pro- and anti-inflammatory cytokines.

Similar to our findings concerning Mφ, the reduction of IL-23 secretion in DC by IR was regulated by ATM and mediated by CREB/CBP interactions. As described before ATM is a serine/threonine kinase protein which co-ordinates cellular responses to repair the damage following IR-induced DNA double strand breaks [620, 714]. In addition, ATM dysfunction has been linked with immune disorders [715]. To illustrate the involvement of ATM in regulation of IL-23 secretion by DC we used an ATM inhibitor, KU55933. Interestingly, the inhibition of ATM with KU55933 resulted in an increase of IL-23 secretion by irradiated but also non-irradiated DC. As described earlier KU55933 is a well-researched and highly specific inhibitor [673]. Although possible off-targets effects of KU55933 should be considered it may be suggested that, similarly to our earlier findings using Mφ, there is a certain level of ATM constitutive activity in DC which regulates IL-23 secretion.

CREB and CBP act as positive transcription factors in the PGE2-induced upregulation of *Il23a* gene in TLR- or TNFα- stimulated bone marrow-derived DC [289]. In this study, we have shown that CREB phosphorylation is a regulator of ATM dependent IL-23 signalling. Following irradiation, ATM phosphorylated CREB at Ser-121 in DC. This is consistent with the finding of Thr-100, Ser-111 and Ser-121 as reported ATM phosphorylation sites in response to genotoxic stress [716]. KU55933 prevented IR-induced activation of ATM and abrogated CREB phosphorylation at Ser-121. It is therefore plausible that activated ATM suppresses IL-23 gene expression upon IR by antagonizing CREB transcriptional activity.

Importantly the reduction in IL-23 in irradiated DC was not caused by decreased viability. These findings agree with earlier report by Merrick *et al.* which demonstrated that human DC are resistant to radiation-induced apoptosis [707]. Furthermore, DC and Mφ show a high level of resistance to DNA damage due to genotoxic stress and are very efficient in repairing SSB and DSB ensuring cell survival [717]. Both these cells produce high level of reactive oxygen species (ROS) as a defence mechanism against infections [718]. This requires an effective repair mechanism preventing self-sustained damage. ATM inhibition did not sensitise DC towards radiation. This was expected as ATM mainly functions in DSB repair through HR and this typically occurs in proliferating cells [719]. Furthermore, DC are competent in repairing SSB through the BER pathway, thus minimizing the generation of DSB that arise from two SSB facing each other [717].

In summary, IR adversely affected IL-23 in human DC through activation of ATM and this impacted on Th17 responses. Pharmacological inhibition of ATM enhanced IL-23 secretion. Importantly, irradiation and inhibition of ATM did not impact on viability. These findings have important implications for designing combined immunotherapy and RT protocols for cancer. Furthermore, manipulating ATM or CREB activation in the immune system creates new opportunities to enhance or repress Th17 responses. This in turn has implications for therapeutic approaches based on immune-modulation in vaccines and in autoimmunity.

Chapter 7. Effect of Fibroblasts on the response of antigen presenting cells to radiation.

7.1 Introduction

Upon activation by stress or danger, APC secrete cytokines and migrate to regional LMN to activate and orchestrate T-cell responses [3]. However, prior to entering lymphatic vessels, APC are in contact with a range of stromal cells including FB. This interplay between APC and stroma plays a crucial role in directing T-cell responses and its dysfunction may lead to the development and maintenance of autoimmune disorders and tumours [3, 720]. In liver, breast and ovarian tumours FB promote tolerogenic DC while TAF from pancreatic cancer skew DC responses towards Th2 [721-723]. Moreover, pancreatic TAF strongly promote Mφ2 responses at the stage of their differentiation from Mo as well as by reverting Mφ1 polarisation into Mφ2 [724]. Lung FB are implicated in the pathology of COPD and asthma through directing DC trafficking to airways and consequently maintaining chronic inflammation *in situ* via CCL2, CCL20 secretion [725]. On the other hand, activated dermal FB promote DC maturation and migration to the LMN [3, 726]. A recent study conducted by Schrimmer and colleagues demonstrated that stromal modulation of IL-23 secretion by DC and resulting promotion of Th17 responses can be implicated in the maintenance of psoriasis [727]. This model proposes that dermal FB respond to TNFα and IL-1β, secreted from activated DC, by producing PGE2. Fibroblast-derived PGE2 acts in a juxtacrine manner to amplify IL-23 release from DC thus supporting the generation of Th17 responses.

FB are numerous in the microenvironment of solid tumours and are involved in progression and metastasis [432-434]. Furthermore, in many tumours, specific subsets

of inflammatory cells are primarily located within the FB-rich stromal compartments of the tumour. Bell *et al*. showed that, although intra-tumoural DC retain an immature phenotype, breast tumour stroma contains mature DC, which are often surrounded by clusters of T-cells and could therefore represent ongoing immune responses [728]. These findings were further confirmed in colorectal cancer and RCC [71, 729]. We speculate that a significant subset of DC interact with FB in the tumour microenvironment. In LMN metastases from melanoma patients, NK cells are observed in close proximity to FB surrounding metastatic lesions [428]. In lung, the number of T-reg in the stromal compartment was greater than in the tumour core and correlated with poor outcome [429]. In contrast, another study showed that higher numbers of CD4+ cells in the cancer stroma, but not within cancer cell nests, correlated with longer survival times in patients with NSCLC [430]. The discrepancy regarding prognosis between these reports may be due to the fact that Kinoshita specifically focused on T-reg populations while Wakabayashi studied the whole CD4+ cohort. Similarly, thymic carcinoma tumour-infiltrating lymphocytes (TIL) were more abundant in the stroma [431]. The patients with low CD4+ and CD20+ lymphocytes within tumour stroma showed poor survival [431]. The differential clinical outcomes correlated with compartmentalisation of leucocytes to tumour beds or stroma demonstrates the importance of leucocytes-stromal interactions in tumour progression. In addition, the presence of activated pro-inflammatory FB was recently demonstrated in early pre-neoplastic lesions in skin carcinogenesis model. This pro-inflammatory phenotype was initiated by immune cells and further maintained by tumour [432]. These reports demonstrate the involvement and importance of FB-immune interactions for cancer progression and suggest the potential of FB to affect APC functions and their responses to therapy.

Although FB-immune interactions have started gaining recognition, the effect of therapeutic modulations on FB and FB-APC cross-talk remains largely un-known. Peiris-Pagès *et al*. demonstrated that standard chemotherapy agents such as Azathioprine, Cisplatin and Doxorubicin activate FB transforming them into TAF [730]. Irradiated FB enter premature senescence altering their phenotype and promoting tumour growth and invasion [731, 732]. IR can induce IL-6 production by lung FB [733]. According to some reports even a single dose of 4Gy can cause prolonged growth arrest in FB [732] leading to senescence although others describe the requirement of repeated IR treatments to induce premature senescence [731]. Such discrepancies may reflect the differences between various types of FB. Therefore, it is tempting to assume that FB-immune interactions undergo profound changes in effect of therapeutic modulations such as RT.

We tested the hypothesis that stromal FB modify cytokine secretion by APC and their response to the inhibitory effects of IR and ultimately affect T-cell responses. We showed that although FB up-regulated IL-23 secretion by MoDC and SlanDC, the effect on Mφ was donor dependent. Furthermore, we aimed to elucidate the factors by which FB promote DC radio-resistance by employing a DC-FB co-culture system in the presence of IR. The addition of irradiated FB to irradiated DC up-regulated IL-23 secretion and increased Th17 responses. Interestingly FB were relatively radio-resistant and IR did not affect their capacity to amplify IL-23 secretion by DC. The enhancing effect of FB could be mimicked by addition of PGE2 or forskolin to irradiated DC and abrogated by treating FB with COX2 inhibitor Indomethacin. This effect occurred despite activation of ATM pathways by irradiated DC. This suggests

that activated FB act through secretion of PGE2 which stimulates cAMP pathway in irradiated DC leading to increased IL-23 secretion irrespective of ATM activation**.**

7.2 Results

7.2.1 Effect of FB on cytokine secretion by MoDC, slanDC and MDMφ

FB were previously established to exhibit regulatory effects on DC and Mφ [3, 724-727]. We therefore sought to confirm the ability of dermal FB to modulate MoDC cytokine secretion. FB cultured alone did not secrete IL-23, IL-12, IL-1β, IL-6 or IL-10. In agreement with previous reports [727], co-culture of activated DC with FB increased IL-23 secretion in all donors. ($n=38$, $p<0.05$ to $p<0.001$) (Figure 7.1). The effect of FB on other cytokines was donor-depended. For all the other cytokines studied, whilst there was the occasional donor who showed a significant effect the overall pattern was one of no substantial change. FB significantly ($p<0.05$ to $p<0.01$) decreased IL-12 secretion in 2 out of 19 donors, up-regulated ($p<0.05$) in one while the rest remained unaffected. IL-1β was significantly ($p<0.05$) decreased in one out of 9 donors, whilst the rest remained unaffected. IL-6 was significantly up-regulated in three out of eight donors tested ($p<0.05$) and down-regulated in another three ($p<0.05$). IL-10 was significantly down-regulated in four and completely abrogated in further three out of thirteen donors tested. One donor up-regulated IL-10 secretion upon co-culture with FB, while it remained unaffected in the rest of the donors.

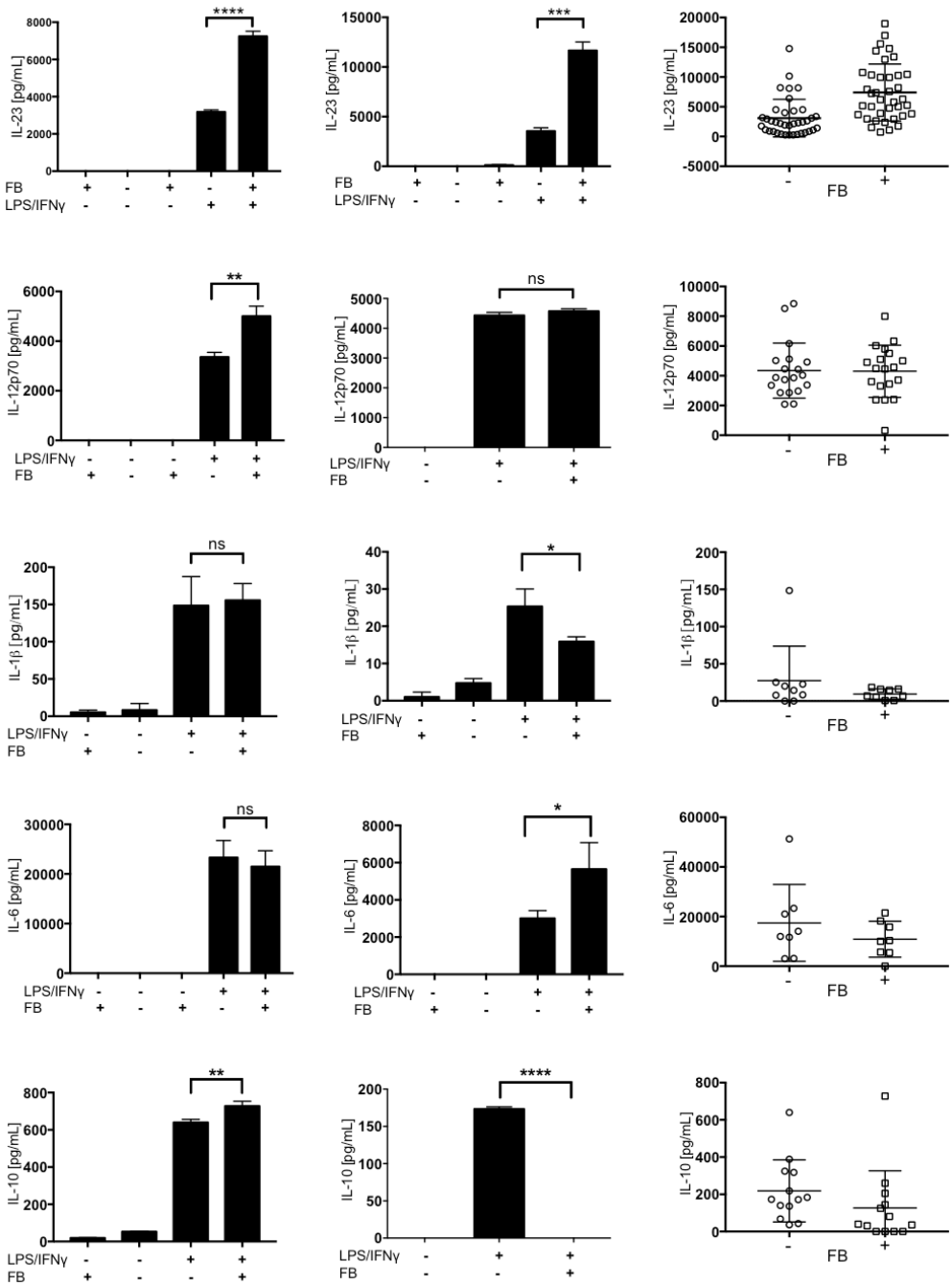


Figure 7.1 Effect of FB-DC co-culture on cytokine secretion by MoDC. FB were seeded in 96-well plates and cultured for 24h. Next iDC were added (proportion FB:DC 1:4 respectively) and co-cultures were incubated for 1h prior to activation with LPS/IFNγ. Supernatants were collected 24h after activation and cytokine secretion was measured by ELISA. Statistical significance was measured by the student t-test.

The effect of FB on cytokine secretion by primary DC was studied. Isolated SlanDC had purity above 95% (Figure 7.2 A). Due to low numbers of SlanDC, their seeding concentration was five times lower than MoDC. There was no detectable secretion of

IL-23 in DC monocultures however, FB stimulated IL-23 secretion by DC in both tested donors (Figure 7.2 B). Furthermore, FB had no impact on IL-12 and IL-10 secretion in one tested donor (Figure 7.2 C). Due to difficulty obtaining sufficient slanDC, further studies were not conducted.

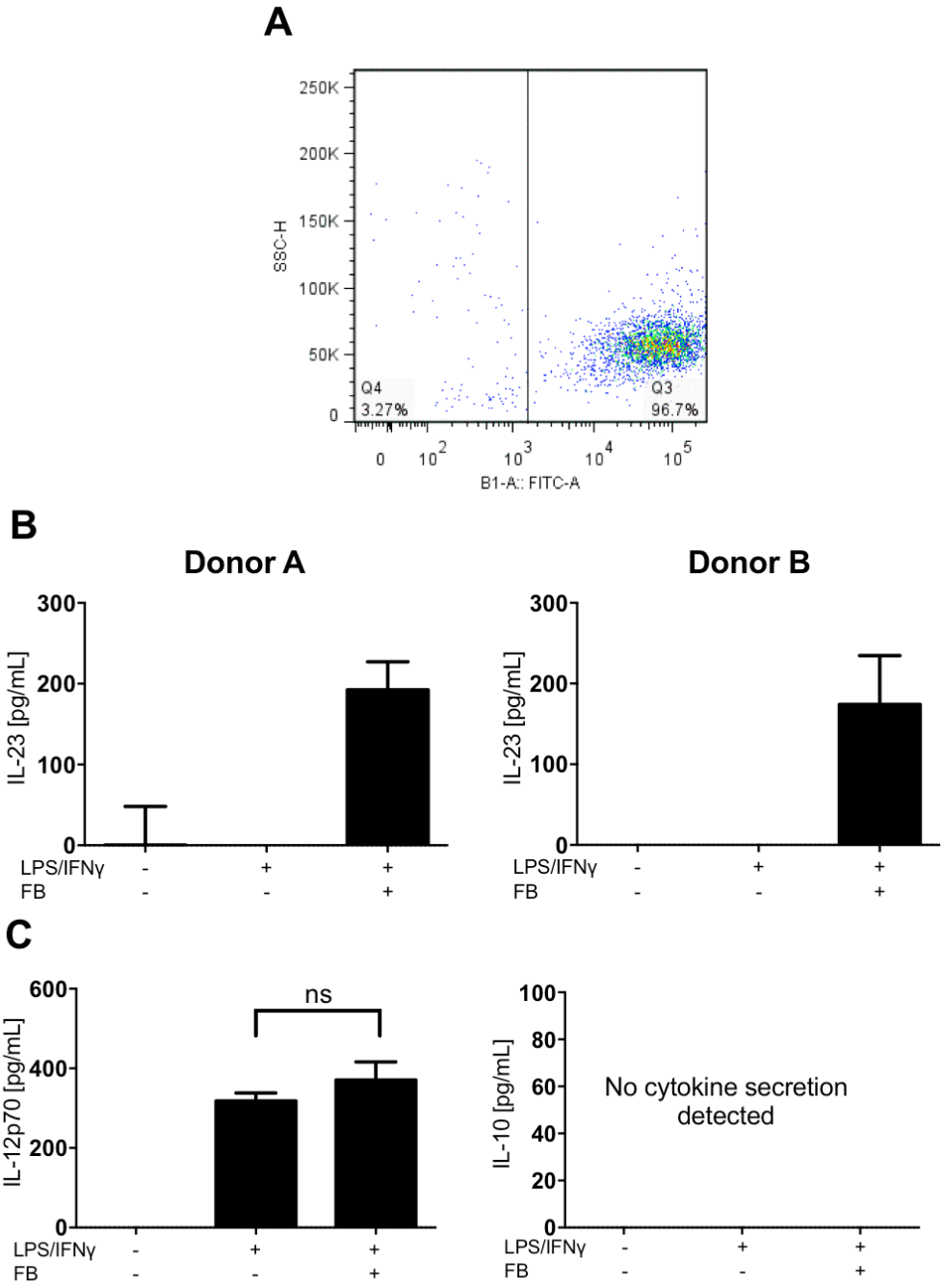


Figure 7.2 Effect of FB on cytokine secretion by slan DC. A- Slan DC purity. SlanDC were isolated from fresh blood and purity was assessed by flow cytometry of cells stained with anti-Slan DC Ab against matched isotype control. Shown is a representative donor. B- IL-23 secretion by FB-SlanDC co-cultures. $2x10^4$ FB were pre-incubated in 96-well plates overnight to reach confluence. Slan DC were rested for 6 hours and added to FB at concentration $1x10^4$. After 1h incubation co-cultures were activated with LPS/IFNγ. IL-23 secretion was assessed by ELISA in supernatants collected 24h post activation. Shown are 2 tested donors. C-IL-12 and IL-10 secretion of FB-Slan DC co-cultures from Donor A assessed by ELISA. Statistical significance was measured by the student t-test.

Multiple reports described the ability of FB to polarise Mφ responses towards Mφ2 [724, 734, 735]. Therefore, next we evaluated the effect of dermal FB on cytokine secretion by Mφ generated in the presence of GM- or M-CSF. FB significantly ($p<0.5$, $p<0,01$) up-regulated IL-23 by GM-Mφ in 6/9 donors and down-regulated IL-12 secretion ($p<0.05$) in 3/9 donors tested while up-regulating it in 2/9 ($p<0.01$). FB down-regulated IL-10 secretion by M-Mφ from 5/8 donors tested. FB did not elicit IL-23 and IL-12 responses from M-Mφ or IL-10 secretion from GM-Mφ.

Since the effect of FB on Mφ was donor-dependent our further work focused on the cross-talk between FB and MoDC.

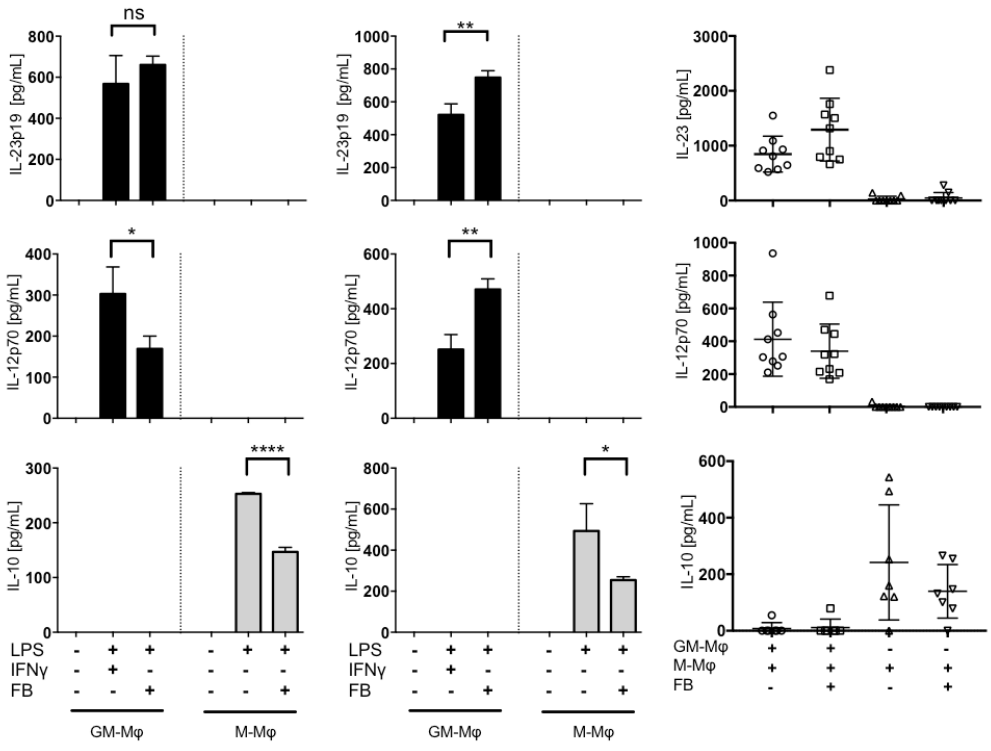


Figure 7.3 Effect of FB on cytokine secretion by Mφ. FB were incubated overnight to reach confluence and Mφ were added (proportion FB: Mφ 1:2.5 respectively). After 1h resting cells were activated with LPS/IFNγ (GM-Mφ) or LPS only (M-Mφ). Cytokines were measured by ELISA in supernatants collected 24h post activation. Shown are 2 representative donors and collective data of al donors tested for IL-23, IL-12 and IL-10. Statistical significance was measured by the student t-test.

7.2.2. FB rescue IL-23 from irradiated DC

The ability of dermal FB to modulate MoDC responses to IR was assessed. The addition of irradiated FB to irradiated MoDC significantly increased IL-23 release by irradiated DC ($p<0.01$) in all donors (Figure 7.4 A, B). The level of IL-23 produced by irradiated DC cultured in the presence of FB was greater or equal to those from non-irradiated DC cultured alone. It should be noted that whilst IL-23 levels secreted by irradiated DC were markedly higher in the presence of FB, its secretion was nevertheless generally lower than those obtained from control co-cultures. Additionally, the ability of FB to increase IL-23 secretion by irradiated DC was maintained regardless of whether BJ6 cell line or primary dermal foreskin FB were used (Figure 7.4 C). Therefore, further experiments were undertaken using the BJ6 cell line unless otherwise stated.

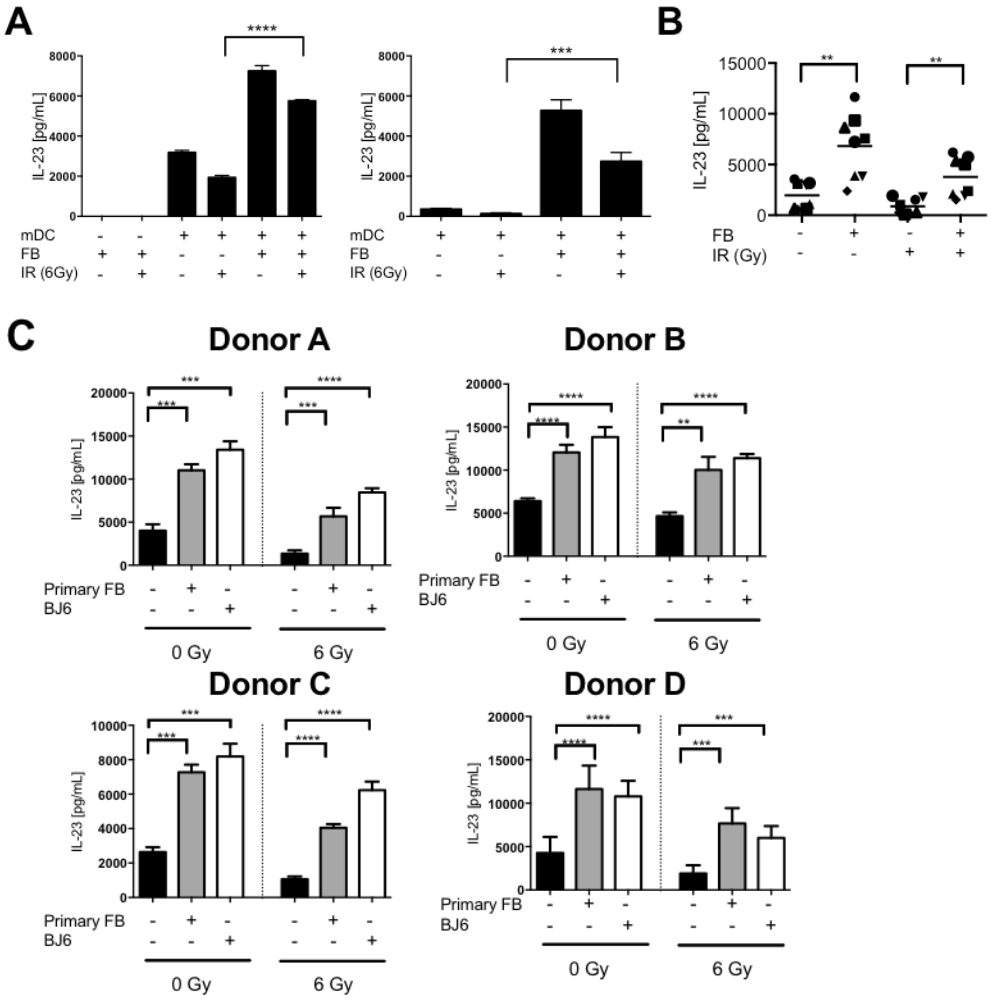


Figure 7.4 FB up-regulate IL-23 secretion by irradiated DC. FB were seeded in 96-well plates and cultured for 24h. Next iDC were added and co-cultures were incubated for 1h to allow cells to settle. Co-cultures were irradiated with 6Gy and immediately treated with LPS/IFNγ. Supernatants were collected 24h after activation and cytokine secretion was measured by ELISA. **A**-shown 2 representative donors. **B**-collective data from all 8 tested donors. **C**- Primary dermal FB demonstrate similar IL-23-enhancing activity to the BJ6 cell line. Shown are 4 out of 5 FB donors tested. Statistical significance was measured by the student t-test.

7.2.3 FB are unaffected by IR

Since irradiated FB up-regulated IL-23 secretion by irradiated DC, we determined the impact of IR on FB. Importantly, when irradiated FB were co-cultured with non-irradiated activated MoDC, there was no impairment in their capacity to promote IL-23 release (Figure 7.5 A, B). This was irrespective of dose of radiation (2-6Gy).

PGE2 positively regulates IL-23 by DC [274, 727], therefore the effect of IR on PGE2 secretion by FB was investigated. IR did not inhibit PGE2 secretion by BJ6 cells or primary FB activated with TNFα and IL-1β (Figure 7.5 C). Furthermore, irradiation had no impact on FB viability as assessed by dye-exclusion (data not shown) and AnnexinV/PI staining (Figure 7.6). The number of viable cells remained >96% regardless of irradiation. It is important to stress that we examined the effects of IR on FB within the first 24h of exposure after which time activated DC would be expected to migrate to regional LMN [727].

IR promotes growth arrest and senescence of FB however the dose and frequency of radiation required varies [731, 732]. Therefore, we assessed the effect of increasing doses of IR on dermal primary FB. Induction of cell senescence by staining with β-galactosidase was measured 7 days after initial insult. Non-irradiated cultures contained only single cells positive for β-galactosidase (Figure 7.7). Number of positive cells increased slightly in cultures treated with 5 and 10 Gy (approximately 5

and 10%) and reached around 80% of cells at 15Gy.

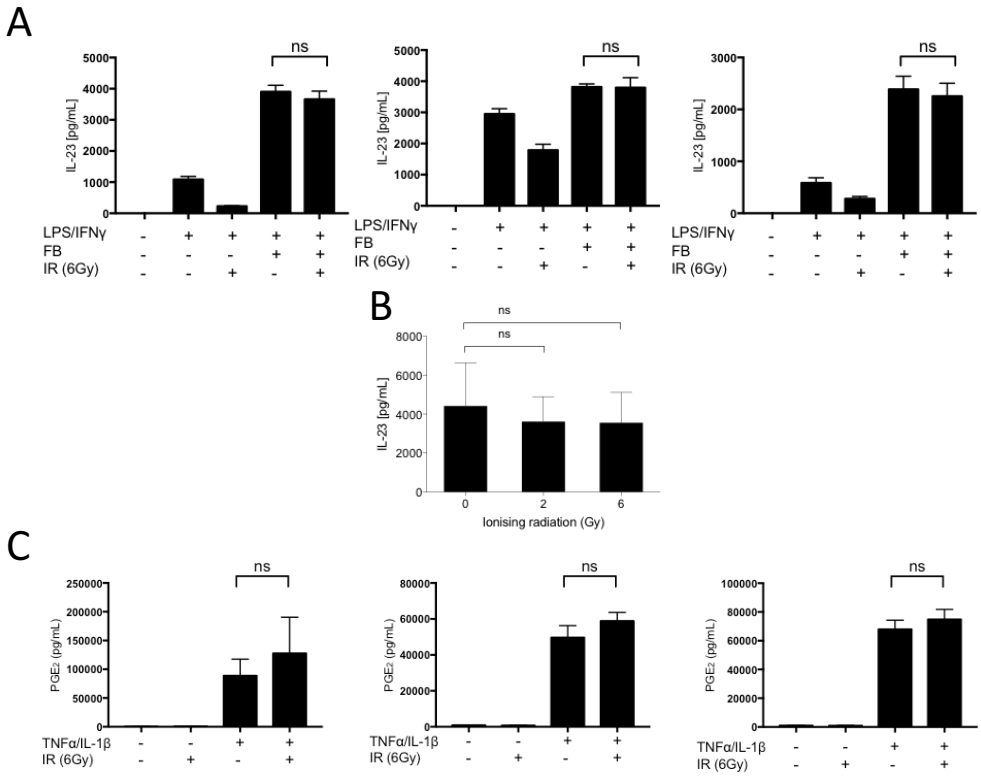


Figure 7.5 FB are functionally impaired by IR. A, B- Irradiated FB retain their ability to up-regulate IL-23 secretion by DC. BJ6 were seeded in 96 well and rested overnight. After reaching full confluence FB were left non-irradiated or submitted to 2 and 6Gy of IR. Immediately after irradiation, activated DC were added and supernatants were collected 24h for assessment of IL-23 by ELISA. **A**-graphs showing 3 individual donors tested. **B**-Collective date from 3 donors. **C**-Irradiated FB retain their ability to respond to TNFα/IL-1β and secrete PGE_2. FB were stimulated with 10ng/ml TNFα and 1ng/ml IL-1β and left non-irradiated or irradiated with 6Gy. PGE_2 secretion was assessed 24 following radiation by ELISA. Graph shows data from 3 different FB cell lines (BJ6 and primary FB from 2 independent donors). Statistical significance was measured by the student t-test.

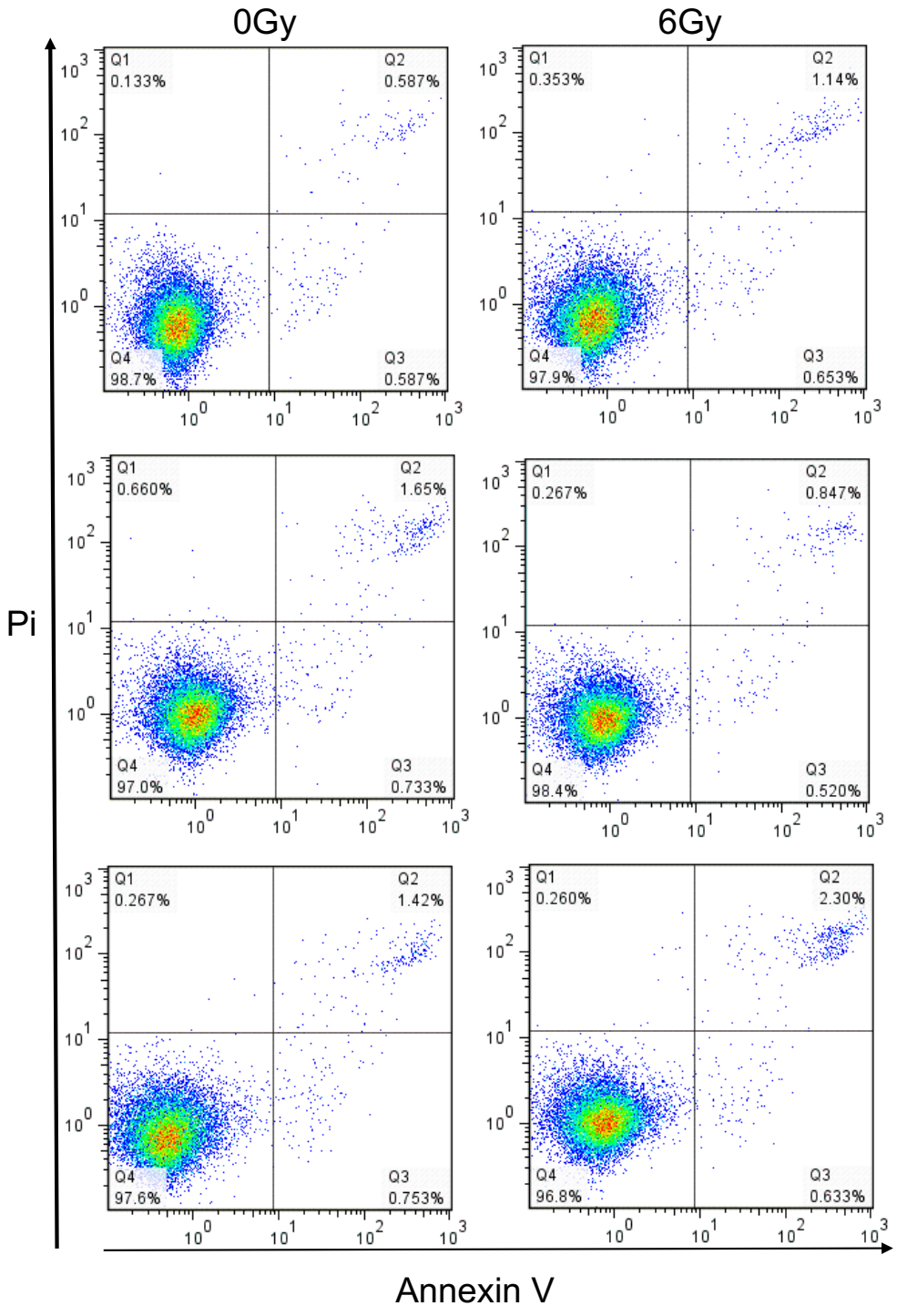


Figure 7.6 Determination of FB viability following exposure to IR. BJ6 were seeded and allowed to attach overnight, next were irradiated with 0-6Gy and viability was assessed 24h post-irradiation by AnnexinV/PI staining. Shown are scatter plots for 3 separate experiments.

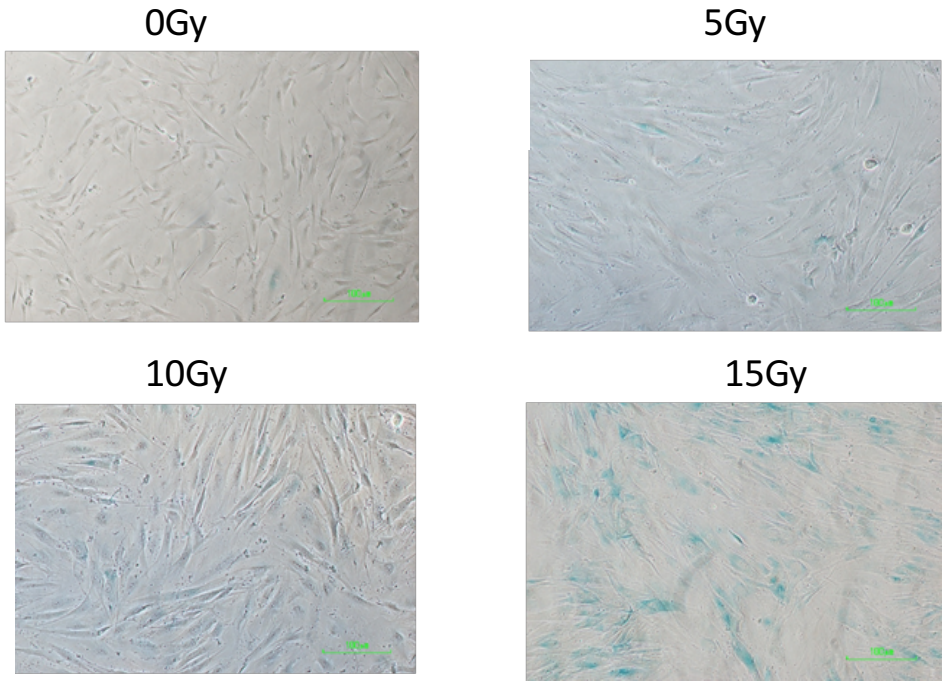


Figure 7.7 β-galactosidase staining of primary FB 7 days after treatment with 0-15 Gy IR. FB were rested overnight and irradiated with indicated doses of IR. 48h later FB were re-seeded in fresh medium. 7 days after radiation FB were permabilised with 4% formaldehyde and stained with β-galactosidase solution. Shown is a representative donor out of three donors tested.

7.2.4 FB promote IL-23 secretion by irradiated DC through COX2-dependent PGE_2 release

The mechanism by which FB restored DC functions following irradiation was investigated. To address the role of soluble factors, DC-FB co-cultures were separated by 0.3μm porous membrane in Transwells. Despite the lack of cell-cell contact, FB retained their capacity to support IL-23 secretion from irradiated DC (Figure 7.8).

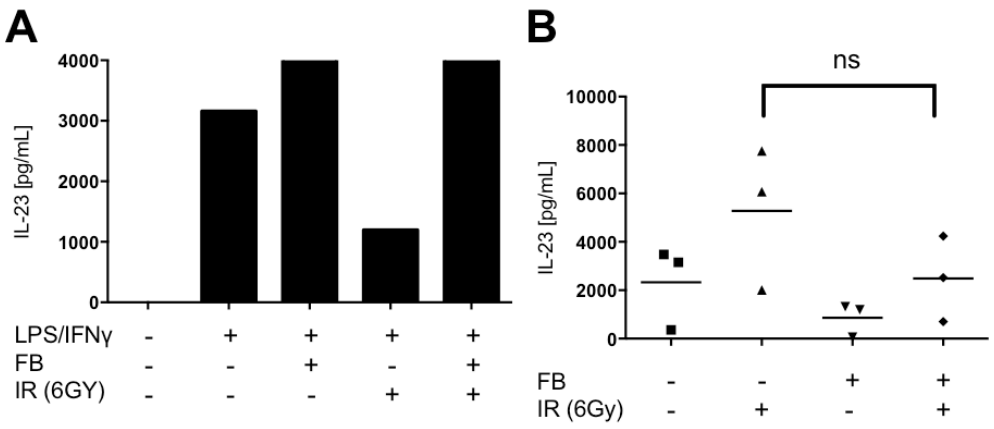


Figure 7.8 FB dependent increase of IL-23 is mediated by soluble factors. Fb and DC co-cultures were separated by 3μm porous membrane in Transwell system. Fb were seeded to the bottom well and left to rest overnight. Next day DCs were added to the upper wells, irradiated and immediately activated with LPS/IFNγ. Supernatants were collected after 24 hours for assessment of IL-23 in ELISA. Experiment was performed in singlicate and repeated for 3 different DC donors. **A**-representative donor. **B**- collective data of all 3 DC donors tested.

Previously Schrimmer *et al*. demonstrated an important role of COX2-dependent PGE2 for communication between FB and DC [727]. Addition of PGE2 to DC immediately after irradiation and activation significantly increased IL-23 secretion ($p<0.05$ in 3 donors and $p<0.001$ in 2 donors) (Figure 7.9 A, B). PGE2 secretion by FB was reported to be COX2-dependent [726, 736], therefore we determined the importance of COX2 for FB-dependent recovery of IL-23 secretion using the COX2 inhibitor, Indomethacin (Indo). In order to reduce the effect of Indomethacin on DC activation, for these experiments MoDC were activated 3 hours before adding to FB and irradiating. Importantly DC irradiated 3 hours after triggering with LPS/IFNγ down-regulated IL-23 ($p<0.01$) (Figure 7.9 C). Furthermore, FB up-regulated IL-23 secretion by DC activated 3h prior to irradiation (Figure 7.9 C). Importantly no reduction in DC or FB viability was seen upon Indomethacin treatment as assessed by trypan blue staining (not shown). In the presence of Indomethacin, IL-23 secretion

from DC-FB co-cultures was reduced to levels similar to those achieved in the absence of FB (Figure 7.9 D, E).

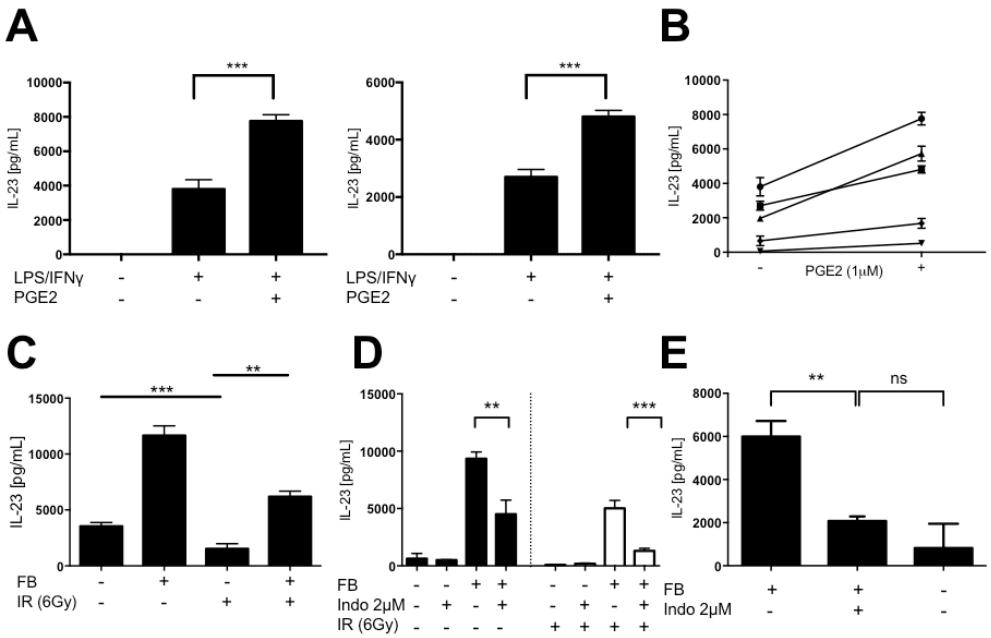


Figure 7.9 FB up-regulate IL-23 secretion by DC through PGE2 in a COX2-dependent manner. A, B Irradiated DC up-regulated IL-23 secretion in response to PGE2. DC were irradiated with 6Gy and immediately activated with LPS/IFNγ and 1μM PGE_2 for 24h. IL-23 levels in supernatants were measured by ELISA. A- 2 representative donors, B-collective data from 5 donors tested. **C-** FB up-regulate IL-23 secretion irrespective of the timing of irradiation of DC. 3h post activation with LPS/IFNγ MoDC were added to confluent FB cultures and immediately irradiated with 6Gy IR. IL-23 was measured by ELISA in supernatants collected 24h post irradiation. Graph shows a representative donor out of 3 tested donors. **D, E**- Up-regulation of IL-23 secretion from irradiated DC by fibroblasts is COX2 dependent. FB were treated with Indomethacin (2μM) for 24h prior to (and throughout) co-culture with irradiated DC. **D**-a representative donor. **E**-collective data from 3 donors tested. Statistical significance was measured by the student t-test and two-way Anova for collective data.

7.2.5 FB promote irradiated DC IL-23 responses through the cAMP pathway

Our earlier results indicated that IR inhibits IL-23 by phosphorylation of ATM. On the other hand, recent reports showed that PGE2 up-regulates IL-23 secretion by activation of cAMP-PKA signalling pathways [275]. Interestingly, Cho *et al*. showed in lung cancer that cAMP signalling inhibits IR-dependent ATM phosphorylation

[737]. Therefore, we dissected the molecular mechanism responsible for up-regulation of IL-23 by irradiated DC co-cultured with FB.

To assess ATM activation in irradiated DC-FB co-cultures cells were separated by 0.3µm porous membrane to ensure that pATM levels were measured only in DC. As expected, irradiation of mDC resulted in ATM phosphorylation after 2h and this persisted for at least 12h as demonstrated by intracellular staining with flow cytometry (Figure 7.10). However, co-culture with FB did not suppress ATM activation in irradiated DC suggesting that the support of DC function by FB was independent of ATM. In contrast, the addition of a cAMP-active analog, forskolin, to irradiated DC resulted in a dose-dependent increase of IL-23 ($p<0.001$ at 100µg, Figure 7.11 A, B).

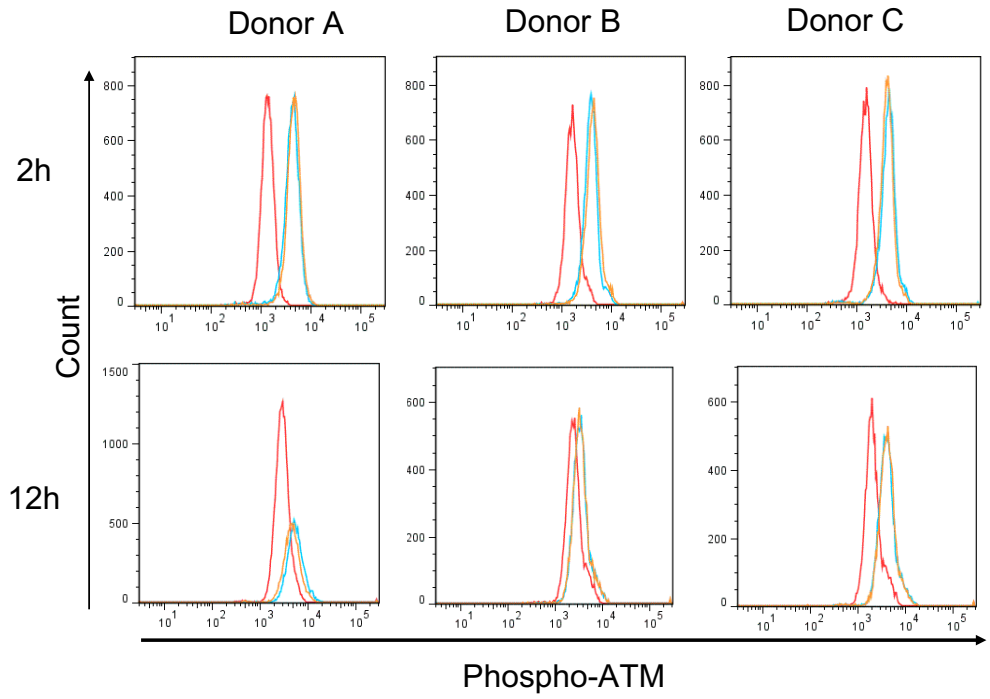


Figure 7.10 Effect of Fb on ATM phosphorylation in irradiated DC. DC were cultured alone or added to FB separated by 3µm membranes in Transwell plates. After resting for 1h cultures were IR with 0-6 Gy X-rays. 2h and 12h post-radiation phospho-ATM was measured by intracellular staining with anti-phospho-ATM antibody and assessed by FACS. Shown are 3 donors tested. Red line- DC alone, 0Gy, orange line- DC alone 6Gy, blue line- DC/FB co-cultures 6Gy.

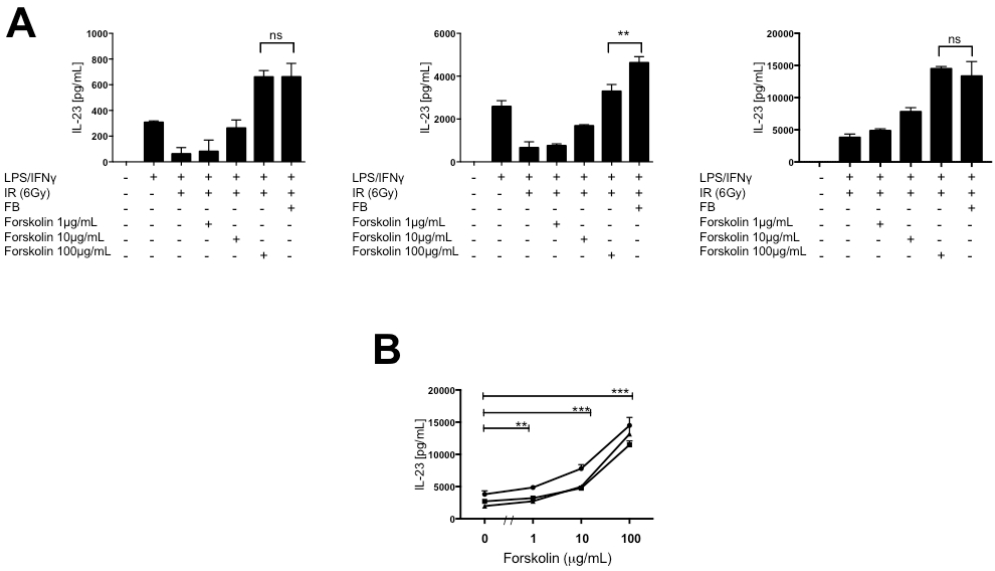


Figure 7.11 Effect of c-AMP activation on IL-23 secretion by IR DC. MoDC were irradiated with 6Gy of IR and immediately activated with LPS/IFNγ and treated with cAMP analog- forskolin (0, 1, 10, 100 μg). Supernatants were collected after 24h and IL-23 was measured by ELISA. **A**- 3 donors tested, **B**-collective data of all donors showing dose-dependent effect. Statistical significance was measured by the student t-test for individual donors and two-way Anova for collective data.

7.2.6 FB permit irradiated DC to promote Th17 responses

The protective effect conferred by FB on Th17 responses was studied. Stimulation of naïve $CD4^+$ T cells through CD3/CD28 in the presence of conditioned supernatants from activated DC elicited Th17 responses as shown by secretion of IL-17A (Figure 7.12 A, B). Furthermore, the addition of FB to DC enhanced IL-17 secretion by T cells irrespective of exposure to IR. Interestingly Th17 responses closely followed increases in IL-23 levels in supernatant of DC with FB. In 2 donors supernatants of DC co-cultured with FB were characterised with 3 fold increases which corresponded to 6 fold increase of IL-17 comparing with stimulation with DC sups alone. Irradiated DC with FB resulted in a 2-fold increase in 2 donors and 1.3 fold increases in 1 donor which corresponded to 4 and 3 fold increase of IL17 respectively.

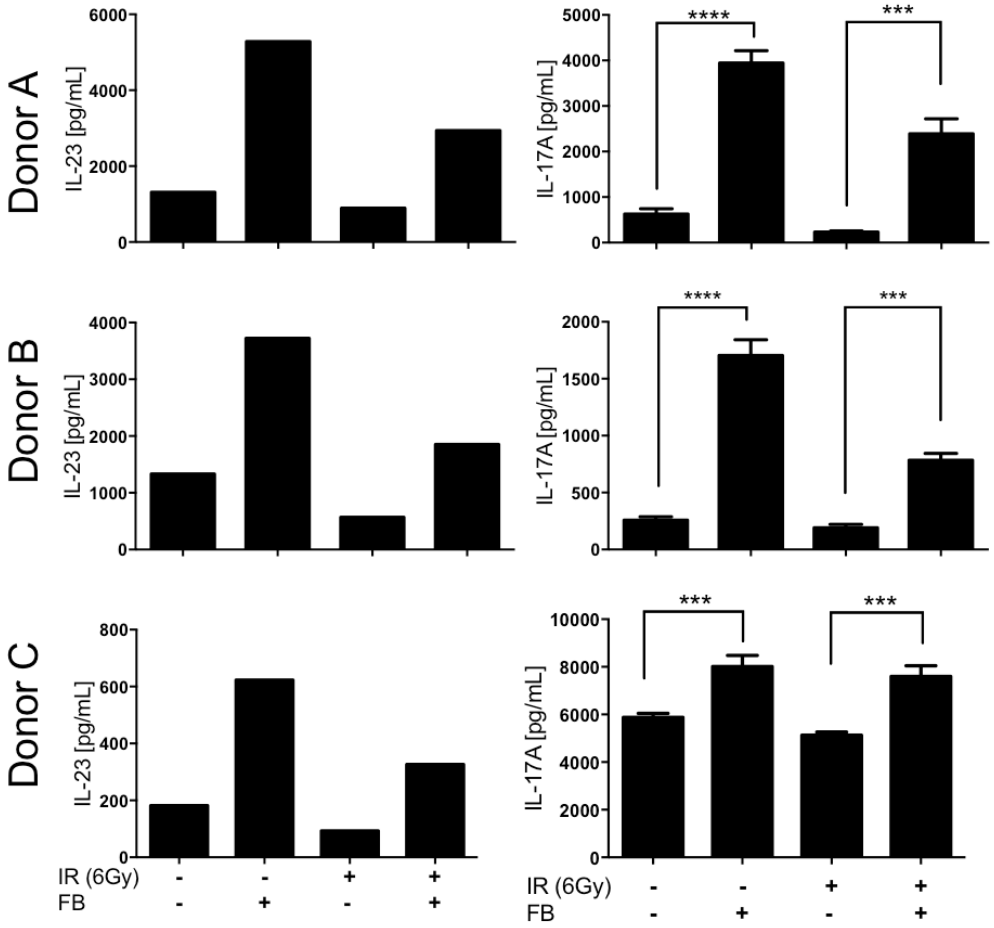


Figure 7.12A FB promote Th17 responses. A – DC monocultures or DC-FB co-cultures were irradiated or left untreated and immediately activated with LPS/IFNγ. 10 hours after activation 90% of the supernatants were replaced with fresh DC media. IL23 levels were measured in supernatants collected 24 hours after DC activation. Naïve CD4Tcells were activated with antiCD3/antiCD28 in the presence of supernatants for 5 days, then rested for 2 days and re-activated with anti-CD3/anti-CD28 for 48h. Supernatants were collected for measurement of IL-17 by ELISA. Graphs show IL-23 levels in DC/FB cultures and corresponding IL-17A levels from T-cells stimulated with respective sups. Statistical significance was measured by the student t-test. **B**- Naive CD4+CD45RA+T-cells were co-stimulated for 5 d with anti-CD28/CD3 in the presence of DC/FB supernatants. After resting for 2 d, cells were re-stimulated with anti-CD3/anti-CD28, treated with Brefeldin A to inhibit cytokine secretion and IL-17A release was determined by FACS. Data shows 2 tested donors.

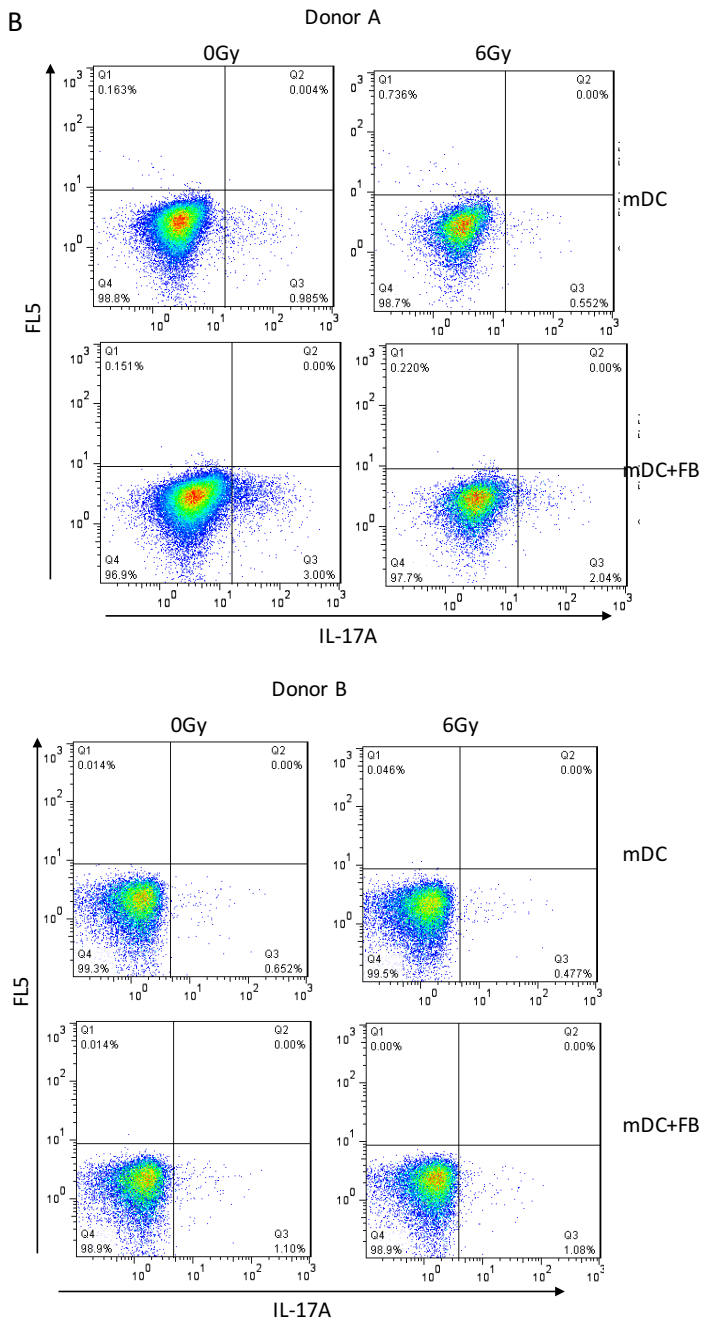


Figure 7.12B. FB promote Th17 responses. A – DC monocultures or DC-FB co-cultures were irradiated or left untreated and immediately activated with LPS/IFNγ. 10 hours after activation 90% of the supernatants were replaced with fresh DC media. IL23 levels were measured in supernatants collected 24 hours after DC activation. Naïve CD4Tcells were activated with antiCD3/antiCD28 in the presence of supernatants for 5 days, then rested for 2 days and re-activated with anti-CD3/anti-CD28 for 48h. Supernatants were collected for measurement of IL-17 by ELISA. Graphs show IL-23 levels in DC/FB cultures and corresponding IL-17A levels from T-cells stimulated with respective sups. Statistical significance was measured by the student t-test.**B**- Naive CD4+CD45RA+T-cells were co-stimulated for 5 d with anti-CD28/CD3 in the presence of DC/FB supernatants. After resting for 2 d, cells were re-stimulated with anti-CD3/anti-CD28, treated with Brefeldin A to inhibit cytokine secretion and IL-17A release was determined by FACS. Data shows 2 tested donors.

7.3 Discussion

Studies on the impact of the stromal microenvironment on immunity are important as immune cells are in constant cross-talk with their stroma during each and every stage of the immune response. Stromal cells can affect a wide range of DC functions including maturation, homing to lymph nodes, and subsequent polarization of T-cell responses [3, 725, 726, 738]. The present study investigated the ability of stroma to modulate the outcome of therapeutic interventions directed towards immune system. Our previous work established that IR inhibits the cytokine response, in particular IL-23, of DC and Mφ. On the other hand, recent report demonstrated that stromal FB support IL-23 production by activated DC [726]. Therefore, we addressed the hypothesis that FB, cells which are known to be relatively radio-resistant [732], continue to support the function of APC in the presence of IR.

In agreement with previous reports [727], FB up-regulated IL-23 secretion by MoDC while effect on IL-12, IL6, IL-1β and IL-10 was inconsistent. Furthermore, FB up-regulated IL-23 secretion by freshly isolated SlanDC. Importantly FB did not secrete detectable levels of IL-23, IL-12, IL6, IL-1β or IL-10 regardless of the presence of TLR4 stimuli. Interestingly several authors reported the presence of TLR on FB and IL-6 secretion by TLR4 stimulated FB has been noted [374, 739]. The difference in our findings may reflect differential anatomic origins of FB [740]. In addition, Wang *et al*. [374] described significantly increased expression of TLR4 receptors on dermal hypertrophic FB compared to FB from healthy skin suggesting a role of prolonged activation for TLR4 expression by dermal FB. We may therefore hypothesize that the levels of FB TLR4 in our system were insufficient for maximal LPS responses. It

would be interesting to assess levels of TLR4 expression on FB cultured separately and with activated DC.

The effect of FB on IL-23 secretion by Mφ was donor-dependent. Mφ express all four EP receptors however similar to DC, PGE2 exert effect through EP2 and EP4 [289, 741]. It is plausible that the inconsistency in FB-dependent modulation of IL-23 secretion may be due to receptor polymorphisms and requirement for a stronger stimulation to affect cytokine secretion [742]. Possibly increasing the ratio of FB:Mφ in co-culture would result in up-regulation of IL-23 secretion by Mφ in all donors. However, it needs to be mentioned that FB were cultured at 100% confluency while further reduction of Mφ numbers in co-culture in order to change the FB:Mφ would result in reduced cytokine levels and increased experimental errors.

Since FB consistently up-regulated IL-23 secretion by MoDC our further work focused on this cell subset. Primary FB and FB cell line demonstrated radio-resistance in regard to viability. It should be noted that this is in the context of early cell death and apoptosis as cell viability was measured at 24h. FB treated with repeated doses of IR were reported to remain viable but enter premature senescence and altered phenotype promoting tumour growth and invasion as well as rendering adjacent tumour more radio-resistant [296, 731, 732]. Even a single dose of 4Gy was shown to cause a prolonged growth arrest in FB [731] leading to senescence. This however was not observed by other researchers [732] who reported the requirement of repeated IR treatments to induce premature senescence and therefore altered phenotype. Our results agree with the latter report showing that primary dermal FB sustain single dose as high as 10Gy without developing senescence.

We also assessed the effect of IR on FB in functional assays. Previous reports show conflicting evidence regarding regulation of PGE2 by gingival FB irradiated with low-level diode laser [743, 744]. FB are characterised by extreme positional diversity and cells from various body locations can respond differentially to similar insults [745]. In our model, irradiation of dermal FB activated with TNFα and IL1β did not affect PGE2 secretion or their capacity to augment IL-23. This suggests that irradiated FB are unimpaired in their ability to produce PGE2 and are capable to respond to environmental signals. Both irradiated and non-irradiated FB did not secrete measurable amounts of IL-6, IL-12, IL-23 or IL-10. The relative resistance of dermal FB to IR within the first 24h is likely due to their important role in initialising and maintaining immune responses to the tissue insult [746].

Schrimmer *et al*. identified the ability of FB to promote IL-23 secretion by DC via COX2-dependent PGE2 release by activated FB [727]. Our results support these findings in the additional setting of IR. Inhibition of COX2 abrogated the enhancing effect of co-cultures after irradiation. It should be noted that Indomethacin is a non-specific inhibitor which affects both COX1 and COX2. Therefore, it is important to consider potential off target effects. For further confirmation, COX2-specific inhibitors such as NS-398 could be used. To evaluate the involvement of PGE2, irradiated DC were stimulated with exogenous PGE2 which resulted in increased IL-23 secretion. Physical isolation of the cells had no impact on IL-23 release and supported the notion that the effect was independent of cell-cell contact.

Interestingly, although FB recovered IL-23 production by irradiated DC, the levels were nevertheless lower than those obtained from non-irradiated co-cultures. FB functions were not affected by IR. Furthermore, as shown earlier, there was no impairment in the ability of irradiated DC to secrete IL-1β and TNFα required for PGE2 secretion by FB. Therefore, irradiated DC are able to sustain their ability to activate FB. We therefore hypothesized that irradiated DC are impaired in their ability to fully respond to PGE2. According to recent reports, PGE2 stimulates IL-23 secretion by DC through cAMP/PKA activation [275]. On the other hand, IR down-regulated IL-23 through phosphorylation of ATM. Interestingly, Cho *et al*. recently demonstrated that cAMP signalling is able to inhibit radiation induced phosphorylation of ATM in lung cancer [737] (Figure 7.12).

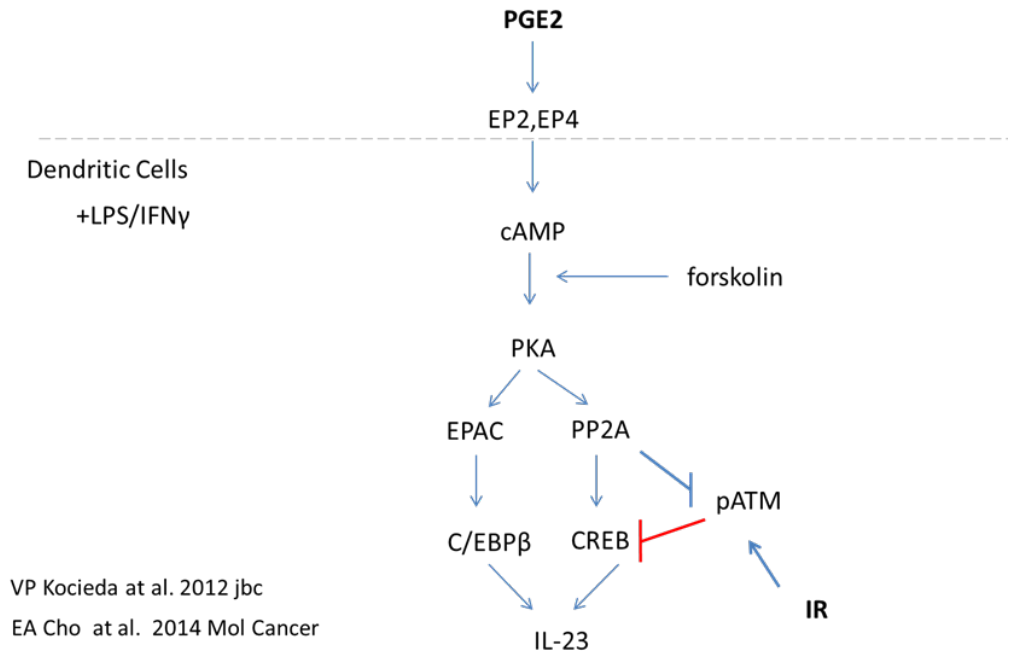


Figure 7.13. Proposed signalling transduction pathways involved in regulation of IL-23 secretion by irradiated DC. PGE2 secreted by stromal cells activates cAMP/PKA signal transduction pathway through EP2/EP4 leading to increased secretion of IL-23 by activated DC. PGE2 effect can be mimicked by cAMP active analog – forskolin. Activation of PP2A blocks phosphorylation of IR-induced ATM. In contrast pATM renders inhibitory effect on CREB causing down-regulation of IL-23 production [289, 737].

Addition of forskolin resulted in an increase of IL-23 secretion by irradiated DC to levels similar to those from irradiated DC co-cultured with FB suggesting the role of cAMP activation in FB-DC co-cultures. However, in contrast to Cho *et al*. co-culture of irradiated DC with FB did not affect DC pATM levels despite up-regulated IL-23 [737]. The disparity between these results may be due to differences in timing of cAMP and ATM activation. In Cho *et al*., cAMP was activated before irradiation allowing blocking of phosphorylation of ATM by activated PP2A [737]. In our model, DC were irradiated before activation and stimulation from FB. Since ATM becomes phosphorylated within 15 minutes of exposure to IR, signals received from FB may reach DC too late to inhibit this process. It is therefore even more important that up-regulation of IL-23 secretion by FB occurs despite ATM activation. We may hypothesise that positive signalling through cAMP is stronger than inhibitory signals from pATM. It remains possible that the main signalling occurs through EPAC/CBP or other unknown down-stream signalling molecules [555] (Figure 7.13).

The supernatants from DC-FB co-cultures enhanced Th17 responses above these obtained with DC alone. PGE2 promoted Th17 directly and in conjunction with IL-23 [747, 748]. In our model the concentration of PGE2 secreted by FB was unaffected by IR. On the other hand, changes in IL-17 reflected differences in IL-23, demonstrating the biological importance of FB-dependent IL-23 secretion in regulation of adaptive immunity. In summary, we show that although IR inhibits IL-23 secretion by TLR-triggered DC, their ability to stimulate stromal FB via TNFα and IL-1β remains unaffected. Activated FB are radio-resistant and counteract inhibitory effect of IR in a COX2 dependent manner which results in up-regulation of IL-23 secretion by DC and enhanced Th17 responses (Figure 7.14).

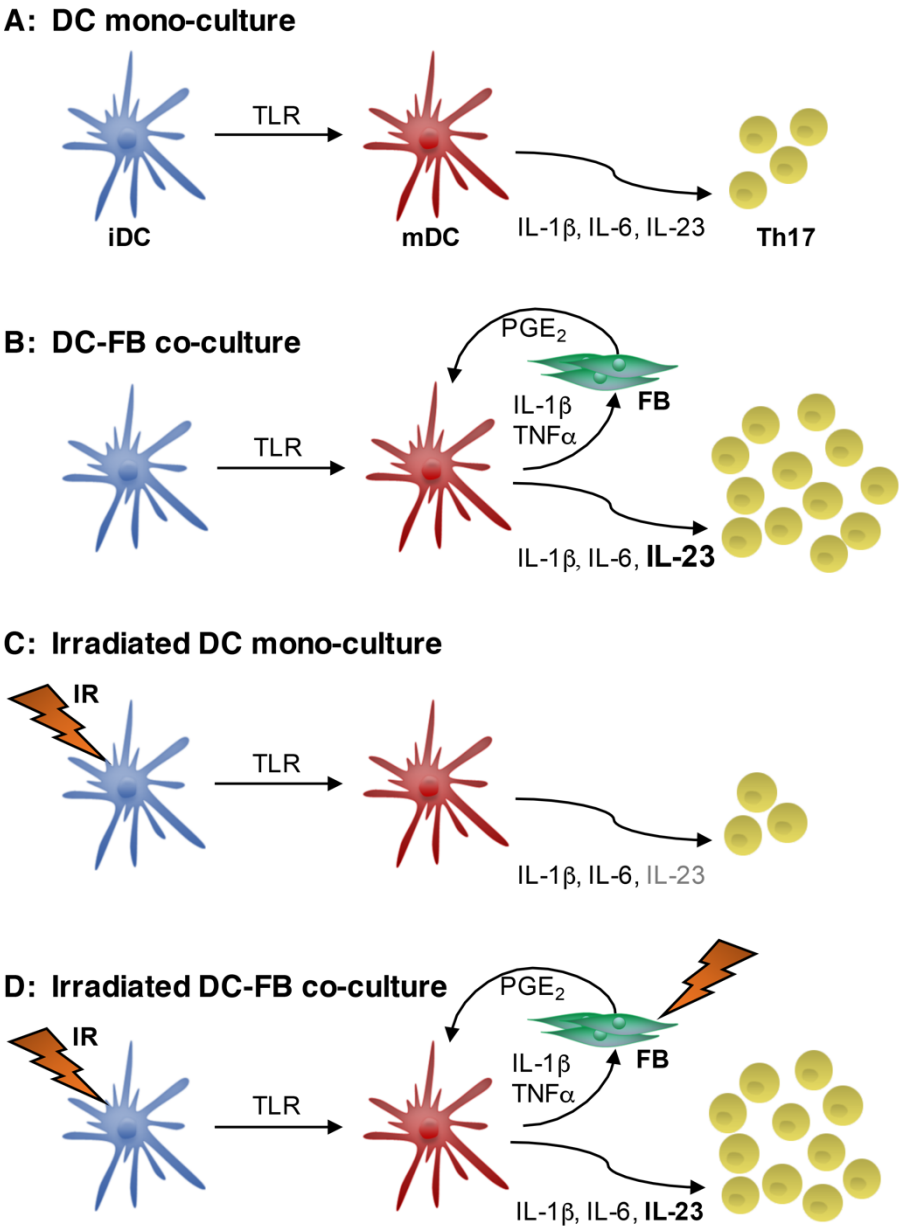


Figure 7.14. Model of DC-FB crosstalk governing IL-23 dependent Th17 responses after irradiation. **A-** TLR-activation of DC in mono-culture elicits secretion of IL-1β, IL-6 and IL-23 which prime Th17 responses. **B-** The addition of FB to DC provides an important feedback loop that serves to enhance IL-23 secretion and thus augments Th17 responses. **C-** Irradiation of DC mono-cultures selectively inhibits IL-23 secretion. **D-** However, the presence of FB ensures that irradiated DC continue to secrete sufficient IL-23 to generate Th17 responses. Importantly, the irradiation of FB does not hinder their reinforcement of IL-23 responses.

Overall, our study confirms the importance of stromal cells such as FB in modulating immune responses and reinforce the fact that APC do not act alone and their effect depends on signals received from their environment. It also highlights the need to use multicellular systems in aim to understand cells behaviour in the context of their microenvironment. Our single cell model showed that irradiation strongly down-regulates IL-23 secretion by DC and Mφ. However, the effect of IR on DC is significantly diminished by addition of FB. This translated to enhanced Th17 responses in a post-RT environment (Figure 7.13). These results support the role of FB as master regulators of IL-23-IL-17 axis where DC initiate responses which are then potentiated by FB. Since IL-17 is a critical factor driving post-radiation fibrosis [24] this work identifies a potential mechanism for the pathological consequences of radiation and chemotherapy [25, 61].

In our system, we modelled the immediate effect of single dose of IR on modulation of DC function by stromal FB from healthy donors. Our next steps will focus on expanding our model to include multiple fractions of IR as well as using TAF and APC from cancer patients. We will also look at the effect of senescent FB on APC and T-cell responses as such interactions may paly important role in tumour promotion of sites subjected to fractionated RT and chemotherapy protocols as well as in older patients [731, 732, 749]. Improved insight into the complex net of interactions between immune system and stroma is necessary for understanding how pathologies develop and are maintained. It will also allow designing more targeted therapies and may provide new targets for therapeutic interventions.

Chapter 8 Final discussion

The role of research on environmental modulation of APC in designing modern immunotherapy

8.1 The importance of environmental factors for designing improved immunotherapy modulations.

The past decade has seen remarkable advances in the development of immunotherapy protocols against a number of diseases raging from cancer [750-755] through autoimmune disorders such as diabetes [756, 757], movement disorders caused by neuroinflammation for example Parkinson's Disease [758] or graft-versus-host disease [759]. These advances were fuelled by discoveries regarding mechanisms governing immune functions as well as novel ways of modulating the behaviour of immune cells. However, the effectiveness of immunotherapies is often underscored by the restrictions in our knowledge concerning the influence of environmental factors on immune cells and their impact on the response of immune cells to the concomitant therapy. For example, only recently TAMφ have been found to play important role in the effectiveness of the PD1–PDL1 axis blockade [760]. Arlauckas *et al.* [761] showed that Mφ can remove anti-PD1 antibodies from T-cells, reducing their response while, Gordon *et al*. [762] demonstrated that Mφ can also express PD1 on their surface, which impairs their phagocytic activity and consequently anti-tumour immunity. Furthermore, unintended effects of traditional therapy such as RT or chemotherapy on immune cells are now thought to play a role in the lack of effectiveness of these therapies and the development of debilitating side effects such as fibrosis [646, 653].

Our work addressed some of the effects of environmental factors on Mφ and DC behaviour. Recently Mφ became one of the targets of immunotherapy utilising various methods including repolarisation efforts, depletion etc. Mφ are renowned for their extreme plasticity, however our findings demonstrate the importance of initial factors in directing Mφ phenotype and restrictions in their ability to fully repolarise upon subsequent challenge. Mφ differentiated in the presence of M-CSF and activated with LPS and IFNγ were deficient in their ability to secrete IL-12 and IL-23 compared to Mφ generated with GM-CSF. The consequences of differentiation conditions were especially striking with regards to IL-23 secretion which was markedly down-regulated. In contrast the outcome of M-CSF on IL-12 secretion was less pronounced. The effect of IFNγ favouring IL-12 responses over IL-23 was shown also in combination with the TLR7/8 agonist, R848. While IFNγ increased IL-12 and decreased IL-10 secretion by R848 activated Mφ, it failed to exert significant effect on IL-23 demonstrating the specificity towards Th1 responses.

Current therapeutic efforts concerning Mφ often focus on a complete change of their polarisation from anti- to pro-inflammatory state in the case of cancer and *vice versa* for autoimmunity. However, a complete change of polarisation requires a change in expression of many genes as demonstrated by Martinez *et al*. [590]. In contrast, our results suggest the possibility to fine-tune Mφ functions, at least with regards to IL-23, rather than completely re-polarise. Solid tumours contain TAMφ of a mixed phenotype and simultaneously utilise their pro and anti-inflammatory properties to promote tumour growth, invade and metastasise. While Mφ1 found in tumours often relate to a more favourable prognosis than Mφ2 [661], they can still promote tumour

growth through factors such as IL-23, which stimulates angiogenesis and chronic inflammation [309, 310]. Therefore, focusing research on the development of strategies promoting desired aspects of Mφ biology may bring considerable advantages over complete repolarisation. So far, our research was restricted to two different methods of Mφ differentiation and a limited number of activating/polarising stimuli. Future work should aim to expand differentiation protocols to more closely imitate immune-inhibitory conditions occurring in tumour micro-environments. Alternately, using IL-17 or immune complexes during differentiation can generate Mφ with phenotypes akin to those in autoimmune disorders.

The aforementioned restrictions in Mφ plasticity constitute yet another important factor requiring consideration for the design of immunotherapy protocols. In addition to the influence of environmental cues during Mφ differentiation from Mo, we also describe the ability of Mφ to develop innate memory in response to LPS and IFNγ. Our results are supported by work by Mackaness *et al*.[608] who showed that Mφ are in a state of higher activity following response to IFNγ secreted by T-cells. Furthermore, in agreement with our results, Goodridge *et al*. [763] postulated that innate memory may develop at the stage of Mφ differentiation from Mo or later during their activation. Moreover, innate memory may persist due to the presence of self-renewing Mφ in tissues or due to the influx of exposed Mo which yield Mφ with altered functions.

The concept of innate memory gained recognition only recently. It has been implicated in some of the positive effects of vaccine adjuvants as well as protection against secondary infections [352, 609, 764]. We may speculate its involvement in the

maintenance of autoimmune disease where initial injury results in APC-mediated stimulation of adoptive responses. These in turn support chronic Mφ activation in a self-perpetuating mechanism. The implications of this for designing of immunotherapy protocols are both positive and negative depending on the context. On one hand, it may not be possible to change resident Mφ to the desired polarisation state. In that case Mφ depletion could be considered together with application of factors promoting more favourable differentiation conditions for newly arrived Mo such as injections of GM-CSF. On the other hand, the phenomenon of innate memory may be used to an advantage by specific priming of Mφ. In this respect, it would be interesting to investigate whether Mφ demonstrate any degree of specificity respectively to the priming factor. We showed different responses of Mφ with regards to treatment with LPS (inhibition) and IFNγ (activation). It is plausible that other factors may also trigger specific phenotypic alterations. Furthermore, it would be interesting to examine whether it is possible to overcome Mφ innate memory with specific triggers. In this regard, we already established that IFNγ can, to certain degree, overcome the inhibitory effect of LPS. Such methods could be used for example in the treatment of autoimmune diseases maintained by activated Mφ.

8.2 The importance of environmental factors in understanding immune-mediated consequences of therapeutic applications

Chemotherapy or RT may reshape the tumour microenvironment which inadvertently modulates the biology of APC. Indeed, Pinto *et al*. [646] showed that RT promotes a pro-inflammatory Mφ phenotype preserving their pro-angiogenic properties. On the other hand, Merrick *et al*. [707] reported immunosuppressive effects of IR on DC characterised by down-regulation of IL-12 and reduced capacity to prime naïve T-

cells. Other studies [765] reported that irradiated DC were impaired in their ability to prime T-cell against endogenously processed Ag but enhanced priming against exogenous Ag demonstrating the complexity of the interplay between radiation and DC function. Our results confirm the immuno-regulatory effect of IR and demonstrate that not all genes are adversely affected. IR selectively down-regulated IL-23 secretion by both Mφ and DC. Although IL-12 secretion by irradiated DC was reduced, this was less pronounced. Furthermore, there was no effect of IR on IL-12 secretion by Mφ suggesting differential regulatory pathways in Mφ and DC.

Recently Schirmer *et al*. [727] reported that FB up-regulate IL-23 secretion by DC. This prompted us to question whether FB continue to up-regulate IL-23 secretion by DC in the presence of IR. Indeed, the attenuating effect of IR on IL-23 secretion by DC was largely diminished in the presence of FB. Since the IL-23-Th17 axis is implicated in the development of idiopathic and radiation-induced fibrosis, our work identifies a potential mechanism for the pathological consequences of radio- and chemotherapy [766-768]. Importantly these results demonstrate how important it is to consider the contribution made by bystander stromal cells when interpreting single-cell studies and the need for comprehensive, multi-cellular, pre-clinical models.

Fibroblasts were relatively radio-resistant to IR. Previous reports have shown that persistent DNA damage due to chemotherapy or RT activates FB and triggers premature senescence characterised by secretion of inflammatory mediators such as IL-6 [730-733]. According to some reports even a single dose of IR may trigger their senescence [732]. It would be interesting to study whether the effect of senescent FB on DC and Mφ is stronger due to higher levels of secreted cytokines. Furthermore,

future studies should address the response of Mφ/DC-FB co-cultures to cumulative doses of IR, using the same fractionated scheme as used in cancer patients. Considering the pro-inflammatory phenotype characteristic for senescent cells it is possible that long-term genotoxic stress may result in an augmentation of APC-FB cross talk and even stronger promotion of IL-23 secretion.

8.3 Employing discoveries from studies on environmental regulation of immune system to create novel targets for immunotherapy

Although the roles of IL-23 in autoimmunity and cancer are well described, the molecular pathways governing its secretion remain largely unknown. Therefore, we investigated the regulatory mechanisms involved in modulation of IL-23 responses.

A key finding was that the effect of IR on IL-23 secretion by Mφ and DC was ATM-dependent. Although the canonical function of ATM is to direct appropriate response to DNA damage, evidence of its involvement in regulation has, in recent years, started to emerge. ATM regulates the production of IL-8, MIP-1α and IL-6 and delays apoptosis in LPS activated neutrophils [769]. Moreover, inhibition of ATM in monocytes increases the levels of IL-1β, IL-6 and TNFα in response to TLR4 activation [769]. On the other hand, A-T patients often display severe immunodeficiency [556]. Furthermore, ATM governs the expression of IFN-γ and IFN-α genes in T-cells [563]. Therefore, the effects of ATM on immune functions seem multifaceted.

Our results support the notion of immunomodulatory functions of ATM. Inhibiting ATM activation with the small molecule inhibitor KU55933 did not adversely affect DC viability therefore presenting ATM as a potential novel target for immunotherapy.

Surprisingly, we found that the mechanism of FB-dependent IL-23 production by irradiated DC was independent of ATM activity and instead mediated through the c-AMP pathway. c-AMP was previously implemented in the induction of IL-23 secretion by PGE_2 [289].

Both ATM and c-AMP exert their effect via CREB-CBP interactions with ATM inhibiting CREB function while c-AMP promotes CREB/CBP interactions [289, 555]. In our multicellular model, we showed simultaneous activation of two autonomous pathways (one governed by ATM and another by c-AMP) required a decision to be made by the receiving APC. Targeting c-AMP in DC therefore may help to reduce the incidence of post-RT fibrosis. However, cAMP activation in FB was linked to reduced fibrosis due to disruption of epithelial–mesenchymal transformation (EMT) and down-regulation of Epac expression by pro-fibrotic agents such as TGF-β or angiotensin II and agents augmenting cAMP activity have been proposed for the treatment of fibrosis [770-772]. In such a setting, multi-cellular models demonstrated potential advantages over single-cell systems by providing additional targets that influence the cross-talk between cells. We show that the cross-talk between FB and DC depends on the ability of DC to secrete TNFα and IL-1β which induce COX2-dependent secretion of PGE_2 by FB. FB-derived PGE2 stimulates cAMP which overcomes the ATM-dependent inhibition of IL-23 secretion. Targeting any of these elements may potentially successfully disrupt the cross-talk between FB and DC resulting in a decrease of IL-23 and consequently IL-17.

Overall our results highlight the importance of studies on the environmental regulation of APC functions and the value of multicellular models for the

development of new therapeutic strategies as well as understanding pathologies. Designing appropriate multicellular systems for testing therapeutic approaches in vitro has tremendous potential to increase the detection of possible failings by the proposed therapy schedule before entering clinical trials. This will improve the economic outcome and allow for faster implementation of possible changes in the protocol therefore positively impacting on the "bench to bed" research.

8.4 Conclusions and future directions

APC continuously interact with microenvironment through their life span, beginning from their generation in a bone marrow and continuing through the evolving disease process. During this time APC are under constant influence by external factors including other cells and therapeutic interventions which also mutually influence each other. Our understanding of APC is largely based on binary premises, for example immature vs mature, or M1 vs M2. However, it is increasingly apparent that, whilst valuable, such concepts fall short of explaining the dynamic, multi-stage changes that occur in the life span of these cells.

Our work addressed a hypothesis that earlier encounters with pro- and anti-inflammatory stimuli modify the response of Mφ to subsequent challenge therefore demonstrating restrictions in their plasticity and presence of innate memory. These findings demonstrate restrictions in Mφ plasticity imposed by initial conditioning and suggest the development of innate memory with a degree of specificity. Our current work examined the key-representative features of pro- and anti-inflammatory Mφ – namely IL-23, IL-12 and IL-10 with a narrow range of co-stimulatory molecules. This should be further expanded to fully address the effect of Mφ conditioning on their instruction of T-cell responses. Furthermore, we described a general model of

pro- and anti-inflammatory Mφ which will form a basis for further work on specific Mφ populations with a focus on TAMφ. Since Mφ are non-proliferating cells, the required number of cells to perform experiments cannot readily be extracted from a tumour tissue. To overcome this challenge, TAMφ are often generated in the presence of cytokines such as IL-6 and IL-10 which are typically abundant in cancer environment. Alternatively, co-cultures of Mφ with cancer cells or in the presence of tumour cell supernatant can be utilised to produce TAMφ specific for a desired type of cancer.

During the progression of a disease process, such as cancer, immune cells residing in the stroma are affected by both microenvironment and therapeutic interventions. We show for the first time, that therapeutic doses of IR suppressed IL-23 secretion by DC and Mφ and consequently the resulting Th17 response. However, the effect on DC was abrogated by the presence of FB. This highlights the importance of employing multi-cellular systems to better understand biology. Our current model is based on APC and healthy FB therefore may reflect the outcome of RT on healthy tissues. Although, it is important to understand the mechanisms of mutual interactions in healthy tissues, it is plausible that using cells from tumour tissues will result in a different outcome. To fully elucidate the effect of RT in cancer, further work should utilize tumour-associated APC and TAF. Furthermore, expanding our two-cell culture model to include cancer cells will allow to better reflect the conditions prevailing in human cancer. In this respect, breast and lung cancer models can present valuable insights into the understanding of short and long-term side-effects of RT such as fibrosis. Although our experiments concluded at 1-2 days following a single RT dose, future work should evaluate later time-points and consider multiple RT doses over a

period of time (5-7 days). Such an experimental regime was utilized by Pinto *at el*. suggesting a pro-inflammatory effect of IR on Mφ. [646]. Furthermore, prolonged culture of FB following radiation or increase in a total cumulative dose may results in the development of senescence therefore profoundly affecting their interactions with other cells. The invention and validation of ever more complex *in vitro* systems that may include relevant matrix components, flow (both vascular and lymphovascular), 3-dimensional culture, microgravity, oxygen-potential, pH etc have the potential to provide physiological models far in advance of current technology. These systems have the potential to increase our understanding of cell-biology and replace current state-of-the-art tools such as patient-derived xenograft (PDX).

Appendices:

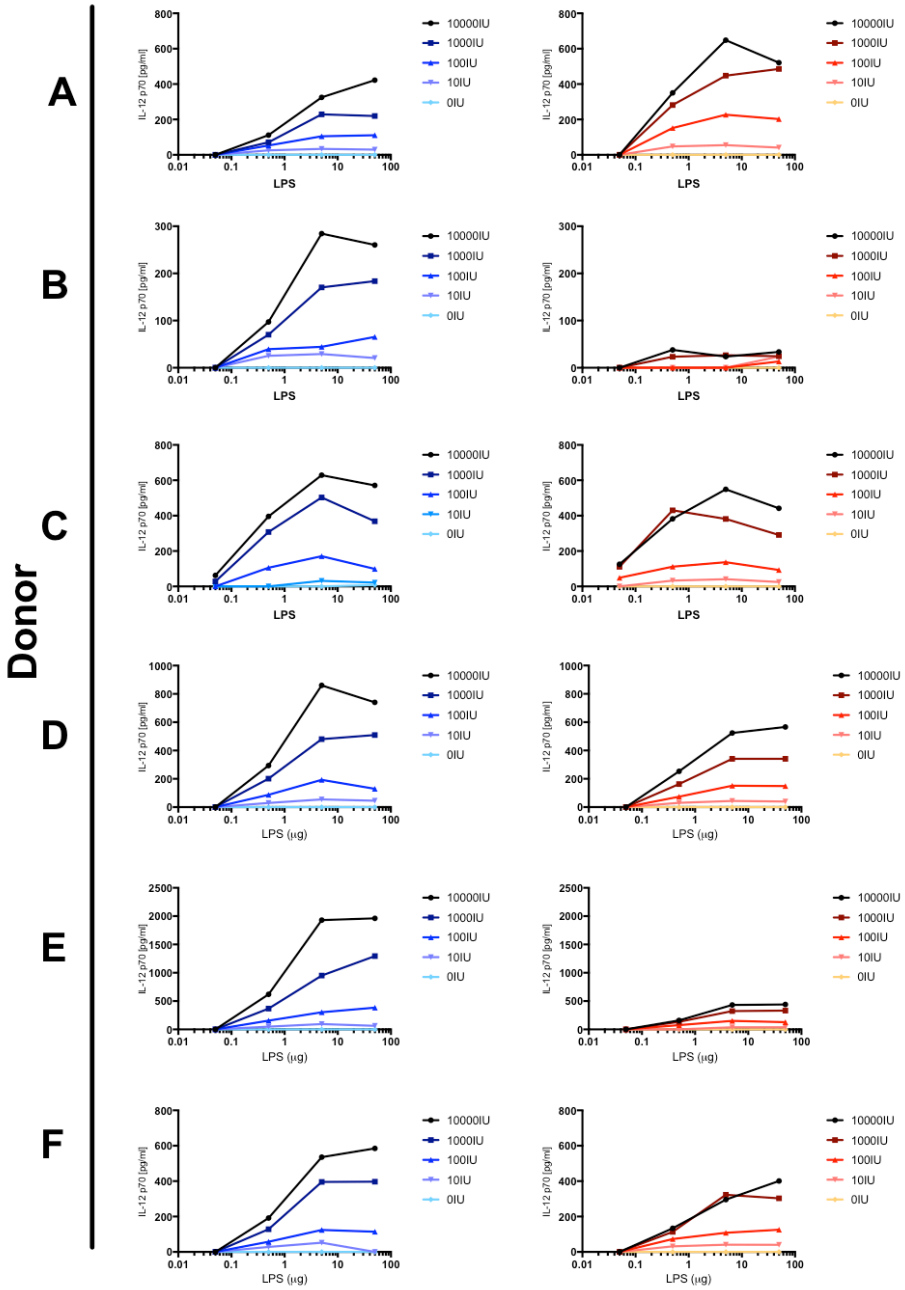


Fig S.1A Effect of LPS and IFNγ dose on IL-12 secretion by MoMφ. GM-Mφ (blue) and M-Mφ (red) from 6 donors were activated with increasing doses of LPS (0.05-50µg) and IFNγ (10-10000 IU). The secretion of IL-12p70, was measured at 24h by ELISA. Each experiment was performed in singlicate.

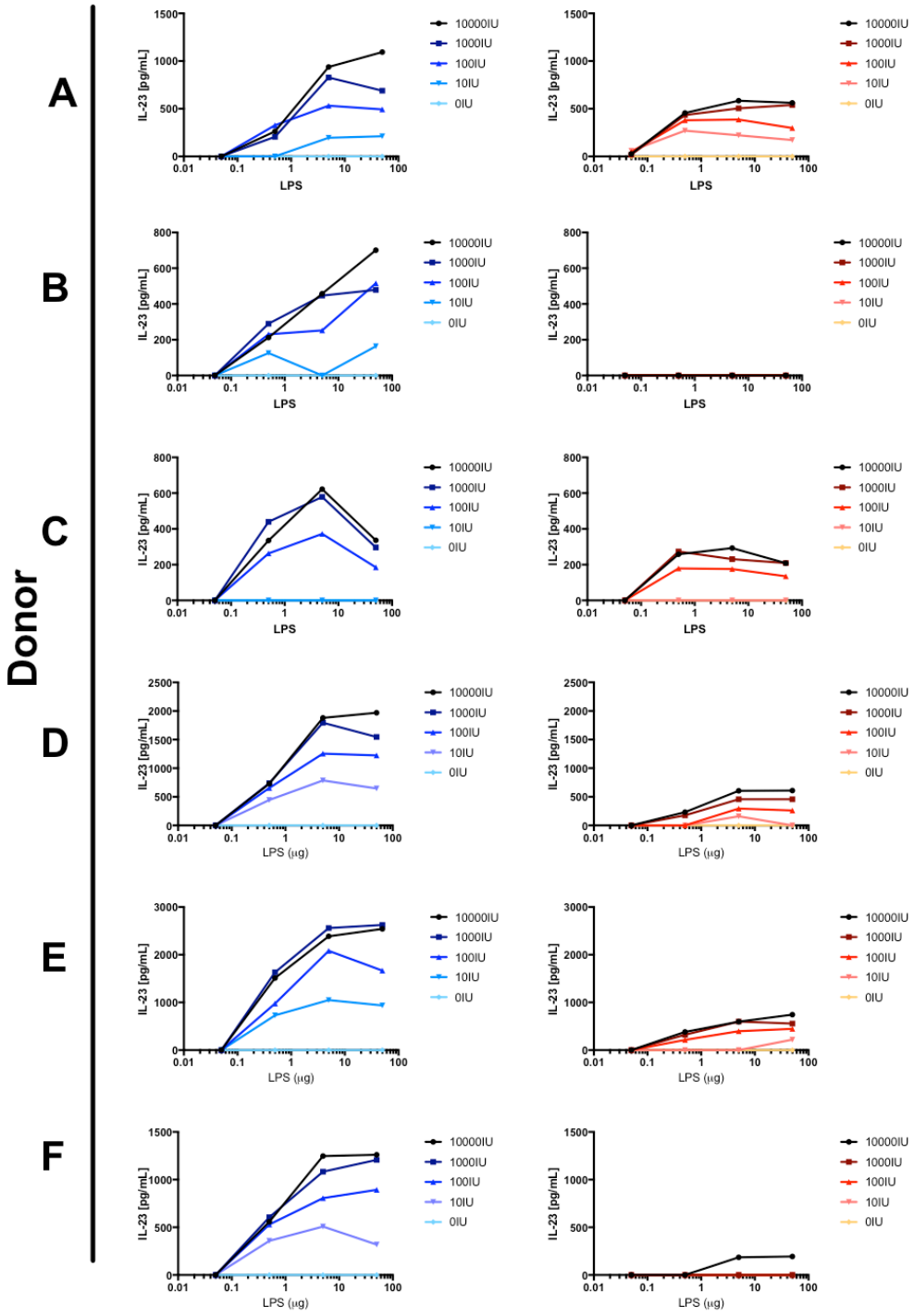


Fig S.1B Effect of LPS and IFNγ dose on IL-23 secretion by MoMφ. GM-Mφ (blue) and M-Mφ (red) from 6 donors were activated with increasing doses of LPS (0.05-50µg) and IFNγ (10-10000 IU). The secretion of IL-23, was measured at 24h by ELISA. Each experiment was performed in singlicate.

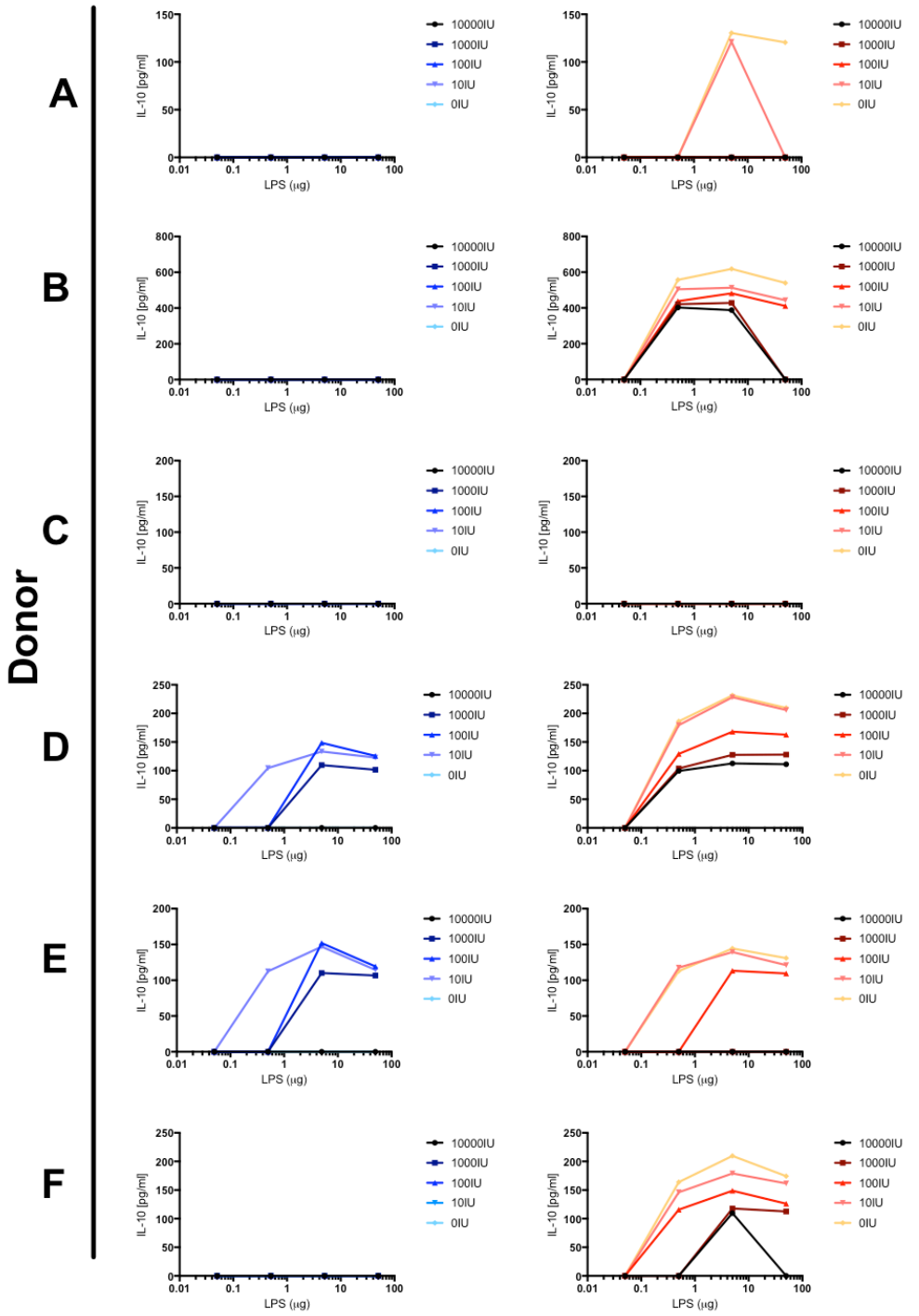


Fig S.1C Effect of LPS and IFNγ dose on IL-10 secretion by MoMφ. GM-Mφ (blue) and M-Mφ (red) from 6 donors were activated with increasing doses of LPS (0.05-50µg) and IFNγ (10-10000 IU). The secretion of IL-10, was measured at 24h by ELISA. Each experiment was performed in singlicate.

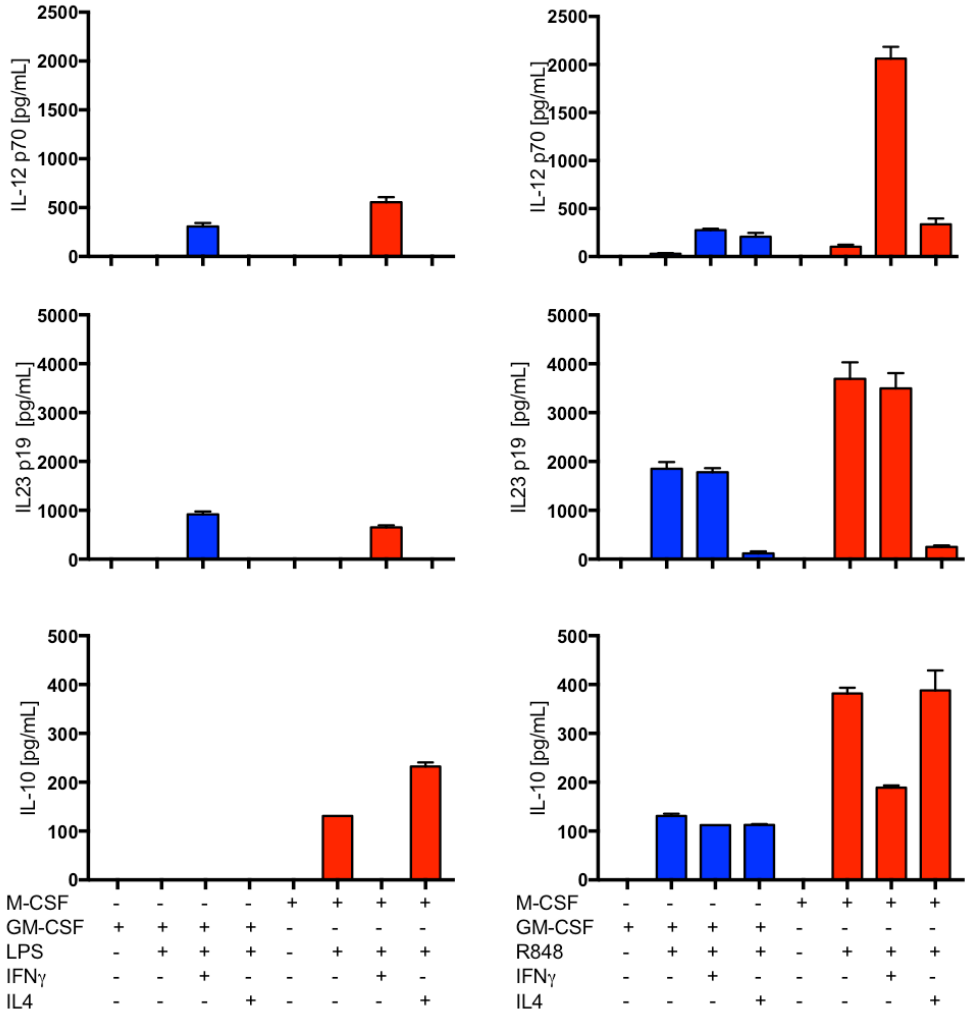


Figure S.2A Comparison of the effect of LPS and R848 stimulation on cytokine secretion by MoMφ. M-Mφ and GM-Mφ from 5 donors (Fig. S2A-E) were stimulated with LPS (5µg/mL) or R848 (40µg/mL) in the presence or absence of IFNγ (1000IU) and IL-4 (20ng/mL). Supernatants were collected 24h later and cytokine levels measured by ELISA. Shown is mean + SD of triplicate stimulation for each condition.

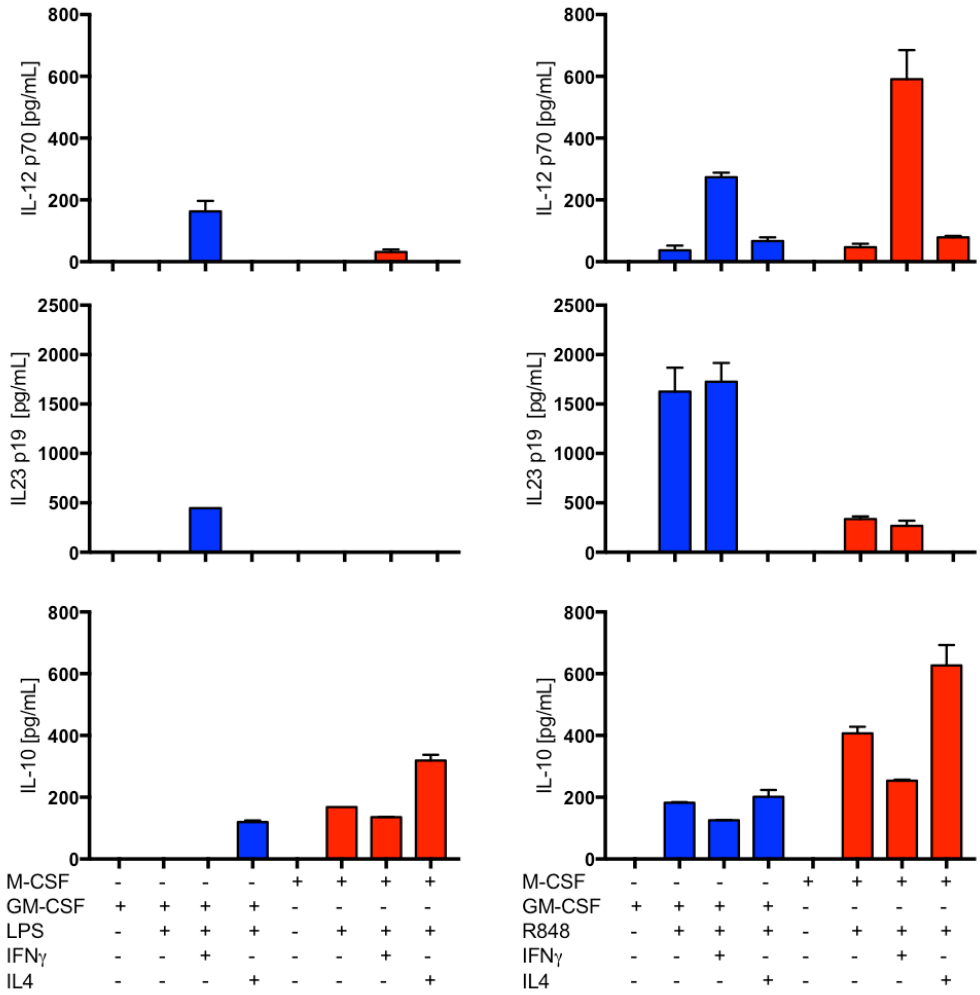


Figure S.2B Comparison of the effect of LPS and R848 stimulation on cytokine secretion by MoMφ. M-Mφ and GM-Mφ from 5 donors (Fig. S2A-E) were stimulated with LPS (5µg/mL) or R848 (40µg/mL) in the presence or absence of IFNγ (1000IU) and IL-4 (20ng/mL). Supernatants were collected 24h later and cytokine levels measured by ELISA. Shown is mean + SD of triplicate stimulation for each condition.

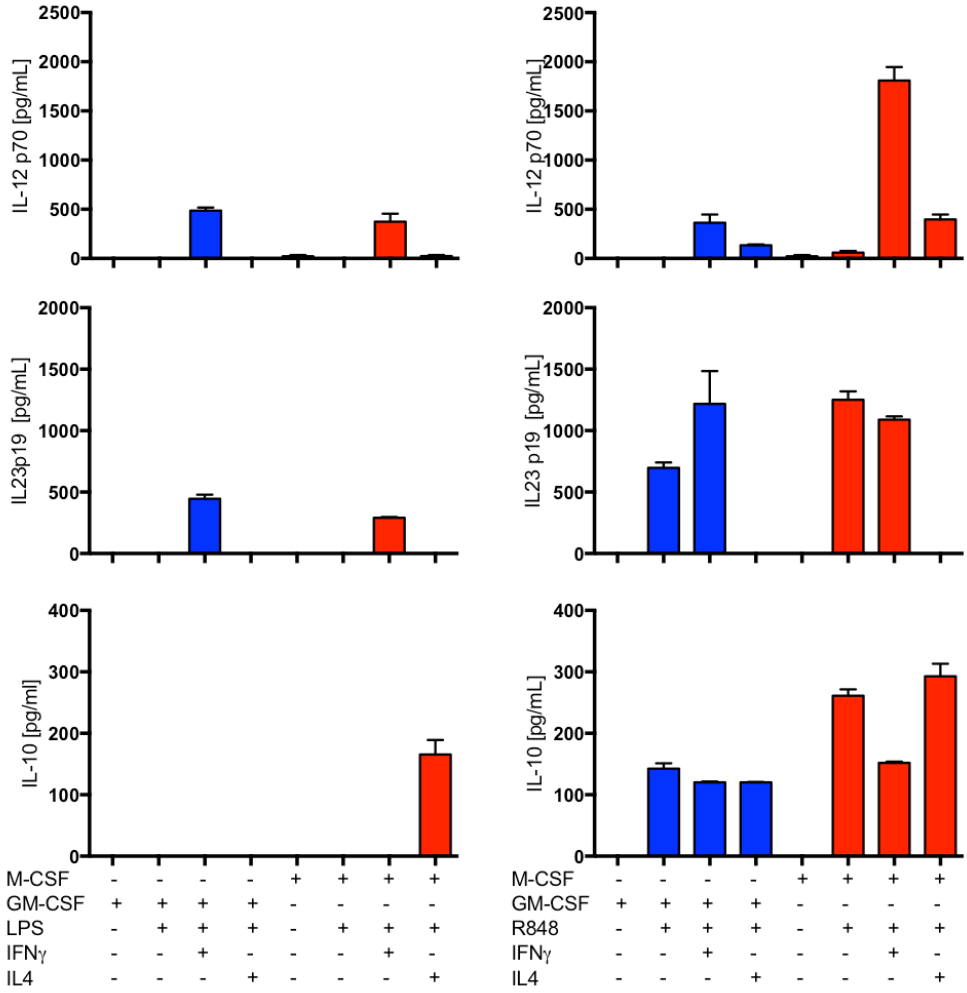


Figure S.2C Comparison of the effect of LPS and R848 stimulation on cytokine secretion by MoMφ. M-Mφ and GM-Mφ from 5 donors (Fig. S2A-E) were stimulated with LPS (5µg/mL) or R848 (40µg/mL) in the presence or absence of IFNγ (1000IU) and IL-4 (20ng/mL). Supernatants were collected 24h later and cytokine levels measured by ELISA. Shown is mean + SD of triplicate stimulation for each condition.

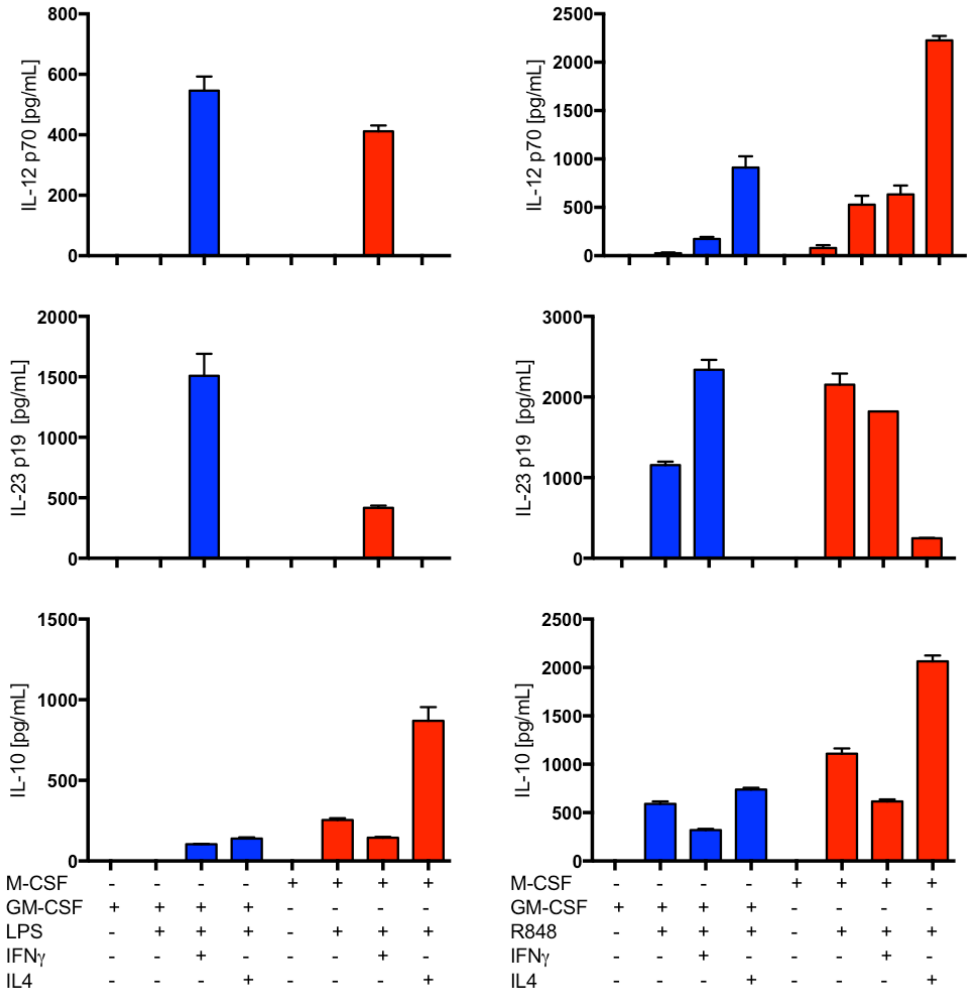


Figure S.2D Comparison of the effect of LPS and R848 stimulation on cytokine secretion by MoMφ. M-Mφ and GM-Mφ from 5 donors (Fig. S2A-E) were stimulated with LPS (5µg/mL) or R848 (40µg/mL) in the presence or absence of IFNγ (1000IU) and IL-4 (20ng/mL). Supernatants were collected 24h later and cytokine levels measured by ELISA. Shown is mean + SD of triplicate stimulation for each condition.

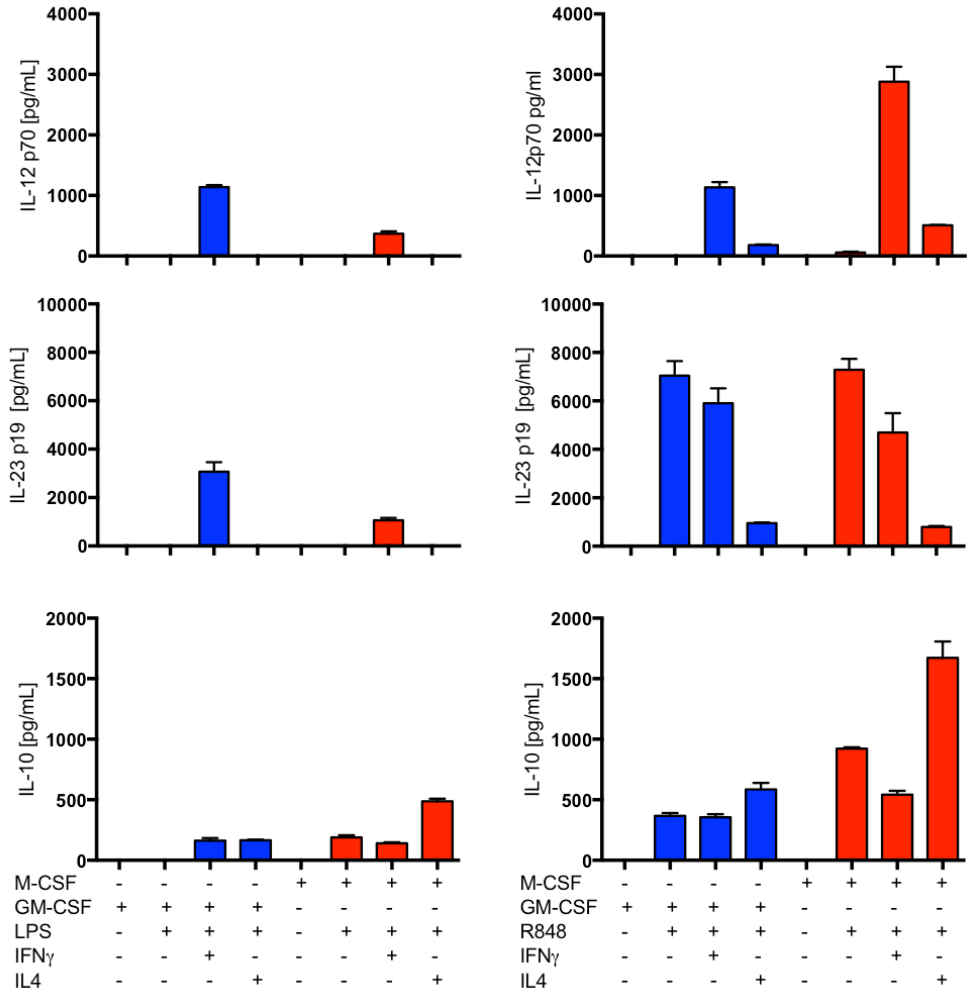


Figure S.2E Comparison of the effect of LPS and R848 stimulation on cytokine secretion by MoMφ. M-Mφ and GM-Mφ from 5 donors (Fig. S2A-E) were stimulated with LPS (5µg/mL) or R848 (40µg/mL) in the presence or absence of IFNγ (1000IU) and IL-4 (20ng/mL). Supernatants were collected 24h later and cytokine levels measured by ELISA. Shown is mean + SD of triplicate stimulation for each condition.

References

1. Hamilos, D.L., *Antigen presenting cells.* Immunol Res, 1989. **8**(2): p. 98-117.
2. Sprent, J., *Antigen-presenting cells: professionals and amateurs.* Current Biology, 1995. **5**(10): p. 1095-1097.
3. Saalbach, A., et al., *Dermal fibroblasts induce maturation of dendritic cells.* The Journal of Immunology, 2007. **178**(8): p. 4966-4974.
4. Saalbach, A., et al., *Fibroblasts support migration of monocyte‐derived dendritic cells by secretion of PGE2 and MMP‐1.* Experimental dermatology, 2015. **24**(8): p. 598-604.
5. Hennen, J., et al., *Cross talk between keratinocytes and dendritic cells: impact on the prediction of sensitization.* Toxicological Sciences, 2011: p. kfr174.
6. Wilson, N.S. and J.A. Villadangos, *Regulation of antigen presentation and cross-presentation in the dendritic cell network: facts, hypothesis, and immunological implications.* Advances in immunology, 2005. **86**: p. 241-305.
7. Bernhard, C.A., et al., *CD169+ macrophages are sufficient for priming of CTLs with specificities left out by cross-priming dendritic cells.* Proceedings of the National Academy of Sciences, 2015. **112**(17): p. 5461-5466.
8. Larson, S., et al., *Ly6C+ monocyte efferocytosis and cross-presentation of cell-associated antigens.* Cell Death & Differentiation, 2016. **23**(6): p. 997-1003.
9. Brode, S. and P.A. Macary, *Cross‐presentation: dendritic cells and macrophages bite off more than they can chew!* Immunology, 2004. **112**(3): p. 345-351.
10. Takeuchi, O. and S. Akira, *Pattern recognition receptors and inflammation.* Cell, 2010. **140**(6): p. 805-820.
11. Takeda, K. and S. Akira, *Toll-like receptors in innate immunity.* International immunology, 2005. **17**(1): p. 1-14.
12. Akira, S., S. Uematsu, and O. Takeuchi, *Pathogen recognition and innate immunity.* Cell, 2006. **124**(4): p. 783-801.
13. Yoneyama, M. and T. Fujita, *Structural mechanism of RNA recognition by the RIG-I-like receptors.* Immunity, 2008. **29**(2): p. 178-181.
14. Janeway, C.A. *Approaching the asymptote? Evolution and revolution in immunology*. in *Cold Spring Harbor symposia on quantitative biology*. 1989. Cold Spring Harbor Laboratory Press.
15. Janeway Jr, C.A. and R. Medzhitov, *Innate immune recognition.* Annual review of immunology, 2002. **20**(1): p. 197-216.
16. Matzinger, P., *Tolerance, danger, and the extended family.* Annual review of immunology, 1994. **12**(1): p. 991-1045.
17. Pulendran, B., *Immune activation: death, danger and dendritic cells.* Current Biology, 2004. **14**(1): p. R30-R32.
18. Shi, Y., J.E. Evans, and K.L. Rock, *Molecular identification of a danger signal that alerts the immune system to dying cells.* Nature, 2003. **425**(6957): p. 516-521.
19. Haag, F., et al., *Extracellular NAD and ATP: Partners in immune cell modulation.* Purinergic signalling, 2007. **3**(1-2): p. 71.

20. Ishikawa, K., et al., *The innate immune system in host mice targets cells with allogenic mitochondrial DNA.* Journal of experimental medicine, 2010. **207**(11): p. 2297-2305.
21. Schaue, D., et al. *Radiation and inflammation*. in *Seminars in radiation oncology*. 2015. Elsevier.
22. Iwasaki, A. and R. Medzhitov, *Toll-like receptor control of the adaptive immune responses.* Nature immunology, 2004. **5**(10): p. 987-995.
23. e Sousa, C.R. *Toll-like receptors and dendritic cells: for whom the bug tolls*. in *Seminars in immunology*. 2004. Elsevier.
24. Majewska, M. and M. Szczepanik, *The role of Toll-like receptors (TLR) in innate and adaptive immune responses and their function in immune response regulation.* Postepy higieny i medycyny doswiadczalnej (Online), 2005. **60**: p. 52-63.
25. Nakano, H., et al., *Blood-derived inflammatory dendritic cells in lymph nodes stimulate acute T helper type 1 immune responses.* Nature immunology, 2009. **10**(4): p. 394-402.
26. West, A.P., et al., *TLR signalling augments macrophage bactericidal activity through mitochondrial ROS.* Nature, 2011. **472**(7344): p. 476-480.
27. Blander, J.M., *Phagocytosis and antigen presentation: a partnership initiated by Toll-like receptors.* Annals of the rheumatic diseases, 2008. **67**(Suppl 3): p. iii44-iii49.
28. Kaliński, P., et al., *T-cell priming by type-1and type-2 polarized dendritic cells: the concept of a third signal.* Immunology today, 1999. **20**(12): p. 561-567.
29. Curtsinger, J.M., D.C. Lins, and M.F. Mescher, *Signal 3 Determines Tolerance versus Full Activation of Naive CD8 T Cells.* Journal of experimental medicine, 2003. **197**(9): p. 1141-1151.
30. Chen, L. and D.B. Flies, *Molecular mechanisms of T cell co-stimulation and co-inhibition.* Nature Reviews Immunology, 2013. **13**(4): p. 227-242.
31. Goral, S., *The three- signal hypothesis of lymphocyte activation/targets for immunosuppression.* Dialysis & Transplantation, 2011. **40**(1): p. 14-16.
32. Greenwald, R.J., G.J. Freeman, and A.H. Sharpe, *The B7 family revisited.* Annu. Rev. Immunol., 2005. **23**: p. 515-548.
33. Carter, L.L., et al., *PD- 1: PD- L inhibitory pathway affects both CD4+ and CD8+ T cells and is overcome by IL- 2.* European journal of immunology, 2002. **32**(3): p. 634-643.
34. Shi, L., et al., *The role of PD-1 and PD-L1 in T-cell immune suppression in patients with hematological malignancies.* Journal of hematology & oncology, 2013. **6**(1): p. 74.
35. Mescher, M.F., et al., *Signals required for programming effector and memory development by CD8+ T cells.* Immunological reviews, 2006. **211**(1): p. 81-92.
36. Curtsinger, J.M. and M.F. Mescher, *Inflammatory cytokines as a third signal for T cell activation.* Current opinion in immunology, 2010. **22**(3): p. 333-340.
37. Zhou, L., M.M. Chong, and D.R. Littman, *Plasticity of CD4+ T cell lineage differentiation.* Immunity, 2009. **30**(5): p. 646-655.
38. Schmidt, C.S. and M.F. Mescher, *Adjuvant effect of IL-12: conversion of peptide antigen administration from tolerizing to immunizing for CD8+ T cells in vivo.* The Journal of Immunology, 1999. **163**(5): p. 2561-2567.

39. Agarwal, P., et al., *Gene regulation and chromatin remodeling by IL-12 and type I IFN in programming for CD8 T cell effector function and memory.* The Journal of Immunology, 2009. **183**(3): p. 1695-1704.
40. Pape, K.A., et al., *Inflammatory cytokines enhance the in vivo clonal expansion and differentiation of antigen-activated CD4+ T cells.* The Journal of Immunology, 1997. **159**(2): p. 591-598.
41. Ben-Sasson, S.Z., et al., *IL-1 acts directly on CD4 T cells to enhance their antigen-driven expansion and differentiation.* Proceedings of the National Academy of Sciences, 2009. **106**(17): p. 7119-7124.
42. Zhu, J. and W.E. Paul, *Peripheral CD4+ T- cell differentiation regulated by networks of cytokines and transcription factors.* Immunological reviews, 2010. **238**(1): p. 247-262.
43. Boyton, R.J. and D.M. Altmann, *Is selection for TCR affinity a factor in cytokine polarization?* Trends in immunology, 2002. **23**(11): p. 526-529.
44. Purvis, H.A., et al., *Low-strength T-cell activation promotes Th17 responses.* Blood, 2010. **116**(23): p. 4829-4837.
45. Wynn, T.A., A. Chawla, and J.W. Pollard, *Origins and Hallmarks of Macrophages: Development, Homeostasis, and Disease.* Nature, 2013. **496**(7446): p. 445-455.
46. Ginhoux, F., et al., *Fate mapping analysis reveals that adult microglia derive from primitive macrophages.* Science, 2010. **330**(6005): p. 841-5.
47. Hoeffel, G. and F. Ginhoux, *Ontogeny of Tissue-Resident Macrophages.* Front Immunol, 2015. **6**: p. 486.
48. van de Laar, L., et al., *Yolk Sac Macrophages, Fetal Liver, and Adult Monocytes Can Colonize an Empty Niche and Develop into Functional Tissue-Resident Macrophages.* Immunity, 2016. **44**(4): p. 755-68.
49. Yona, S., et al., *Fate mapping reveals origins and dynamics of monocytes and tissue macrophages under homeostasis.* Immunity, 2013. **38**(1): p. 79-91.
50. Hoeffel, G., et al., *C-Myb(+) erythro-myeloid progenitor-derived fetal monocytes give rise to adult tissue-resident macrophages.* Immunity, 2015. **42**(4): p. 665-78.
51. Takahashi, K., F. Yamamura, and M. Naito, *Differentiation, maturation, and proliferation of macrophages in the mouse yolk sac: a light-microscopic, enzyme-cytochemical, immunohistochemical, and ultrastructural study.* J Leukoc Biol, 1989. **45**(2): p. 87-96.
52. Scott, C.L., S. Henri, and M. Guilliams, *Mononuclear phagocytes of the intestine, the skin, and the lung.* Immunol Rev, 2014. **262**(1): p. 9-24.
53. Davies, L.C. and P.R. Taylor, *Tissue-resident macrophages: then and now.* Immunology, 2015. **144**(4): p. 541-8.
54. Epelman, S., K.J. Lavine, and G.J. Randolph, *Origin and functions of tissue macrophages.* Immunity, 2014. **41**(1): p. 21-35.
55. Hettinger, J., et al., *Origin of monocytes and macrophages in a committed progenitor.* Nat Immunol, 2013. **14**(8): p. 821-830.
56. Ingersoll, M.A., et al., *Comparison of gene expression profiles between human and mouse monocyte subsets.* Blood, 2010. **115**(3): p. e10-9.
57. Italiani, P. and D. Boraschi, *From Monocytes to M1/M2 Macrophages: Phenotypical vs. Functional Differentiation.* Front Immunol, 2014. **5**: p. 514.
58. Zimmermann, H.W., et al., *Functional contribution of elevated circulating and hepatic non-classical CD14CD16 monocytes to inflammation and human liver fibrosis.* PLoS One, 2010. **5**(6): p. e11049.

59. Auffray, C., M.H. Sieweke, and F. Geissmann, *Blood monocytes: development, heterogeneity, and relationship with dendritic cells.* Annu Rev Immunol, 2009. **27**: p. 669-92.
60. Allan, D.S., et al., *Tetrameric complexes of human histocompatibility leukocyte antigen (HLA)-G bind to peripheral blood myelomonocytic cells.* J Exp Med, 1999. **189**(7): p. 1149-56.
61. Steppich, B., et al., *Selective mobilization of CD14(+)CD16(+) monocytes by exercise.* Am J Physiol Cell Physiol, 2000. **279**(3): p. C578-86.
62. Geissmann, F., S. Jung, and D.R. Littman, *Blood monocytes consist of two principal subsets with distinct migratory properties.* Immunity, 2003. **19**(1): p. 71-82.
63. Nahrendorf, M., et al., *The healing myocardium sequentially mobilizes two monocyte subsets with divergent and complementary functions.* J Exp Med, 2007. **204**(12): p. 3037-47.
64. Landsman, L., C. Varol, and S. Jung, *Distinct differentiation potential of blood monocyte subsets in the lung.* J Immunol, 2007. **178**(4): p. 2000-7.
65. Das, A., et al., *Monocyte and macrophage plasticity in tissue repair and regeneration.* Am J Pathol, 2015. **185**(10): p. 2596-606.
66. Jakubzick, C., et al., *Minimal differentiation of classical monocytes as they survey steady-state tissues and transport antigen to lymph nodes.* Immunity, 2013. **39**(3): p. 599-610.
67. Ancuta, P., et al., *CD16+ monocyte-derived macrophages activate resting T cells for HIV infection by producing CCR3 and CCR4 ligands.* J Immunol, 2006. **176**(10): p. 5760-71.
68. Randolph, G.J., et al., *The CD16(+) (FcγRIII(+)) Subset of Human Monocytes Preferentially Becomes Migratory Dendritic Cells in a Model Tissue Setting.* The Journal of Experimental Medicine, 2002. **196**(4): p. 517-527.
69. Krutzik, S.R., et al., *TLR activation triggers the rapid differentiation of monocytes into macrophages and dendritic cells.* Nat Med, 2005. **11**(6): p. 653-60.
70. Feng, A.L., et al., *CD16(+) monocytes in breast cancer patients: expanded by monocyte chemoattractant protein-1 and may be useful for early diagnosis.* Clinical and Experimental Immunology, 2011. **164**(1): p. 57-65.
71. Saleh, M.N., et al., *CD16+ monocytes in patients with cancer: spontaneous elevation and pharmacologic induction by recombinant human macrophage colony-stimulating factor.* Blood, 1995. **85**(10): p. 2910-7.
72. Erreni, M., et al., *Human glioblastoma tumours and neural cancer stem cells express the chemokine CX3CL1 and its receptor CX3CR1.* Eur J Cancer, 2010. **46**(18): p. 3383-92.
73. Zheng, J., et al., *Chemokine receptor CX3CR1 contributes to macrophage survival in tumor metastasis.* Molecular Cancer, 2013. **12**: p. 141-141.
74. Butovsky, O., et al., *Identification of a Unique TGF-β Dependent Molecular and Functional Signature in Microglia.* Nature neuroscience, 2014. **17**(1): p. 131-143.
75. Gosselin, D., et al., *Environment drives selection and function of enhancers controlling tissue-specific macrophage identities.* Cell, 2014. **159**(6): p. 1327-40.
76. Lavin, Y., et al., *Tissue-Resident Macrophage Enhancer Landscapes Are Shaped by the Local Microenvironment.* Cell, 2014. **159**(6): p. 1312-1326.

77. Epelman, S., et al., *Embryonic and adult-derived resident cardiac macrophages are maintained through distinct mechanisms at steady state and during inflammation.* Immunity, 2014. **40**(1): p. 91-104.
78. Gundra, U.M., et al., *Alternatively activated macrophages derived from monocytes and tissue macrophages are phenotypically and functionally distinct.* Blood, 2014. **123**(20): p. e110-22.
79. Ju, C. and F. Tacke, *Hepatic macrophages in homeostasis and liver diseases: from pathogenesis to novel therapeutic strategies.* Cellular and Molecular Immunology, 2016. **13**(3): p. 316-327.
80. Murphy, J., et al., *The prolonged life-span of alveolar macrophages.* Am J Respir Cell Mol Biol, 2008. **38**(4): p. 380-5.
81. Takahashi, K., *Development and Differentiation of Macrophages and Related Cells*
Historical Review and Current Concepts. Journal of Clinical and Experimental Hematopathology, 2001. **41**(1): p. 1-31.
82. Dixon, L.J., et al., *Kupffer cells in the liver.* Compr Physiol, 2013. **3**(2): p. 785-97.
83. Yu, W., et al., *Macrophage proliferation is regulated through CSF-1 receptor tyrosines 544, 559, and 807.* J Biol Chem, 2012. **287**(17): p. 13694-704.
84. Pixley, F.J. and E.R. Stanley, *CSF-1 regulation of the wandering macrophage: complexity in action.* Trends Cell Biol, 2004. **14**(11): p. 628-38.
85. Tushinski, R.J., et al., *Survival of mononuclear phagocytes depends on a lineage-specific growth factor that the differentiated cells selectively destroy.* Cell, 1982. **28**(1): p. 71-81.
86. Jenkins, S.J., et al., *IL-4 directly signals tissue-resident macrophages to proliferate beyond homeostatic levels controlled by CSF-1.* The Journal of Experimental Medicine, 2013. **210**(11): p. 2477.
87. Jenkins, S.J., et al., *Local macrophage proliferation, rather than recruitment from the blood, is a signature of TH2 inflammation.* Science, 2011. **332**(6035): p. 1284-8.
88. Chen, B.D., C.R. Clark, and T.H. Chou, *Granulocyte/macrophage colony-stimulating factor stimulates monocyte and tissue macrophage proliferation and enhances their responsiveness to macrophage colony-stimulating factor.* Blood, 1988. **71**(4): p. 997-1002.
89. Celada, A. and R.A. Maki, *Transforming growth factor-beta enhances the M-CSF and GM-CSF-stimulated proliferation of macrophages.* J Immunol, 1992. **148**(4): p. 1102-5.
90. Parihar, A., T.D. Eubank, and A.I. Doseff, *Monocytes and macrophages regulate immunity through dynamic networks of survival and cell death.* J Innate Immun, 2010. **2**(3): p. 204-15.
91. Frantz, S. and M. Nahrendorf, *Cardiac macrophages and their role in ischaemic heart disease.* Cardiovasc Res, 2014. **102**(2): p. 240-8.
92. Bain, C.C., et al., *Resident and pro-inflammatory macrophages in the colon represent alternative context-dependent fates of the same Ly6Chi monocyte precursors.* Mucosal Immunol, 2013. **6**(3): p. 498-510.
93. Franklin, R.A., et al., *The cellular and molecular origin of tumor-associated macrophages.* Science, 2014. **344**(6186): p. 921-5.
94. Franklin, R.A. and M.O. Li, *The ontogeny of tumor-associated macrophages: a new understanding of cancer-elicited inflammation.* Oncoimmunology, 2014. **3**(9): p. e955346.

95. Colegio, O.R., et al., *Functional polarization of tumour-associated macrophages by tumour-derived lactic acid.* Nature, 2014. **513**(7519): p. 559-63.
96. Movahedi, K., et al., *Different tumor microenvironments contain functionally distinct subsets of macrophages derived from Ly6C(high) monocytes.* Cancer Res, 2010. **70**(14): p. 5728-39.
97. Pioli, P.A., et al., *TGF-beta regulation of human macrophage scavenger receptor CD163 is Smad3-dependent.* J Leukoc Biol, 2004. **76**(2): p. 500-8.
98. Wynn, T.A., A. Chawla, and J.W. Pollard, *Macrophage biology in development, homeostasis and disease.* Nature, 2013. **496**(7446): p. 445-455.
99. Jantsch, J., et al., *Macrophages in homeostatic immune function.* Frontiers in physiology, 2014. **5**.
100. Nathan, C., *Metchnikoff's Legacy in 2008*. 2008, Nature Publishing Group.
101. Billack, B., *Macrophage activation: role of toll-like receptors, nitric oxide, and nuclear factor kappa B.* American journal of pharmaceutical education, 2006. **70**(5): p. 102.
102. Hilhorst, M., et al., *T cell–macrophage interactions and granuloma formation in vasculitis.* Frontiers in immunology, 2014. **5**.
103. Janeway Jr, C.A., et al., *Macrophage activation by armed CD4 TH1 cells.* 2001.
104. Tiemessen, M.M., et al., *CD4+CD25+Foxp3+ regulatory T cells induce alternative activation of human monocytes/macrophages.* Proc Natl Acad Sci U S A, 2007. **104**(49): p. 19446-51.
105. Parameswaran, N. and S. Patial, *Tumor necrosis factor-α signaling in macrophages.* Critical Reviews™ in Eukaryotic Gene Expression, 2010. **20**(2).
106. Mosser, D.M. and J.P. Edwards, *Exploring the full spectrum of macrophage activation.* Nat Rev Immunol, 2008. **8**(12): p. 958-69.
107. Calandra, T., et al., *MIF as a glucocorticoid-induced modulator of cytokine production.* Nature, 1995. **377**(6544): p. 68-71.
108. Murray, P.J., et al., *Macrophage activation and polarization: nomenclature and experimental guidelines.* Immunity, 2014. **41**(1): p. 14-20.
109. Martinez, F.O. and S. Gordon, *The M1 and M2 paradigm of macrophage activation: time for reassessment.* F1000prime reports, 2014. **6**.
110. Li, J., H.-C. Hsu, and J.D. Mountz, *The Dynamic Duo–Inflammatory M1 macrophages and Th17 cells in Rheumatic Diseases.* Journal of orthopedics & rheumatology, 2013. **1**(1): p. 4.
111. Zhang, W., W. Xu, and S. Xiong, *Macrophage differentiation and polarization via phosphatidylinositol 3-kinase/Akt-ERK signaling pathway conferred by serum amyloid P component.* J Immunol, 2011. **187**(4): p. 1764-77.
112. Arnold, C.E., et al., *The activation status of human macrophages presenting antigen determines the efficiency of Th17 responses.* Immunobiology, 2015. **220**(1): p. 10-19.
113. Olazabal, I.M., et al., *Activation outcomes induced in naïve CD8 T-cells by macrophages primed via "phagocytic" and nonphagocytic pathways.* Molecular biology of the cell, 2008. **19**(2): p. 701-710.
114. Pozzi, L.-A.M., J.W. Maciaszek, and K.L. Rock, *Both dendritic cells and macrophages can stimulate naive CD8 T cells in vivo to proliferate, develop*

effector function, and differentiate into memory cells. The Journal of Immunology, 2005. **175**(4): p. 2071-2081.
115. Ota, H., et al., *Pretreatment of macrophages with the combination of IFN-γ and IL-12 induces resistance to Leishmania major at the early phase of infection.* Journal of Veterinary Medical Science, 2008. **70**(6): p. 589-593.
116. Liew, F., Y. Li, and S. Millott, *Tumor necrosis factor-alpha synergizes with IFN-gamma in mediating killing of Leishmania major through the induction of nitric oxide.* The Journal of Immunology, 1990. **145**(12): p. 4306-4310.
117. Laria, A., et al., *The macrophages in rheumatic diseases.* Journal of inflammation research, 2016. **9**: p. 1.
118. Rőszer, T., *Understanding the mysterious M2 macrophage through activation markers and effector mechanisms.* Mediators of inflammation, 2015. **2015**.
119. Lech, M. and H.-J. Anders, *Macrophages and fibrosis: How resident and infiltrating mononuclear phagocytes orchestrate all phases of tissue injury and repair.* Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease, 2013. **1832**(7): p. 989-997.
120. Ogle, M.E., et al., *Monocytes and macrophages in tissue repair: Implications for immunoregenerative biomaterial design.* Experimental Biology and Medicine, 2016. **241**(10): p. 1084-1097.
121. Kreider, T., et al., *Alternatively activated macrophages in helminth infections.* Current opinion in immunology, 2007. **19**(4): p. 448-453.
122. Kennedy, M.W. and W. Harnett, *Parasitic nematodes: molecular biology, biochemistry and immunology*. 2013: CABI.
123. Haribhai, D., et al., *Alternatively Activated Macrophages Boost Induced Regulatory T and Th17 Cell Responses during Immunotherapy for Colitis.* J Immunol, 2016. **196**(8): p. 3305-17.
124. Mills, C.D., et al., *M-1/M-2 macrophages and the Th1/Th2 paradigm.* J Immunol, 2000. **164**(12): p. 6166-73.
125. Gensel, J.C. and B. Zhang, *Macrophage activation and its role in repair and pathology after spinal cord injury.* Brain Res, 2015. **1619**: p. 1-11.
126. Rojas, J., et al., *Macrophage heterogeneity and plasticity: impact of macrophage biomarkers on atherosclerosis.* Scientifica, 2015. **2015**.
127. Lee, S.J., et al., *Mannose receptor-mediated regulation of serum glycoprotein homeostasis.* Science, 2002. **295**(5561): p. 1898-901.
128. Elenkov, I.J., *Glucocorticoids and the Th1/Th2 balance.* Ann N Y Acad Sci, 2004. **1024**: p. 138-46.
129. DeKruyff, R.H., Y. Fang, and D.T. Umetsu, *Corticosteroids enhance the capacity of macrophages to induce Th2 cytokine synthesis in CD4+ lymphocytes by inhibiting IL-12 production.* J Immunol, 1998. **160**(5): p. 2231-7.
130. Broug-Holub, E. and G. Kraal, *Dose- and time-dependent activation of rat alveolar macrophages by glucocorticoids.* Clin Exp Immunol, 1996. **104**(2): p. 332-6.
131. Gordon, S., *Alternative activation of macrophages.* Nat Rev Immunol, 2003. **3**(1): p. 23-35.
132. Anders, H.J. and M. Ryu, *Renal microenvironments and macrophage phenotypes determine progression or resolution of renal inflammation and fibrosis.* Kidney Int, 2011. **80**(9): p. 915-25.

133. Zizzo, G., et al., *Efficient clearance of early apoptotic cells by human macrophages requires M2c polarization and MerTK induction.* J Immunol, 2012. **189**(7): p. 3508-20.
134. Lu, J., et al., *Discrete functions of M2a and M2c macrophage subsets determine their relative efficacy in treating chronic kidney disease.* Kidney Int, 2013. **84**(4): p. 745-55.
135. Edwards, J.P., et al., *Biochemical and functional characterization of three activated macrophage populations.* J Leukoc Biol, 2006. **80**(6): p. 1298-307.
136. Ito, I., et al., *The Polarization of M2b Monocytes in Cultures of Burn Patient Peripheral CD14+ Cells Treated with a Selected Human CCL1 Antisense Oligodeoxynucleotide.* Nucleic Acid Ther, 2016. **26**(5): p. 269-276.
137. Orme, J. and C. Mohan, *Macrophage subpopulations in systemic lupus erythematosus.* Discov Med, 2012. **13**(69): p. 151-8.
138. Chen, X., et al., *Granulin exacerbates lupus nephritis via enhancing macrophage M2b polarization.* PLoS One, 2013. **8**(6): p. e65542.
139. Ferrante, C.J., et al., *The adenosine-dependent angiogenic switch of macrophages to an M2-like phenotype is independent of interleukin-4 receptor alpha (IL-4Ralpha) signaling.* Inflammation, 2013. **36**(4): p. 921-31.
140. Ferrante, C.J. and S.J. Leibovich, *Regulation of Macrophage Polarization and Wound Healing.* Advances in Wound Care, 2012. **1**(1): p. 10-16.
141. Duluc, D., et al., *Tumor-associated leukemia inhibitory factor and IL-6 skew monocyte differentiation into tumor-associated macrophage-like cells.* Blood, 2007. **110**(13): p. 4319-30.
142. Jenmalm, M.C., et al., *Regulation of myeloid cell function through the CD200 receptor.* J Immunol, 2006. **176**(1): p. 191-9.
143. Ambarus, C.A., et al., *Systematic validation of specific phenotypic markers for in vitro polarized human macrophages.* J Immunol Methods, 2012. **375**(1-2): p. 196-206.
144. Rey-Giraud, F., M. Hafner, and C.H. Ries, *In vitro generation of monocyte-derived macrophages under serum-free conditions improves their tumor promoting functions.* PLoS One, 2012. **7**(8): p. e42656.
145. Vogel, D.Y., et al., *Human macrophage polarization in vitro: maturation and activation methods compared.* Immunobiology, 2014. **219**(9): p. 695-703.
146. Kunisch, E., et al., *Macrophage specificity of three anti-CD68 monoclonal antibodies (KP1, EBM11, and PGM1) widely used for immunohistochemistry and flow cytometry.* Annals of the Rheumatic Diseases, 2004. **63**(7): p. 774-784.
147. Beranek, J.T., *CD68 is not a macrophage-specific antigen.* Ann Rheum Dis, 2005. **64**(2): p. 342-3; author reply 343-4.
148. Pilling, D., et al., *Identification of markers that distinguish monocyte-derived fibrocytes from monocytes, macrophages, and fibroblasts.* PLoS One, 2009. **4**(10): p. e7475.
149. Gottfried, E., et al., *Expression of CD68 in non-myeloid cell types.* Scand J Immunol, 2008. **67**(5): p. 453-63.
150. Raes, G., et al., *Arginase-1 and Ym1 are markers for murine, but not human, alternatively activated myeloid cells.* J Immunol, 2005. **174**(11): p. 6561; author reply 6561-2.
151. Biswas, S.K., A. Sica, and C.E. Lewis, *Plasticity of macrophage function during tumor progression: regulation by distinct molecular mechanisms.* J Immunol, 2008. **180**(4): p. 2011-7.

152. Van den Heuvel, M.M., et al., *Regulation of CD 163 on human macrophages: cross-linking of CD163 induces signaling and activation.* J Leukoc Biol, 1999. **66**(5): p. 858-66.
153. Dupasquier, M., et al., *The dermal microenvironment induces the expression of the alternative activation marker CD301/mMGL in mononuclear phagocytes, independent of IL-4/IL-13 signaling.* J Leukoc Biol, 2006. **80**(4): p. 838-49.
154. Kittan, N.A., et al., *Cytokine induced phenotypic and epigenetic signatures are key to establishing specific macrophage phenotypes.* PLoS One, 2013. **8**(10): p. e78045.
155. Stein, M., et al., *Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation.* J Exp Med, 1992. **176**(1): p. 287-92.
156. Salma, I. and K. Ashok. **- 6**(- 2155-9889): p. - -.
157. Clavel, C., et al., *Among human macrophages polarised to different phenotypes, the M-CSF-oriented cells present the highest pro-inflammatory response to the rheumatoid arthritis-specific immune complexes containing ACPA.* Ann Rheum Dis, 2016. **75**(12): p. 2184-2191.
158. Porcheray, F., et al., *Macrophage activation switching: an asset for the resolution of inflammation.* Clin Exp Immunol, 2005. **142**(3): p. 481-9.
159. Fabriek, B.O., C.D. Dijkstra, and T.K. van den Berg, *The macrophage scavenger receptor CD163.* Immunobiology, 2005. **210**(2-4): p. 153-60.
160. Glim, J.E., et al., *Platelet derived growth factor-CC secreted by M2 macrophages induces alpha-smooth muscle actin expression by dermal and gingival fibroblasts.* Immunobiology, 2013. **218**(6): p. 924-9.
161. Vogel, D.Y., et al., *Macrophages in inflammatory multiple sclerosis lesions have an intermediate activation status.* J Neuroinflammation, 2013. **10**: p. 35.
162. Shortman, K. and S.H. Naik, *Steady-state and inflammatory dendritic-cell development.* Nature Reviews Immunology, 2007. **7**(1): p. 19-30.
163. Steinman, R.M. and Z.A. Cohn, *Identification of a novel cell type in peripheral lymphoid organs of mice.* Journal of Experimental Medicine, 1973. **137**(5): p. 1142-1162.
164. Van Voorhis, W.C., et al., *Human dendritic cells. Enrichment and characterization from peripheral blood.* Journal of Experimental Medicine, 1982. **155**(4): p. 1172-1187.
165. Bratke, K., et al., *Dendritic cell subsets in human bronchoalveolar lavage fluid after segmental allergen challenge.* Thorax, 2007. **62**(2): p. 168-175.
166. Hartmann, E., et al., *Analysis of plasmacytoid and myeloid dendritic cells in nasal epithelium.* Clinical and vaccine immunology, 2006. **13**(11): p. 1278-1286.
167. Jahnsen, F., et al., *Rapid dendritic cell recruitment to the bronchial mucosa of patients with atopic asthma in response to local allergen challenge.* Thorax, 2001. **56**(11): p. 823-826.
168. Lebre, M.C., et al., *Rheumatoid arthritis synovium contains two subsets of CD83− DC-LAMP− dendritic cells with distinct cytokine profiles.* The American journal of pathology, 2008. **172**(4): p. 940-950.
169. Verstege, M.I., et al., *Dendritic cell populations in colon and mesenteric lymph nodes of patients with Crohn's disease.* Journal of Histochemistry & Cytochemistry, 2008. **56**(3): p. 233-241.

170. Drutman, S.B., J.C. Kendall, and E.S. Trombetta, *Inflammatory spleen monocytes can upregulate CD11c expression without converting into dendritic cells.* The Journal of Immunology, 2012. **188**(8): p. 3603-3610.
171. Arnold, I.C., et al., *CD11c+ monocyte/macrophages promote chronic Helicobacter hepaticus-induced intestinal inflammation through the production of IL-23.* Mucosal immunology, 2016. **9**(2): p. 352-363.
172. Gustafson, M.P., et al., *A method for identification and analysis of non-overlapping myeloid immunophenotypes in humans.* PloS one, 2015. **10**(3): p. e0121546.
173. Haniffa, M., et al., *Human tissues contain CD141 hi cross-presenting dendritic cells with functional homology to mouse CD103+ nonlymphoid dendritic cells.* Immunity, 2012. **37**(1): p. 60-73.
174. MacDonald, K.P., et al., *Characterization of human blood dendritic cell subsets.* Blood, 2002. **100**(13): p. 4512-4520.
175. Piccioli, D., et al., *Functional specialization of human circulating CD16 and CD1c myeloid dendritic-cell subsets.* Blood, 2007. **109**(12): p. 5371-5379.
176. Dzionek, A., et al., *BDCA-2, BDCA-3, and BDCA-4: three markers for distinct subsets of dendritic cells in human peripheral blood.* The Journal of Immunology, 2000. **165**(11): p. 6037-6046.
177. Segura, E., et al., *Characterization of resident and migratory dendritic cells in human lymph nodes.* Journal of Experimental Medicine, 2012. **209**(4): p. 653-660.
178. Boltjes, A. and F. Van Wijk, *Human dendritic cell functional specialization in steady-state and inflammation.* Frontiers in immunology, 2014. **5**: p. 131.
179. Jongbloed, S.L., et al., *Human CD141+ (BDCA-3)+ dendritic cells (DCs) represent a unique myeloid DC subset that cross-presents necrotic cell antigens.* Journal of Experimental Medicine, 2010. **207**(6): p. 1247-1260.
180. Cella, M., et al., *Plasmacytoid monocytes migrate to inflamed lymph nodes and produce large amounts of type I interferon.* Nature medicine, 1999. **5**(8): p. 919-923.
181. Hémont, C., et al., *Human blood mDC subsets exhibit distinct TLR repertoire and responsiveness.* Journal of leukocyte biology, 2013. **93**(4): p. 599-609.
182. Mathan, T.S.M., C.G. Figdor, and S.I. Buschow, *Human plasmacytoid dendritic cells: from molecules to intercellular communication network.* Frontiers in immunology, 2013. **4**: p. 372.
183. Sallusto, F. and A. Lanzavecchia, *Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor.* Journal of Experimental Medicine, 1994. **179**(4): p. 1109-1118.
184. Robbins, S.H., et al., *Novel insights into the relationships between dendritic cell subsets in human and mouse revealed by genome-wide expression profiling.* Genome biology, 2008. **9**(1): p. R17.
185. Schäkel, K., et al., *6-Sulfo LacNAc, a novel carbohydrate modification of PSGL-1, defines an inflammatory type of human dendritic cells.* Immunity, 2002. **17**(3): p. 289-301.
186. Cros, J., et al., *Human CD14 dim monocytes patrol and sense nucleic acids and viruses via TLR7 and TLR8 receptors.* Immunity, 2010. **33**(3): p. 375-386.
187. Hänsel, A., et al., *Human slan (6-sulfo LacNAc) dendritic cells are inflammatory dermal dendritic cells in psoriasis and drive strong T H 17/T H*

1 T-cell responses. Journal of Allergy and Clinical Immunology, 2011. **127**(3): p. 787-794. e9.
188. Thomas, K., et al., *Accumulation and therapeutic modulation of 6-sulfo LacNAc+ dendritic cells in multiple sclerosis.* Neurology-Neuroimmunology Neuroinflammation, 2014. **1**(3): p. e33.
189. Schäkel, K., et al., *Human 6-sulfo LacNAc-expressing dendritic cells are principal producers of early interleukin-12 and are controlled by erythrocytes.* Immunity, 2006. **24**(6): p. 767-777.
190. Collin, M., N. McGovern, and M. Haniffa, *Human dendritic cell subsets.* Immunology, 2013. **140**(1): p. 22-30.
191. Valladeau, J., et al., *The monoclonal antibody DCGM4 recognizes Langerin, a protein specific of Langerhans cells, and is rapidly internalized from the cell surface.* European journal of immunology, 1999. **29**(9): p. 2695-2704.
192. Klechevsky, E., et al., *Functional specializations of human epidermal Langerhans cells and CD14+ dermal dendritic cells.* Immunity, 2008. **29**(3): p. 497-510.
193. Liu, K., et al., *In vivo analysis of dendritic cell development and homeostasis.* Science, 2009. **324**(5925): p. 392-397.
194. Onai, N., et al., *Identification of clonogenic common Flt3+ M-CSFR+ plasmacytoid and conventional dendritic cell progenitors in mouse bone marrow.* Nature immunology, 2007. **8**(11): p. 1207-1216.
195. Ginhoux, F., et al., *Langerhans cells arise from monocytes in vivo.* Nature immunology, 2006. **7**(3): p. 265-273.
196. Merad, M. and F. Ginhoux, *Dendritic cell genealogy: a new stem or just another branch? Two papers in this issue report the isolation and characterization of a common clonal bone marrow precursor of plasmacytoid and conventional dendritic cells.* Nature immunology, 2007. **8**(11): p. 1199-1202.
197. Bogunovic, M., et al., *Origin of the lamina propria dendritic cell network.* Immunity, 2009. **31**(3): p. 513-525.
198. Lewis, K.L., et al., *Notch2 receptor signaling controls functional differentiation of dendritic cells in the spleen and intestine.* Immunity, 2011. **35**(5): p. 780-791.
199. Merad, M., et al., *Langerhans cells renew in the skin throughout life under steady-state conditions.* Nature immunology, 2002. **3**(12): p. 1135-1141.
200. Villadangos, J.A. and P. Schnorrer, *Intrinsic and cooperative antigen-presenting functions of dendritic-cell subsets in vivo.* Nature Reviews Immunology, 2007. **7**(7): p. 543-555.
201. Nizzoli, G., et al., *Human CD1c+ dendritic cells secrete high levels of IL-12 and potently prime cytotoxic T-cell responses.* Blood, 2013. **122**(6): p. 932-942.
202. Inaba, K., et al., *Dendritic cells pulsed with protein antigens in vitro can prime antigen-specific, MHC-restricted T cells in situ.* J Exp Med, 1990. **172**(2): p. 631-640.
203. Liu, L. and G. MacPherson, *Antigen acquisition by dendritic cells: intestinal dendritic cells acquire antigen administered orally and can prime naive T cells in vivo.* Journal of Experimental Medicine, 1993. **177**(5): p. 1299-1307.
204. Macatonia, S.E., et al., *Primary stimulation by dendritic cells induces antiviral proliferative and cytotoxic T cell responses in vitro.* J Exp Med, 1989. **169**(4): p. 1255-1264.

205. Nair, S., et al., *Soluble proteins delivered to dendritic cells via pH-sensitive liposomes induce primary cytotoxic T lymphocyte responses in vitro.* J Exp Med, 1992. **175**(2): p. 609-12.
206. Mehta-Damani, A., S. Markowicz, and E.G. Engleman, *Generation of antigen-specific CD8+ CTLs from naive precursors.* The Journal of Immunology, 1994. **153**(3): p. 996-1003.
207. Inaba, K., J.W. Young, and R.M. Steinman, *Direct activation of CD8+ cytotoxic T lymphocytes by dendritic cells.* The Journal of experimental medicine, 1987. **166**(1): p. 182-194.
208. Young, J.W. and R.M. Steinman, *Dendritic cells stimulate primary human cytolytic lymphocyte responses in the absence of CD4+ helper T cells.* J Exp Med, 1990. **171**(4): p. 1315-1332.
209. Heuzé, M.L., et al., *Migration of dendritic cells: physical principles, molecular mechanisms, and functional implications.* Immunological reviews, 2013. **256**(1): p. 240-254.
210. Worbs, T., S.I. Hammerschmidt, and R. Förster, *Dendritic cell migration in health and disease.* Nature Reviews Immunology, 2016.
211. Braun, A., et al., *Afferent lymph-derived T cells and DCs use different chemokine receptor CCR7-dependent routes for entry into the lymph node and intranodal migration.* Nature immunology, 2011. **12**(9): p. 879-887.
212. Mori, S., et al., *Mice lacking expression of the chemokines CCL21-ser and CCL19 (plt mice) demonstrate delayed but enhanced T cell immune responses.* Journal of Experimental Medicine, 2001. **193**(2): p. 207-218.
213. Ito, T., et al., *Interferon-α and interleukin-12 are induced differentially by Toll-like receptor 7 ligands in human blood dendritic cell subsets.* Journal of Experimental Medicine, 2002. **195**(11): p. 1507-1512.
214. Jurk, M., et al., *Human TLR7 or TLR8 independently confer responsiveness to the antiviral compound R-848.* Nature immunology, 2002. **3**(6): p. 499-499.
215. McCaughtry, T.M., et al., *Clonal deletion of thymocytes can occur in the cortex with no involvement of the medulla.* Journal of Experimental Medicine, 2008. **205**(11): p. 2575-2584.
216. Bonasio, R., et al., *Clonal deletion of thymocytes by circulating dendritic cells homing to the thymus.* Nature immunology, 2006. **7**(10): p. 1092-1100.
217. Watanabe, N., et al., *Hassall's corpuscles instruct dendritic cells to induce CD4+ CD25+ regulatory T cells in human thymus.* Nature, 2005. **436**(7054): p. 1181-1185.
218. Hawiger, D., et al., *Dendritic cells induce peripheral T cell unresponsiveness under steady state conditions in vivo.* Journal of Experimental Medicine, 2001. **194**(6): p. 769-780.
219. Popov, A., et al., *Infection of myeloid dendritic cells with Listeria monocytogenes leads to the suppression of T cell function by multiple inhibitory mechanisms.* The Journal of Immunology, 2008. **181**(7): p. 4976-4988.
220. Selenko-Gebauer, N., et al., *B7-H1 (programmed death-1 ligand) on dendritic cells is involved in the induction and maintenance of T cell anergy.* The Journal of Immunology, 2003. **170**(7): p. 3637-3644.
221. Ghiringhelli, F., et al., *Tumor cells convert immature myeloid dendritic cells into TGF-β–secreting cells inducing CD4+ CD25+ regulatory T cell proliferation.* Journal of Experimental Medicine, 2005. **202**(7): p. 919-929.

222. Schmidt, S.V., A.C. Nino-Castro, and J.L. Schultze, *Regulatory dendritic cells: there is more than just immune activation.* 2012.
223. Vignali, D.A. and V.K. Kuchroo, *IL-12 family cytokines: immunological playmakers.* Nature immunology, 2012. **13**(8): p. 722.
224. Oppmann, B., et al., *Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12.* Immunity, 2000. **13**(5): p. 715-725.
225. Pflanz, S., et al., *IL-27, a heterodimeric cytokine composed of EBI3 and p28 protein, induces proliferation of naive CD4+ T cells.* Immunity, 2002. **16**(6): p. 779-790.
226. Niedbala, W., et al., *IL- 35 is a novel cytokine with therapeutic effects against collagen- induced arthritis through the expansion of regulatory T cells and suppression of Th17 cells.* European journal of immunology, 2007. **37**(11): p. 3021-3029.
227. Presky, D.H., et al., *A functional interleukin 12 receptor complex is composed of two β-type cytokine receptor subunits.* Proceedings of the National Academy of Sciences, 1996. **93**(24): p. 14002-14007.
228. Chua, A.O., et al., *Cloning and characterization of a mouse IL-12 receptor-beta component.* The Journal of Immunology, 1995. **155**(9): p. 4286-4294.
229. Parham, C., et al., *A receptor for the heterodimeric cytokine IL-23 is composed of IL-12Rβ1 and a novel cytokine receptor subunit, IL-23R.* The Journal of Immunology, 2002. **168**(11): p. 5699-5708.
230. Thierfelder, W.E., et al., *Requirement for Stat4 in interleukin-12-mediated responses of natural killer and T cells.* Nature, 1996. **382**(6587): p. 171-174.
231. Collison, L.W., et al., *The composition and signaling of the IL-35 receptor are unconventional.* Nature immunology, 2012. **13**(3): p. 290-299.
232. Hibbert, L., et al., *IL-27 and IFN-α signal via Stat1 and Stat3 and induce T-Bet and IL-12R β 2 in naive T cells.* Journal of interferon & cytokine research, 2003. **23**(9): p. 513-522.
233. Lucas, S., et al., *IL-27 regulates IL-12 responsiveness of naive CD4+ T cells through Stat1-dependent and-independent mechanisms.* Proceedings of the National Academy of Sciences, 2003. **100**(25): p. 15047-15052.
234. Pflanz, S., et al., *WSX-1 and glycoprotein 130 constitute a signal-transducing receptor for IL-27.* The Journal of Immunology, 2004. **172**(4): p. 2225-2231.
235. Collison, L.W. and D.A. Vignali, *Interleukin- 35: odd one out or part of the family?* Immunological reviews, 2008. **226**(1): p. 248-262.
236. Stritesky, G.L., N. Yeh, and M.H. Kaplan, *IL-23 promotes maintenance but not commitment to the Th17 lineage.* The Journal of Immunology, 2008. **181**(9): p. 5948-5955.
237. O'Shea, J.J. and W.E. Paul, *Regulation of THI differentiation-controlling the controllers.* Nature immunology, 2002. **3**(6): p. 506-509.
238. Murphy, C.A., et al., *Divergent pro-and antiinflammatory roles for IL-23 and IL-12 in joint autoimmune inflammation.* Journal of Experimental Medicine, 2003. **198**(12): p. 1951-1957.
239. Segal, B.M., B.K. Dwyer, and E.M. Shevach, *An interleukin (IL)-10/IL-12 immunoregulatory circuit controls susceptibility to autoimmune disease.* Journal of Experimental Medicine, 1998. **187**(4): p. 537-546.
240. Segal, B.M., D.M. Klinman, and E.M. Shevach, *Microbial products induce autoimmune disease by an IL-12-dependent pathway.* The Journal of Immunology, 1997. **158**(11): p. 5087-5090.

241. Constantinescu, C.S., et al., *Antibodies against IL-12 prevent superantigen-induced and spontaneous relapses of experimental autoimmune encephalomyelitis.* The Journal of Immunology, 1998. **161**(9): p. 5097-5104.
242. Cua, D.J., et al., *Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain.* Nature, 2003. **421**(6924): p. 744-748.
243. Langrish, C.L., et al., *IL-23 drives a pathogenic T cell population that induces autoimmune inflammation.* Journal of Experimental Medicine, 2005. **201**(2): p. 233-240.
244. Collison, L.W., et al., *The inhibitory cytokine IL-35 contributes to regulatory T-cell function.* Nature, 2007. **450**(7169): p. 566-569.
245. Collison, L.W., et al., *IL-35-mediated induction of a potent regulatory T cell population.* Nature immunology, 2010. **11**(12): p. 1093-1101.
246. Chaturvedi, V., et al., *Cutting edge: human regulatory T cells require IL-35 to mediate suppression and infectious tolerance.* The Journal of Immunology, 2011. **186**(12): p. 6661-6666.
247. Owaki, T., et al., *A role for IL-27 in early regulation of Th1 differentiation.* The Journal of Immunology, 2005. **175**(4): p. 2191-2200.
248. Batten, M. and N. Ghilardi, *The biology and therapeutic potential of interleukin 27.* Journal of Molecular Medicine, 2007. **85**(7): p. 661-672.
249. Hamano, S., et al., *WSX-1 is required for resistance to Trypanosoma cruzi infection by regulation of proinflammatory cytokine production.* Immunity, 2003. **19**(5): p. 657-667.
250. Artis, D., et al., *Cutting edge: early IL-4 production governs the requirement for IL-27-WSX-1 signaling in the development of protective Th1 cytokine responses following Leishmania major infection.* The Journal of Immunology, 2004. **172**(8): p. 4672-4675.
251. Pearl, J.E., et al., *IL-27 signaling compromises control of bacterial growth in mycobacteria-infected mice.* The Journal of Immunology, 2004. **173**(12): p. 7490-7496.
252. Zahn, S., et al., *Impaired Th1 responses in mice deficient in Epstein‐Barr virus‐induced gene 3 and challenged with physiological doses of Leishmania major.* European journal of immunology, 2005. **35**(4): p. 1106-1112.
253. Hölscher, C., et al., *The IL-27 receptor chain WSX-1 differentially regulates antibacterial immunity and survival during experimental tuberculosis.* The Journal of Immunology, 2005. **174**(6): p. 3534-3544.
254. Rosas, L.E., et al., *Interleukin-27R (WSX-1/T-cell cytokine receptor) gene-deficient mice display enhanced resistance to leishmania donovani infection but develop severe liver immunopathology.* The American journal of pathology, 2006. **168**(1): p. 158-169.
255. Villarino, A., et al., *The IL-27R (WSX-1) is required to suppress T cell hyperactivity during infection.* Immunity, 2003. **19**(5): p. 645-655.
256. Stumhofer, J.S., et al., *Interleukin 27 negatively regulates the development of interleukin 17–producing T helper cells during chronic inflammation of the central nervous system.* Nature immunology, 2006. **7**(9): p. 937-945.
257. Yoshimura, T., et al., *Two-sided roles of IL-27: induction of Th1 differentiation on naive CD4+ T cells versus suppression of proinflammatory cytokine production including IL-23-induced IL-17 on activated CD4+ T cells partially through STAT3-dependent mechanism.* The Journal of Immunology, 2006. **177**(8): p. 5377-5385.

258. Batten, M., et al., *Interleukin 27 limits autoimmune encephalomyelitis by suppressing the development of interleukin 17–producing T cells.* Nature immunology, 2006. **7**(9): p. 929-936.
259. Do, J., et al., *An IL-27/Lag3 axis enhances Foxp3+ regulatory T cell–suppressive function and therapeutic efficacy.* Mucosal immunology, 2016. **9**(1): p. 137-145.
260. Huber, M., et al., *IL-27 inhibits the development of regulatory T cells via STAT3.* International immunology, 2007. **20**(2): p. 223-234.
261. Murugaiyan, G. and B. Saha, *IL-27 in tumor immunity and immunotherapy.* Trends in molecular medicine, 2013. **19**(2): p. 108-116.
262. Pennock, N.D., L. Gapin, and R.M. Kedl, *IL-27 is required for shaping the magnitude, affinity distribution, and memory of T cells responding to subunit immunization.* Proceedings of the National Academy of Sciences, 2014. **111**(46): p. 16472-16477.
263. Kunze, A., et al., *Autocrine TNF- α and IL- 1β prime 6- sulfo LacNAc+ dendritic cells for high- level production of IL- 23.* Experimental dermatology, 2017. **26**(4): p. 314-316.
264. Verreck, F.A., et al., *Human IL-23-producing type 1 macrophages promote but IL-10-producing type 2 macrophages subvert immunity to (myco) bacteria.* Proceedings of the National Academy of Sciences of the United States of America, 2004. **101**(13): p. 4560-4565.
265. Wu, Q., et al., *IL-23-dependent IL-17 production is essential in neutrophil recruitment and activity in mouse lung defense against respiratory Mycoplasma pneumoniae infection.* Microbes and infection, 2007. **9**(1): p. 78-86.
266. Pirhonen, J., S. Matikainen, and I. Julkunen, *Regulation of virus-induced IL-12 and IL-23 expression in human macrophages.* The Journal of Immunology, 2002. **169**(10): p. 5673-5678.
267. Siren, J., et al., *Cytokine and contact-dependent activation of natural killer cells by influenza A or Sendai virus-infected macrophages.* Journal of general virology, 2004. **85**(8): p. 2357-2364.
268. Butchar, J.P., et al., *Francisella tularensis induces IL-23 production in human monocytes.* The Journal of Immunology, 2007. **178**(7): p. 4445-4454.
269. Correa, F., et al., *The endocannabinoid anandamide downregulates IL-23 and IL-12 subunits in a viral model of multiple sclerosis: evidence for a cross-talk between IL-12p70/IL-23 axis and IL-10 in microglial cells.* Brain, behavior, and immunity, 2011. **25**(4): p. 736-749.
270. Li, Y., et al., *Increased IL-23p19 expression in multiple sclerosis lesions and its induction in microglia.* Brain, 2006. **130**(2): p. 490-501.
271. Brentano, F., et al., *Abundant expression of the interleukin (IL) 23 subunit p19, but low levels of bioactive IL23 in the rheumatoid synovium: differential expression and Toll-like receptor-(TLR) dependent regulation of the IL23 subunits, p19 and p40, in rheumatoid arthritis.* Annals of the rheumatic diseases, 2009. **68**(1): p. 143-150.
272. Kim, H.-R., et al., *Up-regulation of IL-23p19 expression in rheumatoid arthritis synovial fibroblasts by IL-17 through PI3-kinase-, NF-κB-and p38 MAPK-dependent signalling pathways.* Rheumatology, 2006. **46**(1): p. 57-64.
273. Liu, F.-L., et al., *Interleukin (IL)-23 p19 expression induced by IL-1β in human fibroblast-like synoviocytes with rheumatoid arthritis via active*

nuclear factor-κB and AP-1 dependent pathway. Rheumatology, 2007. **46**(8): p. 1266-1273.
274. Sheibanie, A.F., et al., *Prostaglandin E2 induces IL-23 production in bone marrow-derived dendritic cells.* The FASEB Journal, 2004. **18**(11): p. 1318-1320.
275. Shi, Q., et al., *PGE2 elevates IL-23 production in human dendritic cells via a cAMP dependent pathway.* Mediators of inflammation, 2015. **2015**.
276. Barton, G.M. and R. Medzhitov, *Toll-like receptor signaling pathways.* Science, 2003. **300**(5625): p. 1524-1525.
277. Rogers, N.C., et al., *Syk-dependent cytokine induction by Dectin-1 reveals a novel pattern recognition pathway for C type lectins.* Immunity, 2005. **22**(4): p. 507-517.
278. Goodridge, H.S. and D.M. Underhill, *Fungal recognition by TLR2 and Dectin-1*, in *Toll-Like Receptors (TLRs) and Innate Immunity*. 2008, Springer. p. 87-109.
279. Dennehy, K.M., et al., *Reciprocal regulation of IL‐23 and IL‐12 following co‐activation of Dectin‐1 and TLR signaling pathways.* European journal of immunology, 2009. **39**(5): p. 1379-1386.
280. LeibundGut-Landmann, S., et al., *Syk-and CARD9-dependent coupling of innate immunity to the induction of T helper cells that produce interleukin 17.* Nature immunology, 2007. **8**(6): p. 630-638.
281. Geijtenbeek, T.B. and S.I. Gringhuis, *Signalling through C-type lectin receptors: shaping immune responses.* Nature Reviews Immunology, 2009. **9**(7): p. 465-479.
282. Gringhuis, S.I., et al., *Dectin-1 directs T helper cell differentiation by controlling noncanonical NF-κB activation through Raf-1 and Syk.* Nature immunology, 2009. **10**(2): p. 203-213.
283. Bonifazi, P., et al., *Intranasally delivered siRNA targeting PI3K/Akt/mTOR inflammatory pathways protects from aspergillosis.* Mucosal immunology, 2010. **3**(2): p. 193-205.
284. Kuwabara, T., et al., *CCR7 ligands up-regulate IL-23 through PI3-kinase and NF-κB pathway in dendritic cells.* Journal of leukocyte biology, 2012. **92**(2): p. 309-318.
285. Jang, M.S., et al., *Synergistic production of interleukin-23 by dendritic cells derived from cord blood in response to costimulation with LPS and IL-12.* Journal of leukocyte biology, 2009. **86**(3): p. 691-699.
286. Utsugi, M., et al., *Rac1 negatively regulates lipopolysaccharide-induced IL-23 p19 expression in human macrophages and dendritic cells and NF-κB p65 trans activation plays a novel role.* The Journal of Immunology, 2006. **177**(7): p. 4550-4557.
287. Chang, J., et al., *Interleukin-23 production in dendritic cells is negatively regulated by protein phosphatase 2A.* Proceedings of the National Academy of Sciences, 2010. **107**(18): p. 8340-8345.
288. Mühlbauer, M., et al., *Impaired Bcl3 Up-regulation Leads to Enhanced Lipopolysaccharide-induced Interleukin (IL)-23P19 Gene Expression in IL-10–/–Mice.* Journal of Biological Chemistry, 2008. **283**(21): p. 14182-14189.
289. Kocieda, V.P., et al., *Prostaglandin E2-induced IL-23p19 is regulated by CREB and C/EBPβin bone marrow derived dendritic cells.* Journal of Biological Chemistry, 2012: p. jbc. M112. 402958.

290. Mise-Omata, S., et al., *A proximal κB site in the IL-23 p19 promoter is responsible for RelA-and c-Rel-dependent transcription.* The Journal of Immunology, 2007. **179**(10): p. 6596-6603.
291. Liu, W., et al., *AP-1 activated by toll-like receptors regulates expression of IL-23 p19.* Journal of Biological Chemistry, 2009. **284**(36): p. 24006-24016.
292. Xu, M., et al., *IL-12/IL-23 production induced by Toll-like receptor agonist primed myeloid dendritic cells is differentially regulated by prostaglandin E2.* The FASEB Journal, 2008. **22**(1 Supplement): p. 1078.5-1078.5.
293. Ollivier, V., et al., *Elevated cyclic AMP inhibits NF-κB-mediated transcription in human monocytic cells and endothelial cells.* Journal of Biological Chemistry, 1996. **271**(34): p. 20828-20835.
294. Parry, G. and N. Mackman, *Role of cyclic AMP response element-binding protein in cyclic AMP inhibition of NF-kappaB-mediated transcription.* The Journal of Immunology, 1997. **159**(11): p. 5450-5456.
295. Li, J., et al., *Interleukin 23 regulates proliferation of lung cancer cells in a concentration-dependent way in association with the interleukin-23 receptor.* Carcinogenesis, 2012: p. bgs384.
296. Suzuki, H., et al., *IL-23 directly enhances the proliferative and invasive activities of colorectal carcinoma.* Oncology letters, 2012. **4**(2): p. 199-204.
297. Nasti, T.H., et al., *IL-23 Inhibits Melanoma Development by Augmenting DNA Repair and Modulating T Cell Subpopulations.* The Journal of Immunology, 2017. **198**(2): p. 950-961.
298. Elson, C.O., et al., *Monoclonal anti–interleukin 23 reverses active colitis in a T Cell–mediated model in mice.* Gastroenterology, 2007. **132**(7): p. 2359-2370.
299. Bettelli, E., et al., *Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells.* Nature, 2006. **441**(7090): p. 235-238.
300. Mangan, P.R., et al., *Transforming growth factor-β induces development of the TH17 lineage.* Nature, 2006. **441**(7090): p. 231-234.
301. Aggarwal, S., et al., *Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17.* Journal of Biological Chemistry, 2003. **278**(3): p. 1910-1914.
302. Lo, C.-H., et al., *Antitumor and antimetastatic activity of IL-23.* The Journal of Immunology, 2003. **171**(2): p. 600-607.
303. Sieve, A.N., et al., *A novel immunoregulatory function for IL- 23: Inhibition of IL- 12- dependent IFN- γ production.* European journal of immunology, 2010. **40**(8): p. 2236-2247.
304. Frucht, D.M., *IL-23: a cytokine that acts on memory T cells.* Sci Stke, 2002. **114**: p. 1-3.
305. van de Wetering, D., et al., *IL-23 modulates CD56+/CD3− NK cell and CD56+/CD3+ NK-like T cell function differentially from IL-12.* International immunology, 2008. **21**(2): p. 145-153.
306. van de Wetering, D., et al., *Salmonella Induced IL-23 and IL-1β Allow for IL-12 Production by Monocytes and Mφ1 through Induction of IFN-γ in CD56+ NK/NK-Like T Cells.* PLoS One, 2009. **4**(12): p. e8396.
307. Fukuda, M., et al., *IL-23 promotes growth and proliferation in human squamous cell carcinoma of the oral cavity.* International journal of oncology, 2010. **36**(6): p. 1355.

308. Lan, F., et al., *IL-23/IL-23R: potential mediator of intestinal tumor progression from adenomatous polyps to colorectal carcinoma.* International journal of colorectal disease, 2011. **26**(12): p. 1511-1518.
309. Langowski, J.L., R.A. Kastelein, and M. Oft, *Swords into plowshares: IL-23 repurposes tumor immune surveillance.* Trends in immunology, 2007. **28**(5): p. 207-212.
310. Langowski, J.L., et al., *IL-23 promotes tumour incidence and growth.* Nature, 2006. **442**(7101): p. 461-465.
311. Teng, M.W., et al., *IL-23 suppresses innate immune response independently of IL-17A during carcinogenesis and metastasis.* Proceedings of the National Academy of Sciences, 2010. **107**(18): p. 8328-8333.
312. Numasaki, M., et al., *Interleukin-17 promotes angiogenesis and tumor growth.* Blood, 2003. **101**(7): p. 2620-2627.
313. Zhang, J.-P., et al., *Increased intratumoral IL-17-producing cells correlate with poor survival in hepatocellular carcinoma patients.* Journal of hepatology, 2009. **50**(5): p. 980-989.
314. Tosolini, M., et al., *Clinical impact of different classes of infiltrating T cytotoxic and helper cells (Th1, th2, treg, th17) in patients with colorectal cancer.* Cancer research, 2011. **71**(4): p. 1263-1271.
315. Wu, C., et al., *Increased frequencies of T helper type 17 cells in the peripheral blood of patients with acute myeloid leukaemia.* Clinical & Experimental Immunology, 2009. **158**(2): p. 199-204.
316. Prabhala, R.H., et al., *Elevated IL-17 produced by TH17 cells promotes myeloma cell growth and inhibits immune function in multiple myeloma.* Blood, 2010. **115**(26): p. 5385-5392.
317. Downs-Canner, S., et al., *Suppressive IL-17A+ Foxp3+ and ex-Th17 IL-17AnegFoxp3+ Treg cells are a source of tumour-associated Treg cells.* Nature Communications, 2017. **8**.
318. Hinrichs, C.S., et al., *Type 17 CD8+ T cells display enhanced antitumor immunity.* Blood, 2009. **114**(3): p. 596-599.
319. Muranski, P., et al., *Tumor-specific Th17-polarized cells eradicate large established melanoma.* Blood, 2008. **112**(2): p. 362-373.
320. Meeks, K.D., et al., *IL-23 is required for protection against systemic infection with Listeria monocytogenes.* The Journal of Immunology, 2009. **183**(12): p. 8026-8034.
321. Xu, S., et al., *IL-17A–Producing γδT Cells Promote CTL Responses against Listeria monocytogenes Infection by Enhancing Dendritic Cell Cross-Presentation.* The Journal of Immunology, 2010. **185**(10): p. 5879-5887.
322. Kleinschek, M.A., et al., *IL-23 enhances the inflammatory cell response in Cryptococcus neoformans infection and induces a cytokine pattern distinct from IL-12.* The Journal of Immunology, 2006. **176**(2): p. 1098-1106.
323. Happel, K.I., et al., *Divergent roles of IL-23 and IL-12 in host defense against Klebsiella pneumoniae.* Journal of Experimental Medicine, 2005. **202**(6): p. 761-769.
324. Khader, S.A., et al., *IL-23 is required for long-term control of Mycobacterium tuberculosis and B cell follicle formation in the infected lung.* The Journal of Immunology, 2011. **187**(10): p. 5402-5407.
325. Bär, E., et al., *IL-17 regulates systemic fungal immunity by controlling the functional competence of NK cells.* Immunity, 2014. **40**(1): p. 117-127.

326. Dubin, P.J. and J.K. Kolls, *IL-23 mediates inflammatory responses to mucoid Pseudomonas aeruginosa lung infection in mice.* American Journal of Physiology-Lung Cellular and Molecular Physiology, 2007. **292**(2): p. L519-L528.
327. Uhlig, H.H., et al., *Differential activity of IL-12 and IL-23 in mucosal and systemic innate immune pathology.* Immunity, 2006. **25**(2): p. 309-318.
328. Sutton, C., et al., *A crucial role for interleukin (IL)-1 in the induction of IL-17–producing T cells that mediate autoimmune encephalomyelitis.* Journal of Experimental Medicine, 2006. **203**(7): p. 1685-1691.
329. Thakker, P., et al., *IL-23 is critical in the induction but not in the effector phase of experimental autoimmune encephalomyelitis.* The Journal of Immunology, 2007. **178**(4): p. 2589-2598.
330. Lubberts, E., *The IL-23-IL-17 axis in inflammatory arthritis.* Nature Reviews Rheumatology, 2015. **11**(7): p. 415-429.
331. Maxwell, J.R., et al., *Differential roles for interleukin-23 and interleukin-17 in intestinal immunoregulation.* Immunity, 2015. **43**(4): p. 739-750.
332. Netea, M.G., J. Quintin, and J.W. van der Meer, *Trained immunity: a memory for innate host defense.* Cell host & microbe, 2011. **9**(5): p. 355-361.
333. Blanden, R., M. Lefford, and G. Mackaness, *The host response to Calmette-Guérin bacillus infection in mice.* Journal of experimental medicine, 1969. **129**(5): p. 1079-1107.
334. Howard, J., et al., *The effect of Mycobacterium tuberculosis (BCG) infection on the resistance of mice to bacterial endotoxin and Salmonella enteritidis infection.* British journal of experimental pathology, 1959. **40**(3): p. 281.
335. Sher, N., et al., *Effects of BCG, Corynebacterium parvum, and methanol-extration residue in the reduction of mortality from Staphylococcus aureus and Candida albicans infections in immunosuppressed mice.* Infection and immunity, 1975. **12**(6): p. 1325-1330.
336. Tribouley, J., J. Tribouley-Duret, and M. Appriou, *Effect of Bacillus Callmette Guerin (BCG) on the receptivity of nude mice to Schistosoma mansoni.* Comptes rendus des seances de la Societe de biologie et de ses filiales, 1978. **172**(5): p. 902-904.
337. Wout, J., R. Poell, and R. Furth, *The Role of BCG/PPD- Activated Macrophages in Resistance against Systemic Candidiasis in Mice.* Scandinavian journal of immunology, 1992. **36**(5): p. 713-720.
338. Bistoni, F., et al., *Evidence for macrophage-mediated protection against lethal Candida albicans infection.* Infection and immunity, 1986. **51**(2): p. 668-674.
339. Bistoni, F., et al., *Immunomodulation by a low-virulence, agerminative variant of Candida albicans. Further evidence for macrophage activation as one of the effector mechanisms of nonspecific anti-infectious protection.* Journal of medical and veterinary mycology, 1988. **26**(5): p. 285-299.
340. Chen, F., et al., *Neutrophils prime a long-lived effector macrophage phenotype that mediates accelerated helminth expulsion.* Nature immunology, 2014. **15**(10): p. 938.
341. Ishii, K.J., et al., *CpG- activated Thy1. 2+ dendritic cells protect against lethal Listeria monocytogenes infection.* European journal of immunology, 2005. **35**(8): p. 2397-2405.
342. Zhang, B., et al., *Prevention and cure of rotavirus infection via TLR5/NLRC4–mediated production of IL-22 and IL-18.* Science, 2014. **346**(6211): p. 861-865.

343. Benn, C.S., et al., *A small jab–a big effect: nonspecific immunomodulation by vaccines.* Trends in immunology, 2013. **34**(9): p. 431-439.
344. Aaby, P., et al., *The optimal age of measles immunisation in low-income countries: a secondary analysis of the assumptions underlying the current policy.* BMJ open, 2012. **2**(4): p. e000761.
345. Steenhuis, T., et al., *Bacille–Calmette–Guerin vaccination and the development of allergic disease in children: a randomized, prospective, single- blind study.* Clinical & Experimental Allergy, 2008. **38**(1): p. 79-85.
346. Mills, K.H., *TLR-dependent T cell activation in autoimmunity.* Nature Reviews Immunology, 2011. **11**(12): p. 807.
347. Ueda, Y., M. Kondo, and G. Kelsoe, *Inflammation and the reciprocal production of granulocytes and lymphocytes in bone marrow.* Journal of Experimental Medicine, 2005. **201**(11): p. 1771-1780.
348. Welner, R.S., et al., *Lymphoid precursors are directed to produce dendritic cells as a result of TLR9 ligation during herpes infection.* Blood, 2008. **112**(9): p. 3753-3761.
349. Sioud, M. and Y. Fløisand, *TLR agonists induce the differentiation of human bone marrow CD34+ progenitors into CD11c+ CD80/86+ DC capable of inducing a Th1- type response.* European journal of immunology, 2007. **37**(10): p. 2834-2846.
350. Yona, S., et al., *Fate mapping reveals origins and dynamics of monocytes and tissue macrophages under homeostasis.* Immunity, 2013. **38**(1): p. 79-91.
351. Kleinnijenhuis, J., et al., *Bacille Calmette-Guerin induces NOD2-dependent nonspecific protection from reinfection via epigenetic reprogramming of monocytes.* Proceedings of the National Academy of Sciences, 2012. **109**(43): p. 17537-17542.
352. Hamon, M.A. and J. Quintin. *Innate immune memory in mammals*. in *Seminars in immunology*. 2016. Elsevier.
353. Shalova, I.N., et al., *Human monocytes undergo functional re-programming during sepsis mediated by hypoxia-inducible factor-1α.* Immunity, 2015. **42**(3): p. 484-498.
354. Monneret, G., et al., *The anti-inflammatory response dominates after septic shock: association of low monocyte HLA-DR expression and high interleukin-10 concentration.* Immunology letters, 2004. **95**(2): p. 193-198.
355. Zhang, X. and D.C. Morrison, *Lipopolysaccharide-induced selective priming effects on tumor necrosis factor alpha and nitric oxide production in mouse peritoneal macrophages.* Journal of Experimental Medicine, 1993. **177**(2): p. 511-516.
356. Deng, H., et al., *Molecular mechanism responsible for the priming of macrophage activation.* Journal of Biological Chemistry, 2013. **288**(6): p. 3897-3906.
357. Netea, M.G., et al., *Trained immunity: a program of innate immune memory in health and disease.* Science, 2016. **352**(6284): p. aaf1098.
358. Gatfield, D., et al., *Integration of microRNA miR-122 in hepatic circadian gene expression.* Genes & development, 2009. **23**(11): p. 1313-1326.
359. Montecalvo, A., et al., *Mechanism of transfer of functional microRNAs between mouse dendritic cells via exosomes.* Blood, 2012. **119**(3): p. 756-766.
360. Zhang, Y., et al., *Secreted monocytic miR-150 enhances targeted endothelial cell migration.* Molecular cell, 2010. **39**(1): p. 133-144.

361. Monticelli, S. and G. Natoli, *Short-term memory of danger signals and environmental stimuli in immune cells.* Nature immunology, 2013. **14**(8): p. 777.
362. Lebre, M.C., et al., *Human keratinocytes express functional Toll-like receptor 3, 4, 5, and 9.* Journal of Investigative Dermatology, 2007. **127**(2): p. 331-341.
363. Song, P.I., et al., *Human keratinocytes express functional CD14 and toll-like receptor 4.* Journal of investigative dermatology, 2002. **119**(2): p. 424-432.
364. Mempel, M., et al., *Toll-like receptor expression in human keratinocytes: nuclear factor κB controlled gene activation by Staphylococcus aureus is Toll-like receptor 2 but not Toll-like receptor 4 or platelet activating factor receptor dependent.* Journal of Investigative Dermatology, 2003. **121**(6): p. 1389-1396.
365. Baker, B., et al., *Normal keratinocytes express toll‐like receptors (TLRs) 1, 2 and 5: modulation of TLR expression in chronic plaque psoriasis.* British Journal of Dermatology, 2003. **148**(4): p. 670-679.
366. Kawai, K., et al., *Expression of functional Toll-like receptor 2 on human epidermal keratinocytes.* Journal of dermatological science, 2002. **30**(3): p. 185-194.
367. Karikó, K., et al., *Small interfering RNAs mediate sequence-independent gene suppression and induce immune activation by signaling through toll-like receptor 3.* The Journal of Immunology, 2004. **172**(11): p. 6545-6549.
368. Miller, L.S. and R.L. Modlin, *Human keratinocyte Toll-like receptors promote distinct immune responses.* Journal of Investigative Dermatology, 2007. **127**(2): p. 262-263.
369. Begon, É., et al., *Expression, subcellular localization and cytokinic modulation of Toll-like receptors (TLRs) in normal human keratinocytes: TLR2 up-regulation in psoriatic skin.* European Journal of Dermatology, 2007. **17**(6): p. 497-506.
370. Curry, J.L., et al., *Innate immune-related receptors in normal and psoriatic skin.* Archives of pathology & laboratory medicine, 2003. **127**(2): p. 178-186.
371. Miller, L.S., et al., *TGF-α regulates TLR expression and function on epidermal keratinocytes.* The Journal of Immunology, 2005. **174**(10): p. 6137-6143.
372. Lebre, M.C., et al., *Double-stranded RNA-exposed human keratinocytes promote Th1 responses by inducing a type-1 polarized phenotype in dendritic cells: role of keratinocyte-derived tumor necrosis factor α, type I interferons, and interleukin-18.* Journal of Investigative Dermatology, 2003. **120**(6): p. 990-997.
373. Souza, P.P.C.d., et al., *Stimulation of IL-6 cytokines in fibroblasts by toll-like receptors 2.* Journal of dental research, 2010. **89**(8): p. 802-807.
374. Wang, J., et al., *Toll‐like receptors expressed by dermal fibroblasts contribute to hypertrophic scarring.* Journal of cellular physiology, 2011. **226**(5): p. 1265-1273.
375. Hu, F., et al., *Toll-like receptors expressed by synovial fibroblasts perpetuate Th1 and th17 cell responses in rheumatoid arthritis.* PLoS One, 2014. **9**(6): p. e100266.
376. Opitz, B., et al., *Role of Toll-like receptors, NOD-like receptors and RIG-I-like receptors in endothelial cells and systemic infections.* Thromb Haemost, 2009. **102**(6): p. 1103-1109.

377. Mai, J., et al., *An evolving new paradigm: endothelial cells–conditional innate immune cells.* Journal of hematology & oncology, 2013. **6**(1): p. 61.
378. Krishnaswamy, G., et al., *Human endothelium as a source of multifunctional cytokines: molecular regulation and possible role in human disease.* Journal of interferon & cytokine research, 1999. **19**(2): p. 91-104.
379. Howland, S.W., C.M. Poh, and L. Rénia, *Activated brain endothelial cells cross-present malaria antigen.* PLoS Pathog, 2015. **11**(6): p. e1004963.
380. Abrahimi, P., et al., *Blocking MHC class II on human endothelium mitigates acute rejection.* JCI insight, 2016. **1**(1).
381. Pober, J.S. and W.C. Sessa, *Evolving functions of endothelial cells in inflammation.* Nature Reviews Immunology, 2007. **7**(10): p. 803-815.
382. Ma, W. and J.S. Pober, *Human endothelial cells effectively costimulate cytokine production by, but not differentiation of, naive CD4+ T cells.* The Journal of Immunology, 1998. **161**(5): p. 2158-2167.
383. Fan, L., et al., *Antigen presentation by keratinocytes directs autoimmune skin disease.* Proceedings of the National Academy of Sciences, 2003. **100**(6): p. 3386-3391.
384. Leggatt, G.R., et al., *Interferon-gamma enhances cytotoxic T lymphocyte recognition of endogenous peptide in keratinocytes without lowering the requirement for surface peptide.* Immunology and cell biology, 2002. **80**(5): p. 415-424.
385. Xiao, L., et al., *Large adipocytes function as antigen-presenting cells to activate CD4+ T cells via upregulating MHCII in obesity.* International journal of obesity, 2013.
386. Deng, T., et al., *Class II major histocompatibility complex plays an essential role in obesity-induced adipose inflammation.* Cell metabolism, 2013. **17**(3): p. 411-422.
387. Koyama, M., et al., *Recipient nonhematopoietic antigen-presenting cells are sufficient to induce lethal acute graft-versus-host disease.* Nature medicine, 2012. **18**(1): p. 135-142.
388. Ransohoff, R.M. and M.L. Estes, *Astrocyte expression of major histocompatibility complex gene products in multiple sclerosis brain tissue obtained by stereotactic biopsy.* Archives of neurology, 1991. **48**(12): p. 1244-1246.
389. Zeinstra, E., et al., *Astrocytes in chronic active multiple sclerosis plaques express MHC class II molecules.* Neuroreport, 2000. **11**(1): p. 89-91.
390. Firestein, G.S., M.M. Paine, and B.H. Littman, *Gene Expression (Collagenase, Tissue Inhibitor of Metalloproteinases, Complement, and HLA–DR) in Rheumatoid Arthritis and Osteoarthritis Synovium. Quantitative Analysis and Effect of Intraarticular Corticosteroids.* Arthritis & Rheumatology, 1991. **34**(9): p. 1094-1105.
391. Burmester, G.R., et al., *Differential expression of Ia antigens by rheumatoid synovial lining cells.* Journal of Clinical Investigation, 1987. **80**(3): p. 595.
392. Boots, A., A. Wimmers-Bertens, and A. Rijnders, *Antigen-presenting capacity of rheumatoid synovial fibroblasts.* Immunology, 1994. **82**(2): p. 268.
393. Laning, J.C., et al., *In Vitro Analysis Of Cd40-cd154 And Cd28-cd80/86 Interactions In The Primary T-cell Response To Allogeneic "non Professional" Antigen Presenting Cells.* Transplantation, 2001. **71**(10): p. 1467-1474.

394. Bal, V., et al., *Antigen presentation by keratinocytes induces tolerance in human T cells.* European journal of immunology, 1990. **20**(9): p. 1893-1897.
395. Tay, S.S., et al., *IFN-γ reverses the stop signal allowing migration of antigen-specific T cells into inflammatory sites.* The Journal of Immunology, 2003. **170**(6): p. 3315-3322.
396. Marelli-Berg, F.M., et al., *Antigen recognition influences transendothelial migration of CD4+ T cells.* The Journal of Immunology, 1999. **162**(2): p. 696-703.
397. Manes, T.D., et al., *TCR signaling antagonizes rapid IP-10-mediated transendothelial migration of effector memory CD4+ T cells.* The Journal of Immunology, 2007. **178**(5): p. 3237-3243.
398. Ohyama, H., et al., *Counter-antigen presentation: fibroblasts produce cytokines by signalling through HLA class II molecules without inducing T-cell proliferation.* Cytokine, 2002. **17**(4): p. 175-181.
399. Kato-Kogoe, N., et al., *Fibroblasts stimulated via HLA-II molecules produce prostaglandin E2 and regulate cytokine production from helper T cells.* Laboratory Investigation, 2010. **90**(12): p. 1747-1756.
400. Carman, C.V. and R. Martinelli, *T Lymphocyte–endothelial interactions: emerging Understanding of Trafficking and Antigen-Specific immunity.* Frontiers in immunology, 2015. **6**.
401. Grabie, N., et al., *Endothelial programmed death-1 ligand 1 (PD-L1) regulates CD8+ T-cell–mediated injury in the heart.* Circulation, 2007. **116**(18): p. 2062-2071.
402. Hollenbaugh, D., et al., *Expression of functional CD40 by vascular endothelial cells.* Journal of Experimental Medicine, 1995. **182**(1): p. 33-40.
403. Marelli-Berg, F., et al., *Major histocompatibility complex class II-expressing endothelial cells induce allospecific nonresponsiveness in naive T cells.* Journal of Experimental Medicine, 1996. **183**(4): p. 1603-1612.
404. Turesson, C., *Endothelial expression of MHC class II molecules in autoimmune disease.* Current pharmaceutical design, 2004. **10**(2): p. 129-143.
405. Kambayashi, T. and T.M. Laufer, *Atypical MHC class II-expressing antigen-presenting cells: can anything replace a dendritic cell?* Nature Reviews Immunology, 2014. **14**(11): p. 719-730.
406. Khayyamian, S., et al., *ICOS-ligand, expressed on human endothelial cells, costimulates Th1 and Th2 cytokine secretion by memory CD4+ T cells.* Proceedings of the National Academy of Sciences, 2002. **99**(9): p. 6198-6203.
407. Keir, M.E., et al., *Tissue expression of PD-L1 mediates peripheral T cell tolerance.* Journal of Experimental Medicine, 2006. **203**(4): p. 883-895.
408. Black, A.P., et al., *Human keratinocyte induction of rapid effector function in antigen‐specific memory CD4+ and CD8+ T cells.* European journal of immunology, 2007. **37**(6): p. 1485-1493.
409. Youngnak-Piboonratanakit, P., et al., *The expression of B7-H1 on keratinocytes in chronic inflammatory mucocutaneous disease and its regulatory role.* Immunology letters, 2004. **94**(3): p. 215-222.
410. Otten, H., et al., *Alloantigen‐specific T‐cell anergy induced by human keratinocytes is abrogated upon loss of cell‐cell contact.* Immunology, 1996. **88**(2): p. 214-219.
411. Beswick, E.J., et al., *Expression of the programmed death ligand 1, B7-H1, on gastric epithelial cells after Helicobacter pylori exposure promotes*

development of CD4+ CD25+ FoxP3+ regulatory T cells. Infection and immunity, 2007. **75**(9): p. 4334-4341.
412. Beswick, E.J., et al., *Role of gastric epithelial cell-derived transforming growth factor β in reduced CD4+ T cell proliferation and development of regulatory T cells during Helicobacter pylori infection.* Infection and immunity, 2011. **79**(7): p. 2737-2745.
413. Pinchuk, I.V., et al., *Stromal cells induce Th17 during Helicobacter pylori infection and in the gastric tumor microenvironment.* PLoS One, 2013. **8**(1): p. e53798.
414. Bernard, F.-X., et al., *Keratinocytes under fire of proinflammatory cytokines: bona fide innate immune cells involved in the physiopathology of chronic atopic dermatitis and psoriasis.* Journal of allergy, 2012. **2012**.
415. Purwar, R., T. Werfel, and M. Wittmann, *IL-13-stimulated human keratinocytes preferentially attract CD4+ CCR4+ T cells: possible role in atopic dermatitis.* Journal of investigative dermatology, 2006. **126**(5): p. 1043-1051.
416. Werfel, T., *The role of leukocytes, keratinocytes, and allergen-specific IgE in the development of atopic dermatitis.* Journal of Investigative Dermatology, 2009. **129**(8): p. 1878-1891.
417. Kinoshita, H., et al., *Cytokine milieu modulates release of thymic stromal lymphopoietin from human keratinocytes stimulated with double-stranded RNA.* Journal of Allergy and Clinical Immunology, 2009. **123**(1): p. 179-186.
418. Wittmann, M., et al., *Human keratinocytes respond to interleukin-18: implication for the course of chronic inflammatory skin diseases.* Journal of Investigative Dermatology, 2005. **124**(6): p. 1225-1233.
419. Kanda, N., et al., *IL- 18 enhances IFN- γ- induced production of CXCL9, CXCL10, and CXCL11 in human keratinocytes.* European journal of immunology, 2007. **37**(2): p. 338-350.
420. Tykocinski, L., et al., *A1. 28 Synovial fibroblasts suppress TH1, but not TH2 or TH17 cells, through tryptophan metabolism.* Annals of the Rheumatic Diseases, 2015. **74**(Suppl 1): p. A12-A12.
421. Tykocinski, L.-O., et al., *Synovial Fibroblasts Selectively Suppress Th1 Cell Responses through IDO1-Mediated Tryptophan Catabolism.* The Journal of Immunology, 2017. **198**(8): p. 3109-3117.
422. Komatsu, N., et al., *Pathogenic conversion of Foxp3+ T cells into TH17 cells in autoimmune arthritis.* Nature medicine, 2014. **20**(1): p. 62-68.
423. Clark, R.A. and T.S. Kupper, *IL-15 and dermal fibroblasts induce proliferation of natural regulatory T cells isolated from human skin.* Blood, 2007. **109**(1): p. 194-202.
424. Miranda-Carús, M.-E., et al., *IL-15 and the initiation of cell contact-dependent synovial fibroblast-T lymphocyte cross-talk in rheumatoid arthritis: effect of methotrexate.* The Journal of Immunology, 2004. **173**(2): p. 1463-1476.
425. Philip, M., D.A. Rowley, and H. Schreiber. *Inflammation as a tumor promoter in cancer induction*. in *Seminars in cancer biology*. 2004. Elsevier.
426. Lu, H., W. Ouyang, and C. Huang, *Inflammation, a key event in cancer development.* Molecular Cancer Research, 2006. **4**(4): p. 221-233.
427. Houghton, J., et al., *Gastric cancer originating from bone marrow-derived cells.* Science, 2004. **306**(5701): p. 1568-1571.

428. Balsamo, M., et al., *Melanoma-associated fibroblasts modulate NK cell phenotype and antitumor cytotoxicity.* Proceedings of the National Academy of Sciences, 2009. **106**(49): p. 20847-20852.
429. Wu, Y., et al., *CCL3-CCR5 axis regulates intratumoral accumulation of leukocytes and fibroblasts and promotes angiogenesis in murine lung metastasis process.* The Journal of Immunology, 2008. **181**(9): p. 6384-6393.
430. Wakabayashi, O., et al., *CD4+ T cells in cancer stroma, not CD8+ T cells in cancer cell nests, are associated with favorable prognosis in human non-small cell lung cancers.* Cancer science, 2003. **94**(11): p. 1003-1009.
431. Shim, H.S., et al., *Prognostic effect of stromal lymphocyte infiltration in thrymic carcinoma.* Lung Cancer, 2011. **74**(2): p. 338-343.
432. Zhang, J., et al., *FSP1+ fibroblasts promote skin carcinogenesis by maintaining MCP-1-mediated macrophage infiltration and chronic inflammation.* The American journal of pathology, 2011. **178**(1): p. 382-390.
433. Shimoda, M., K.T. Mellody, and A. Orimo. *Carcinoma-associated fibroblasts are a rate-limiting determinant for tumour progression*. in *Seminars in cell & developmental biology*. 2010. Elsevier.
434. Martinez-Outschoorn, U.E., et al., *Oxidative stress in cancer associated fibroblasts drives tumor-stroma co-evolution: A new paradigm for understanding tumor metabolism, the field effect and genomic instability in cancer cells.* Cell cycle, 2010. **9**(16): p. 3276-3296.
435. Hanahan, D. and R.A. Weinberg, *The hallmarks of cancer.* cell, 2000. **100**(1): p. 57-70.
436. Hanahan, D. and R.A. Weinberg, *Hallmarks of cancer: the next generation.* cell, 2011. **144**(5): p. 646-674.
437. Pohl, C., A. Hombach, and W. Kruis, *Chronic inflammatory bowel disease and cancer.* Hepato-gastroenterology, 1999. **47**(31): p. 57-70.
438. Rakoff-Nahoum, S., *Cancer Issue: Why Cancer and Inflammation?* The Yale journal of biology and medicine, 2006. **79**(3-4): p. 123.
439. Kinoshita, T., et al., *Forkhead box P3 regulatory T cells coexisting with cancer associated fibroblasts are correlated with a poor outcome in lung adenocarcinoma.* Cancer science, 2013. **104**(4): p. 409-415.
440. Erez, N., et al., *Cancer-Associated Fibroblasts Are Activated in Incipient Neoplasia to Orchestrate Tumor-Promoting Inflammation in an NF-kappaB-Dependent Manner.* Cancer Cell, 2010. **17**(2): p. 135-47.
441. DiGiovanni, J., *Multistage carcinogenesis in mouse skin.* Pharmacology & therapeutics, 1992. **54**(1): p. 63-128.
442. Steinhauser, M.L., et al., *Macrophage/fibroblast coculture induces macrophage inflammatory protein-1alpha production mediated by intercellular adhesion molecule-1 and oxygen radicals.* J Leukoc Biol, 1998. **64**(5): p. 636-41.
443. Popivanova, B.K., et al., *Blockade of a chemokine, CCL2, reduces chronic colitis-associated carcinogenesis in mice.* Cancer Res, 2009. **69**(19): p. 7884-92.
444. Sasaki, S., et al., *Crucial involvement of the CCL3-CCR5 axis-mediated fibroblast accumulation in colitis-associated carcinogenesis in mice.* Int J Cancer, 2014. **135**(6): p. 1297-306.
445. Dvorak, H.F., *Tumors: wounds that do not heal: similarities between tumor stroma generation and wound healing.* The New England journal of medicine, 1986. **315**(26): p. 1650-1659.

446. Ploeger, D., et al., *Cell plasticity in wound healing: paracrine factors of M1/M2 polarized macrophages influence the phenotypical state of dermal fibroblasts.* Cell Commun Signal, 2013. **11**(1): p. 29.
447. Bingle, L., N. Brown, and C. Lewis, *The role of tumour‐associated macrophages in tumour progression: implications for new anticancer therapies.* The Journal of pathology, 2002. **196**(3): p. 254-265.
448. Comito, G., et al., *Cancer-associated fibroblasts and M2-polarized macrophages synergize during prostate carcinoma progression.* Oncogene, 2014. **33**(19): p. 2423-31.
449. Stuelten, C.H., et al., *Transient tumor-fibroblast interactions increase tumor cell malignancy by a TGF-β mediated mechanism in a mouse xenograft model of breast cancer.* PLoS One, 2010. **5**(3): p. e9832.
450. Mantovani, A., et al., *Cancer-related inflammation.* Nature, 2008. **454**(7203): p. 436-444.
451. Spahn, J.H. and D. Kreisel, *Monocytes in sterile inflammation: recruitment and functional consequences.* Archivum immunologiae et therapiae experimentalis, 2014. **62**(3): p. 187-194.
452. Feng, A.L., et al., *CD16+ monocytes in breast cancer patients: expanded by monocyte chemoattractant protein‐1 and may be useful for early diagnosis.* Clinical & Experimental Immunology, 2011. **164**(1): p. 57-65.
453. Subimerb, C., et al., *Circulating CD14+ CD16+ monocyte levels predict tissue invasive character of cholangiocarcinoma.* Clinical & Experimental Immunology, 2010. **161**(3): p. 471-479.
454. Saleh, M., et al., *CD16+ monocytes in patients with cancer: spontaneous elevation and pharmacologic induction by recombinant human macrophage colony-stimulating factor.* Blood, 1995. **85**(10): p. 2910-2917.
455. Sanford, D.E., et al., *Inflammatory monocyte mobilization decreases patient survival in pancreatic cancer: a role for targeting the CCL2/CCR2 axis.* Clinical Cancer Research, 2013. **19**(13): p. 3404-3415.
456. Weber, C., et al., *Differential chemokine receptor expression and function in human monocyte subpopulations.* Journal of leukocyte biology, 2000. **67**(5): p. 699-704.
457. Lee, H.-W., et al., *Recruitment of monocytes/macrophages in different tumor microenvironments.* Biochimica et Biophysica Acta (BBA)-Reviews on Cancer, 2013. **1835**(2): p. 170-179.
458. Augsten, M., et al., *CXCL14 is an autocrine growth factor for fibroblasts and acts as a multi-modal stimulator of prostate tumor growth.* Proceedings of the National Academy of Sciences, 2009. **106**(9): p. 3414-3419.
459. Frederick, M.J., et al., *In vivo expression of the novel CXC chemokine BRAK in normal and cancerous human tissue.* The American journal of pathology, 2000. **156**(6): p. 1937-1950.
460. Shurin, G.V., et al., *Loss of new chemokine CXCL14 in tumor tissue is associated with low infiltration by dendritic cells (DC), while restoration of human CXCL14 expression in tumor cells causes attraction of DC both in vitro and in vivo.* The Journal of Immunology, 2005. **174**(9): p. 5490-5498.
461. Kurth, I., et al., *Monocyte selectivity and tissue localization suggests a role for breast and kidney–expressed chemokine (BRAK) in macrophage development.* Journal of Experimental Medicine, 2001. **194**(6): p. 855-862.
462. O'Neill, G., et al., *Overexpression of human prostaglandin G/H synthase-1 and-2 by recombinant vaccinia virus: inhibition by nonsteroidal anti-*

inflammatory drugs and biosynthesis of 15-hydroxyeicosatetraenoic acid. Molecular Pharmacology, 1994. **45**(2): p. 245-254.
463. Xie, W., D.L. Robertson, and D.L. Simmons, *Mitogen‐inducible prostaglandin G/H synthase: A new target for nonsteroidal antiinflammatory drugs.* Drug Development Research, 1992. **25**(4): p. 249-265.
464. Bos, C.L., et al., *Prostanoids and prostanoid receptors in signal transduction.* The international journal of biochemistry & cell biology, 2004. **36**(7): p. 1187-1205.
465. Xi, P.-p., et al., *Role of the prostaglandin E2 receptor agonists in TGF-β1-induced mesangial cell damage.* Bioscience reports, 2016. **36**(5): p. e00383.
466. Breyer, M.D. and R.M. Breyer, *G Protein–coupled prostanoid receptors and the kidney.* Annual review of physiology, 2001. **63**(1): p. 579-605.
467. Shiraishi, N., et al., *Prostaglandin E2-EP3 axis in fine-tuning excessive skin inflammation by restricting dendritic cell functions.* PloS one, 2013. **8**(7): p. e69599.
468. Amano, H., et al., *Host prostaglandin E2-EP3 signaling regulates tumor-associated angiogenesis and tumor growth.* Journal of Experimental Medicine, 2003. **197**(2): p. 221-232.
469. Hara, S., et al., *Prostaglandin E synthases: understanding their pathophysiological roles through mouse genetic models.* Biochimie, 2010. **92**(6): p. 651-659.
470. Wallace, J.L., *Prostaglandian biology in inflammatory bowel disease.* Gastroenterology Clinics, 2001. **30**(4): p. 971-980.
471. Sheibanie, A.F., et al., *Prostaglandin E2 exacerbates collagen‐induced arthritis in mice through the inflammatory interleukin‐23/interleukin‐17 axis.* Arthritis & Rheumatology, 2007. **56**(8): p. 2608-2619.
472. Qian, X., et al., *Increased Th17 cells in the tumor microenvironment is mediated by IL-23 via tumor-secreted prostaglandin E2.* The Journal of Immunology, 2013. **190**(11): p. 5894-5902.
473. Kalinski, P., *Regulation of immune responses by prostaglandin E2.* The Journal of Immunology, 2012. **188**(1): p. 21-28.
474. Snijdewint, F., et al., *Prostaglandin E2 differentially modulates cytokine secretion profiles of human T helper lymphocytes.* The Journal of Immunology, 1993. **150**(12): p. 5321-5329.
475. Betz, M. and B. Fox, *Prostaglandin E2 inhibits production of Th1 lymphokines but not of Th2 lymphokines.* The Journal of Immunology, 1991. **146**(1): p. 108-113.
476. Bergmann, C., et al., *Expansion of human T regulatory type 1 cells in the microenvironment of cyclooxygenase 2 overexpressing head and neck squamous cell carcinoma.* Cancer research, 2007. **67**(18): p. 8865-8873.
477. Montero, J., et al., *Prostaglandin E2 promotes M2 polarization of macrophages via a cAMP/CREB signaling pathway and deactivates granulocytes in teleost fish.* Fish & shellfish immunology, 2016. **55**: p. 632-641.
478. Zasłona, Z., et al., *Prostaglandin E2 restrains macrophage maturation via E prostanoid receptor 2/protein kinase A signaling.* Blood, 2012. **119**(10): p. 2358-2367.
479. Aronoff, D.M., C. Canetti, and M. Peters-Golden, *Prostaglandin E2 inhibits alveolar macrophage phagocytosis through an E-prostanoid 2 receptor-*

mediated increase in intracellular cyclic AMP. The Journal of Immunology, 2004. **173**(1): p. 559-565.
480. Chulada, P.C., et al., *Genetic disruption of Ptgs-1, as well as of Ptgs-2, reduces intestinal tumorigenesis in Min mice.* Cancer research, 2000. **60**(17): p. 4705-4708.
481. Sumitani, K., et al., *Specific inhibition of cyclooxygenase‐2 results in inhibition of proliferation of oral cancer cell lines via suppression of prostaglandin E2 production.* Journal of oral pathology & medicine, 2001. **30**(1): p. 41-47.
482. Gately, S., *The contributions of cyclooxygenase-2 to tumor angiogenesis.* Cancer and metastasis reviews, 2000. **19**(1-2): p. 19-27.
483. Fischer, S.M., E.T. Hawk, and R.A. Lubet, *Coxibs and other nonsteroidal anti-inflammatory drugs in animal models of cancer chemoprevention.* Cancer prevention research, 2011. **4**(11): p. 1728-1735.
484. Rosenberg, L., et al., *A hypothesis: nonsteroidal anti-inflammatory drugs reduce the incidence of large-bowel cancer.* JNCI: Journal of the National Cancer Institute, 1991. **83**(5): p. 355-358.
485. Thun, M.J., M.M. Namboodiri, and C.W. Heath Jr, *Aspirin use and reduced risk of fatal colon cancer.* New England Journal of Medicine, 1991. **325**(23): p. 1593-1596.
486. Waddell, W.R., et al., *Sulindac for polyposis of the colon.* The American journal of surgery, 1989. **157**(1): p. 175-179.
487. Waddell, W.R. and R.W. Loughry, *Sulindac for polyposis of the colon.* Journal of surgical oncology, 1983. **24**(1): p. 83-87.
488. Schmidt, A., et al., *Direct growth-inhibitory effects of prostaglandin E2 in pancreatic cancer cells in vitro through an EP4/PKA-mediated mechanism.* Surgery, 2017. **161**(6): p. 1570-1578.
489. Wilson, J.W. and C.S. Potten, *The effect of exogenous prostaglandin administration on tumor size and yield in Min/+ mice.* Cancer research, 2000. **60**(16): p. 4645-4653.
490. Begg, A.C., F.A. Stewart, and C. Vens, *Strategies to improve radiotherapy with targeted drugs.* Nature Reviews Cancer, 2011. **11**(4): p. 239-253.
491. Baskar, R., et al., *Biological response of cancer cells to radiation treatment.* Frontiers in molecular biosciences, 2014. **1**.
492. Connell, P.P. and S. Hellman, *Advances in radiotherapy and implications for the next century: a historical perspective.* Cancer research, 2009. **69**(2): p. 383-392.
493. Vysín, L., et al., *Proton-induced direct and indirect damage of plasmid DNA.* Radiation and environmental biophysics, 2015. **54**(3): p. 343.
494. Barcellos-Hoff, M.H., C. Park, and E.G. Wright, *Radiation and the microenvironment–tumorigenesis and therapy.* Nature Reviews Cancer, 2005. **5**(11): p. 867-875.
495. Little, M.P., et al., *Risks associated with low doses and low dose rates of ionizing radiation: why linearity may be (almost) the best we can do 1.* Radiology, 2009. **251**(1): p. 6-12.
496. Mettler, F.A., *Medical effects and risks of exposure to ionising radiation.* Journal of Radiological Protection, 2012. **32**(1): p. N9.
497. Nikolic, B., et al., *Patient Radiation Dose Associated with Uterine Artery Embolization 1.* Radiology, 2000. **214**(1): p. 121-125.

498. Koenig, T.R., et al., *Skin injuries from fluoroscopically guided procedures: part 1, characteristics of radiation injury.* American journal of roentgenology, 2001. **177**(1): p. 3-11.
499. Fry, R.M., *Deterministic effects.* Health physics, 2001. **80**(4): p. 338-343.
500. Hellevik, T. and I. Martinez-Zubiaurre, *Radiotherapy and the tumor stroma: the importance of dose and fractionation.* Frontiers in oncology, 2014. **4**: p. 1.
501. Malaker, K., et al., *Retrospective analysis of treatment of unresectable keloids with primary radiation over 25 years.* Clinical Oncology, 2004. **16**(4): p. 290-298.
502. Sclafani, A.P., et al., *Prevention of Earlobe Keloid Recurrence with Postoperative Corticosteroid Injections Versus Radiation Therapy A Randomized, Prospective Study and Review of the Literature.* Dermatologic surgery, 1996. **22**(6): p. 569-574.
503. Wunderlich, R., et al., *Low and moderate doses of ionizing radiation up to 2 Gy modulate transmigration and chemotaxis of activated macrophages, provoke an anti‐inflammatory cytokine milieu, but do not impact upon viability and phagocytic function.* Clinical & Experimental Immunology, 2015. **179**(1): p. 50-61.
504. Fowler, J.F., *The linear-quadratic formula and progress in fractionated radiotherapy.* The British journal of radiology, 1989. **62**(740): p. 679-694.
505. Bourke, V.A., et al., *Correlation of radiation response with tumor oxygenation in the Dunning prostate R3327-AT1 tumor.* International Journal of Radiation Oncology* Biology* Physics, 2007. **67**(4): p. 1179-1186.
506. Carlson, D.J., K.M. Yenice, and C.G. Orton, *Tumor hypoxia is an important mechanism of radioresistance in hypofractionated radiotherapy and must be considered in the treatment planning process.* Medical physics, 2011. **38**(12): p. 6347-6350.
507. Mottram, J., *A factor of importance in the radio sensitivity of tumours.* The British Journal of Radiology, 1936. **9**(105): p. 606-614.
508. Barker, H.E., et al., *The tumour microenvironment after radiotherapy: mechanisms of resistance and recurrence.* Nature reviews Cancer, 2015. **15**(7): p. 409-425.
509. Feng, H., et al., *Hypoxia-induced autophagy as an additional mechanism in human osteosarcoma radioresistance.* Journal of Bone Oncology, 2016. **5**(2): p. 67-73.
510. Iliakis, G. *Cell cycle regulation in irradiated and nonirradiated cells*. in *Seminars in oncology*. 1997.
511. Tobey, R.A., *Different drugs arrest cells at a number of distinct stages in G2.* Nature, 1975. **254**: p. 245-247.
512. Iliakis, G., et al., *DNA damage checkpoint control in cells exposed to ionizing radiation.* Oncogene, 2003. **22**(37): p. 5834-5847.
513. Wei, L., et al., *Rapid recruitment of BRCA1 to DNA double-strand breaks is dependent on its association with Ku80.* Molecular and cellular biology, 2008. **28**(24): p. 7380-7393.
514. Zhang, J. and S.N. Powell, *The role of the BRCA1 tumor suppressor in DNA double-strand break repair.* Molecular Cancer Research, 2005. **3**(10): p. 531-539.
515. Roy, R., J. Chun, and S.N. Powell, *BRCA1 and BRCA2: different roles in a common pathway of genome protection.* Nature Reviews Cancer, 2012. **12**(1): p. 68-78.

516. Shen, Z. and J.A. Nickoloff, *Mammalian homologous recombination repair and cancer intervention.* DNA Repair, Genetic Instability, and Cancer, 2007: p. 119-156.
517. Moynahan, M.E. and M. Jasin, *Mitotic homologous recombination maintains genomic stability and suppresses tumorigenesis.* Nature reviews Molecular cell biology, 2010. **11**(3): p. 196-207.
518. Abraham, R.T., *Cell cycle checkpoint signaling through the ATM and ATR kinases.* Genes & development, 2001. **15**(17): p. 2177-2196.
519. Yazdi, P.T., et al., *SMC1 is a downstream effector in the ATM/NBS1 branch of the human S-phase checkpoint.* Genes & development, 2002. **16**(5): p. 571-582.
520. Lovejoy, C.A. and D. Cortez, *Common mechanisms of PIKK regulation.* DNA repair, 2009. **8**(9): p. 1004-1008.
521. Kastan, M.B. and D.-s. Lim, *The many substrates and functions of ATM.* Nature reviews Molecular cell biology, 2000. **1**(3): p. 179-186.
522. Perry, J. and N. Kleckner, *The ATRs, ATMs, and TORs are giant HEAT repeat proteins.* Cell, 2003. **112**(2): p. 151-155.
523. Bakkenist, C.J. and M.B. Kastan, *DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation.* Nature, 2003. **421**(6922): p. 499.
524. Dodson, G.E. and R.S. Tibbetts, *DNA replication stress-induced phosphorylation of cyclic AMP response element-binding protein mediated by ATM.* Journal of Biological Chemistry, 2006. **281**(3): p. 1692-1697.
525. Williams, G.J., S.P. Lees-Miller, and J.A. Tainer, *Mre11–Rad50–Nbs1 conformations and the control of sensing, signaling, and effector responses at DNA double-strand breaks.* DNA repair, 2010. **9**(12): p. 1299-1306.
526. Kurz, E.U. and S.P. Lees-Miller, *DNA damage-induced activation of ATM and ATM-dependent signaling pathways.* DNA repair, 2004. **3**(8): p. 889-900.
527. You, Z., et al., *ATM activation and its recruitment to damaged DNA require binding to the C terminus of Nbs1.* Molecular and cellular biology, 2005. **25**(13): p. 5363-5379.
528. Falck, J., J. Coates, and S.P. Jackson, *Conserved modes of recruitment of ATM, ATR and DNA-PKcs to sites of DNA damage.* Nature, 2005. **434**(7033): p. 605-611.
529. Goodarzi, A.A., et al., *Autophosphorylation of ataxia-telangiectasia mutated is regulated by protein phosphatase 2A.* The EMBO journal, 2004. **23**(22): p. 4451-4461.
530. Guo, C.Y., D.L. Brautigan, and J.M. Larner, *ATM-dependent dissociation of B55 regulatory subunit from nuclear PP2A in response to ionizing radiation.* Journal of Biological Chemistry, 2002. **277**(7): p. 4839-4844.
531. Guo, Z., et al., *ATM activation by oxidative stress.* Science, 2010. **330**(6003): p. 517-521.
532. Lim, D.-S., et al., *ATM phosphorylates p95/nbs1 in an S-phase checkpoint pathway.* Nature, 2000. **404**(6778): p. 613-617.
533. Buscemi, G., et al., *Chk2 activation dependence on Nbs1 after DNA damage.* Molecular and Cellular Biology, 2001. **21**(15): p. 5214-5222.
534. Lee, J.H., et al., *53BP1 promotes ATM activity through direct interactions with the MRN complex.* The EMBO journal, 2010. **29**(3): p. 574-585.
535. Gatei, M., et al., *Role for ATM in DNA damage-induced phosphorylation of BRCA1.* Cancer research, 2000. **60**(12): p. 3299-3304.

536. Gatei, M., et al., *Ataxia Telangiectasia Mutated (ATM) Kinase and ATM and Rad3 Related Kinase Mediate Phosphorylation of Brca1 at Distinct and Overlapping Sites IN VIVO ASSESSMENT USING PHOSPHO-SPECIFIC ANTIBODIES.* Journal of Biological Chemistry, 2001. **276**(20): p. 17276-17280.
537. Xu, B., et al., *Phosphorylation of serine 1387 in Brca1 is specifically required for the Atm-mediated S-phase checkpoint after ionizing irradiation.* Cancer research, 2002. **62**(16): p. 4588-4591.
538. Li, S., et al., *Functional link of BRCA1 and ataxia telangiectasia gene product in DNA damage response.* Nature, 2000. **406**(6792): p. 210-215.
539. Lee, J.-S., et al., *hCds1-mediated phosphorylation of BRCA1 regulates the DNA damage response.* Nature, 2000. **404**(6774): p. 201-204.
540. Banin, S., et al., *Enhanced phosphorylation of p53 by ATM in response to DNA damage.* Science, 1998. **281**(5383): p. 1674-1677.
541. Canman, C.E., et al., *Activation of the ATM kinase by ionizing radiation and phosphorylation of p53.* Science, 1998. **281**(5383): p. 1677-1679.
542. Khosravi, R., et al., *Rapid ATM-dependent phosphorylation of MDM2 precedes p53 accumulation in response to DNA damage.* Proceedings of the National Academy of Sciences, 1999. **96**(26): p. 14973-14977.
543. Maya, R., et al., *ATM-dependent phosphorylation of Mdm2 on serine 395: role in p53 activation by DNA damage.* Genes & development, 2001. **15**(9): p. 1067-1077.
544. Kim, S.H., et al., *Tunable regulation of CREB DNA binding activity couples genotoxic stress response and metabolism.* Nucleic Acids Research, 2016. **44**(20): p. 9667-9680.
545. Oda, E., et al., *Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis.* Science, 2000. **288**(5468): p. 1053-1058.
546. Dogu, Y. and J. Díaz, *Mathematical model of a network of interaction between p53 and Bcl-2 during genotoxic-induced apoptosis.* Biophysical chemistry, 2009. **143**(1): p. 44-54.
547. Kuribayashi, K., et al., *The relative contribution of pro-apoptotic p53-target genes in the triggering of apoptosis following DNA damage in vitro and in vivo.* Cell Cycle, 2011. **10**(14): p. 2380-2389.
548. Morrison, C., et al., *The controlling role of ATM in homologous recombinational repair of DNA damage.* The EMBO journal, 2000. **19**(3): p. 463-471.
549. Shiloh, Y., *ATM and related protein kinases: safeguarding genome integrity.* Nature Reviews Cancer, 2003. **3**(3): p. 155-168.
550. Falck, J., et al., *The ATM–Chk2–Cdc25A checkpoint pathway guards against radioresistant DNA synthesis.* Nature, 2001. **410**(6830): p. 842-847.
551. Mailand, N., et al., *Regulation of G2/M events by Cdc25A through phosphorylation- dependent modulation of its stability.* The EMBO journal, 2002. **21**(21): p. 5911-5920.
552. Zhao, H., J.L. Watkins, and H. Piwnica-Worms, *Disruption of the checkpoint kinase 1/cell division cycle 25A pathway abrogates ionizing radiation-induced S and G2 checkpoints.* Proceedings of the National Academy of Sciences, 2002. **99**(23): p. 14795-14800.
553. Sakamoto, K.M. and D.A. Frank, *CREB in the pathophysiology of cancer: implications for targeting transcription factors for cancer therapy.* Clinical Cancer Research, 2009. **15**(8): p. 2583-2587.

554. Chrivia, J.C., et al., *Phosphorylated CREB binds specifically to the nuclear protein CBP.* Nature, 1993. **365**(6449): p. 855-859.
555. Shi, Y., et al., *Direct regulation of CREB transcriptional activity by ATM in response to genotoxic stress.* Proceedings of the National Academy of Sciences of the United States of America, 2004. **101**(16): p. 5898-5903.
556. Nowak-Wegrzyn, A., et al., *Immunodeficiency and infections in ataxia-telangiectasia.* The Journal of pediatrics, 2004. **144**(4): p. 505-511.
557. Staples, E., et al., *Immunodeficiency in ataxia telangiectasia is correlated strongly with the presence of two null mutations in the ataxia telangiectasia mutated gene.* Clinical & Experimental Immunology, 2008. **153**(2): p. 214-220.
558. Bredemeyer, A.L., et al., *ATM stabilizes DNA double-strand-break complexes during V (D) J recombination.* Nature, 2006. **442**(7101): p. 466-470.
559. Peterson, R.A., W. Kelly, and R. Good, *Ataxia-telangiectasia its association with a defective thymus, immunological-deficiency disease, and malignancy.* The Lancet, 1964. **283**(7344): p. 1189-1193.
560. Paganelli, R., et al., *Selective deficiency of CD4+/CD45RA+ lymphocytes in patients with ataxia-telangiectasia.* Journal of clinical immunology, 1992. **12**(2): p. 84-91.
561. Gasser, S., et al., *The DNA damage pathway regulates innate immune system ligands of the NKG2D receptor.* nature, 2005. **436**(7054): p. 1186-1190.
562. Soriani, A., et al., *ATM-ATR–dependent up-regulation of DNAM-1 and NKG2D ligands on multiple myeloma cells by therapeutic agents results in enhanced NK-cell susceptibility and is associated with a senescent phenotype.* Blood, 2009. **113**(15): p. 3503-3511.
563. Brzostek-Racine, S., et al., *The DNA damage response induces IFN.* The Journal of Immunology, 2011. **187**(10): p. 5336-5345.
564. Weintz, G., et al., *The phosphoproteome of toll‐like receptor‐activated macrophages.* Molecular systems biology, 2010. **6**(1): p. 371.
565. Wen, A.Y., K.M. Sakamoto, and L.S. Miller, *The role of the transcription factor CREB in immune function.* The Journal of Immunology, 2010. **185**(11): p. 6413-6419.
566. Kim, H.-P. and W.J. Leonard, *CREB/ATF-dependent T cell receptor–induced FoxP3 gene expression: a role for DNA methylation.* Journal of Experimental Medicine, 2007. **204**(7): p. 1543-1551.
567. Alvarez, Y., et al., *The induction of IL-10 by zymosan in dendritic cells depends on CREB activation by the coactivators CREB-binding protein and TORC2 and autocrine PGE2.* The Journal of Immunology, 2009. **183**(2): p. 1471-1479.
568. Gosset, P., et al., *Prostaglandin D2 affects the maturation of human monocyte-derived dendritic cells: consequence on the polarization of naive Th cells.* The Journal of Immunology, 2003. **170**(10): p. 4943-4952.
569. Ruffell, D., et al., *A CREB-C/EBPβ cascade induces M2 macrophage-specific gene expression and promotes muscle injury repair.* Proceedings of the National Academy of Sciences, 2009. **106**(41): p. 17475-17480.
570. Hu, X., et al., *IFN-γ suppresses IL-10 production and synergizes with TLR2 by regulating GSK3 and CREB/AP-1 proteins.* Immunity, 2006. **24**(5): p. 563-574.

571. Avni, D., et al., *Role of CREB in modulation of TNFα and IL-10 expression in LPS-stimulated RAW264. 7 macrophages.* Molecular immunology, 2010. **47**(7): p. 1396-1403.
572. Wang, Q., et al., *Novel approach for interleukin- 23 up- regulation in human dendritic cells and the impact on T helper type 17 generation.* Immunology, 2011. **134**(1): p. 60-72.
573. Antypas, H., B. Libberton, and K. Melican, *Reducing background cytokine expression in epithelial cells without serum starvation.* MethodsX, 2014. **1**: p. 251-253.
574. Mills, C.D. and K. Ley, *M1 and M2 macrophages: the chicken and the egg of immunity.* J Innate Immun, 2014. **6**(6): p. 716-26.
575. Vermes, I., et al., *A novel assay for apoptosis flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled annexin V.* Journal of immunological methods, 1995. **184**(1): p. 39-51.
576. Chung, M.P., et al., *Adhesion-induced generation of oxygen free radical from human alveolar macrophages and its mechanisms.* Tuberculosis and Respiratory Diseases, 1996. **43**(2): p. 210-220.
577. Shattock, R.J., J. Friedland, and G.E. Griffin, *Release of human immunodeficiency virus by THP-1 cells and human macrophages is regulated by cellular adherence and activation.* Journal of virology, 1993. **67**(6): p. 3569-3575.
578. Shen, M. and T.A. Horbett, *The effects of surface chemistry and adsorbed proteins on monocyte/macrophage adhesion to chemically modified polystyrene surfaces.* Journal of Biomedical Materials Research Part A, 2001. **57**(3): p. 336-345.
579. Ryan, J.A., *Evolution of cell culture surfaces.* BioFiles, 2008. **3**(8): p. 21.
580. Amstein, C.F. and P.A. Hartman, *Adaptation of plastic surfaces for tissue culture by glow discharge.* Journal of clinical microbiology, 1975. **2**(1): p. 46-54.
581. Ramsey, W., et al., *Surface treatments and cell attachment.* In Vitro Cellular & Developmental Biology-Plant, 1984. **20**(10): p. 802-808.
582. Hamilton, J.A., *Colony stimulating factors and macrophage heterogeneity.* Inflammation and Regeneration, 2011. **31**(3): p. 228-236.
583. Lacey, D.C., et al., *Defining GM-CSF–and macrophage-CSF–dependent macrophage responses by in vitro models.* The Journal of Immunology, 2012. **188**(11): p. 5752-5765.
584. Li, J., H.-C. Hsu, and J.D. Mountz, *Managing macrophages in rheumatoid arthritis by reform or removal.* Current rheumatology reports, 2012. **14**(5): p. 445-454.
585. Lombardo, E., et al., *TLR4-mediated survival of macrophages is MyD88 dependent and requires TNF-α autocrine signalling.* The Journal of Immunology, 2007. **178**(6): p. 3731-3739.
586. Kimura, F., *Granulocyte-macrophage colony-stimulating factor (GM-CSF).* Nihon rinsho. Japanese journal of clinical medicine, 1999. **57**: p. 762.
587. Sica, A. and A. Mantovani, *Macrophage plasticity and polarization: in vivo veritas.* The Journal of clinical investigation, 2012. **122**(3): p. 787.
588. Smith, T.D., et al., *Regulation of macrophage polarization and plasticity by complex activation signals.* Integrative Biology, 2016. **8**(9): p. 946-955.

589. Stout, R.D., S.K. Watkins, and J. Suttles, *Functional plasticity of macrophages: in situ reprogramming of tumor-associated macrophages.* Journal of leukocyte biology, 2009. **86**(5): p. 1105-1109.
590. Martinez, F.O., et al., *Transcriptional profiling of the human monocyte-to-macrophage differentiation and polarization: new molecules and patterns of gene expression.* J Immunol, 2006. **177**(10): p. 7303-11.
591. Xu, W., et al., *Reversible differentiation of pro-and anti-inflammatory macrophages.* Molecular immunology, 2013. **53**(3): p. 179-186.
592. Xue, J., et al., *Transcriptome-based network analysis reveals a spectrum model of human macrophage activation.* Immunity, 2014. **40**(2): p. 274-288.
593. Major, J., J.E. Fletcher, and T.A. Hamilton, *IL-4 pretreatment selectively enhances cytokine and chemokine production in lipopolysaccharide-stimulated mouse peritoneal macrophages.* The Journal of Immunology, 2002. **168**(5): p. 2456-2463.
594. Roy, S., et al., *Interleukin-4 regulates macrophage interleukin-12 protein synthesis through a c-fos mediated mechanism.* Surgery, 2000. **128**(2): p. 219-224.
595. Varin, A., et al., *Alternative activation of macrophages by IL-4 impairs phagocytosis of pathogens but potentiates microbial-induced signalling and cytokine secretion.* Blood, 2010. **115**(2): p. 353-362.
596. Watkins, S.K., et al., *IL-12 rapidly alters the functional profile of tumor-associated and tumor-infiltrating macrophages in vitro and in vivo.* The Journal of Immunology, 2007. **178**(3): p. 1357-1362.
597. Duluc, D., et al., *Interferon-γ reverses the immunosuppressive and protumoral properties and prevents the generation of human tumor-associated macrophages.* International journal of cancer, 2009. **125**(2): p. 367-373.
598. Tang, X., et al., *Anti-tumour strategies aiming to target tumour-associated macrophages.* Immunology, 2013. **138**(2): p. 93-104.
599. Hagemann, T., et al., *"Re-educating" tumor-associated macrophages by targeting NF-κB.* Journal of Experimental Medicine, 2008. **205**(6): p. 1261-1268.
600. Cluff, C.W., *Monophosphoryl lipid A (MPL) as an adjuvant for anti-cancer vaccines: clinical results*, in *Lipid A in Cancer Therapy*. 2009, Springer. p. 111-123.
601. Smith, K., S. Hamza, and H. Skelton, *Topical imidazoquinoline therapy of cutaneous squamous cell carcinoma polarizes lymphoid and monocyte/macrophage populations to a Th1 and M1 cytokine pattern.* Clinical and experimental dermatology, 2004. **29**(5): p. 505-512.
602. Wolfs, I.M., et al., *Reprogramming macrophages to an anti-inflammatory phenotype by helminth antigens reduces murine atherosclerosis.* The FASEB Journal, 2014. **28**(1): p. 288-299.
603. Chana, K.K., et al., *Lack of macrophage plasticity in COPD*. 2012, Eur Respiratory Soc.
604. Genin, M., et al., *M1 and M2 macrophages derived from THP-1 cells differentially modulate the response of cancer cells to etoposide.* BMC cancer, 2015. **15**(1): p. 577.
605. Tsukada, K., et al., *Macrophage tolerance induced by stimulation with Toll-like receptor 7/8 ligands.* Immunology letters, 2007. **111**(1): p. 51-56.

606. Nahid, M.A., et al., *TLR4, TLR7/8 agonist-induced miR-146a promotes macrophage tolerance to MyD88-dependent TLR agonists.* Journal of leukocyte biology, 2016. **100**(2): p. 339-349.
607. Liu, Y.-C., et al., *Macrophage polarization in inflammatory diseases.* International journal of biological sciences, 2014. **10**(5): p. 520.
608. Mackaness, G., *The influence of immunologically committed lymphoid cells on macrophage activity in vivo.* Journal of experimental medicine, 1969. **129**(5): p. 973-992.
609. Töpfer, E., D. Boraschi, and P. Italiani, *Innate immune memory: the latest frontier of adjuvanticity.* Journal of immunology research, 2015. **2015**.
610. Saeed, S., et al., *Epigenetic programming of monocyte-to-macrophage differentiation and trained innate immunity.* Science, 2014. **345**(6204): p. 1251086.
611. Foster, S.L., D.C. Hargreaves, and R. Medzhitov, *Gene-specific control of inflammation by TLR-induced chromatin modifications.* Nature, 2007. **447**(7147): p. 972-978.
612. Quintin, J., et al., *Candida albicans infection affords protection against reinfection via functional reprogramming of monocytes.* Cell host & microbe, 2012. **12**(2): p. 223-232.
613. Schön, M. and M. Schön, *TLR7 and TLR8 as targets in cancer therapy.* Oncogene, 2008. **27**(2): p. 190-199.
614. Weeratna, R.D., et al., *TLR agonists as vaccine adjuvants: comparison of CpG ODN and Resiquimod (R-848).* Vaccine, 2005. **23**(45): p. 5263-5270.
615. Doyle, S.L. and L.A. O'Neill, *Toll-like receptors: from the discovery of NFκB to new insights into transcriptional regulations in innate immunity.* Biochemical pharmacology, 2006. **72**(9): p. 1102-1113.
616. Roses, R.E., et al., *Differential production of IL-23 and IL-12 by myeloid-derived dendritic cells in response to TLR agonists.* The Journal of Immunology, 2008. **181**(7): p. 5120-5127.
617. Overgaard, J., et al., *Five compared with six fractions per week of conventional radiotherapy of squamous-cell carcinoma of head and neck: DAHANCA 6&7 randomised controlled trial.* The Lancet, 2003. **362**(9388): p. 933-940.
618. Pierce, S.M., et al., *Long-term radiation complications following conservative surgery (CS) and radiation therapy (RT) in patients with early stage breast cancer.* International Journal of Radiation Oncology* Biology* Physics, 1992. **23**(5): p. 915-923.
619. Gaspar, L.E., et al., *The role of whole brain radiation therapy in the management of newly diagnosed brain metastases: a systematic review and evidence-based clinical practice guideline.* Journal of neuro-oncology, 2010. **96**(1): p. 17-32.
620. Bauer, M., et al., *Human monocytes are severely impaired in base and DNA double-strand break repair that renders them vulnerable to oxidative stress.* Proceedings of the National Academy of Sciences, 2011. **108**(52): p. 21105-21110.
621. Lee, Y., et al., *Therapeutic effects of ablative radiation on local tumor require CD8+ T cells: changing strategies for cancer treatment.* Blood, 2009. **114**(3): p. 589-595.

622. Shiao, S.L. and L.M. Coussens, *The tumor-immune microenvironment and response to radiation therapy.* Journal of mammary gland biology and neoplasia, 2010. **15**(4): p. 411-421.
623. Teitz-Tennenbaum, S., et al., *Mechanisms involved in radiation enhancement of intratumoral dendritic cell therapy.* Journal of immunotherapy (Hagerstown, Md.: 1997), 2008. **31**(4): p. 345.
624. Chakraborty, M., et al., *Irradiation of tumor cells up-regulates Fas and enhances CTL lytic activity and CTL adoptive immunotherapy.* The Journal of Immunology, 2003. **170**(12): p. 6338-6347.
625. Ifeadi, V. and C. Garnett-Benson, *Sub-lethal irradiation of human colorectal tumor cells imparts enhanced and sustained susceptibility to multiple death receptor signaling pathways.* PloS one, 2012. **7**(2): p. e31762.
626. Vereecque, R., et al., *γ- Ray irradiation induces B7. 1 expression in myeloid leukaemic cells.* British journal of haematology, 2000. **108**(4): p. 825-831.
627. Baluna, R.G., T.Y. Eng, and C.R. Thomas Jr, *Adhesion molecules in radiotherapy.* Radiation research, 2006. **166**(6): p. 819-831.
628. GAUGLER, C.S., A. VAN DER MEEREN, J.-M. BERTHO, M. VANDAMME and M.-H. M.-A. MOUTHON, *Late and persistent up-regulation of intercellular adhesion molecule-1 (ICAM-1) expression by ionizing radiation in human endothelial cells in vitro.* International journal of radiation biology, 1997. **72**(2): p. 201-209.
629. Zamai, L., et al., *Lymphocyte binding to K562 cells: effect of target cell irradiation and correlation with ICAM-1 and LFA-3 expression.* Eur J Histochem, 1994. **38**(Suppl 1): p. 53-60.
630. Ehlers, G. and M. Fridman, *Abscopal effect of radiation in papillary adenocarcinoma.* The British journal of radiology, 1973. **46**(543): p. 220-222.
631. Ohba, K., et al., *Abscopal regression of hepatocellular carcinoma after radiotherapy for bone metastasis.* Gut, 1998. **43**(4): p. 575-577.
632. Rees, G. and C. Ross, *Abscopal regression following radiotherapy for adenocarcinoma.* The British journal of radiology, 1983. **56**(661): p. 63-66.
633. Wersäll, P.J., et al., *Regression of non-irradiated metastases after extracranial stereotactic radiotherapy in metastatic renal cell carcinoma.* Acta oncologica, 2006. **45**(4): p. 493-497.
634. Chang, M.-C., J.C.-H. Cheng, and Y.-C. Chiang, *Local irradiation promotes antitumor immunity and abscopal effect of antigen-specific immunotherapy by enhancing dendritic cell maturation*. 2017, AACR.
635. Min, Y., et al., *Antigen-capturing nanoparticles improve the abscopal effect and cancer immunotherapy.* Nature nanotechnology, 2017. **12**(9): p. 877.
636. Teitz-Tennenbaum, S., et al., *Radiotherapy potentiates the therapeutic efficacy of intratumoral dendritic cell administration.* Cancer research, 2003. **63**(23): p. 8466-8475.
637. Kim, K.W., et al., *Direct injection of immature dendritic cells into irradiated tumor induces efficient antitumor immunity.* International journal of cancer, 2004. **109**(5): p. 685-690.
638. Mason, K.A., et al., *Targeting toll-like receptor 9 with CpG oligodeoxynucleotides enhances tumor response to fractionated radiotherapy.* Clinical Cancer Research, 2005. **11**(1): p. 361-369.
639. Milas, L., et al., *CpG oligodeoxynucleotide enhances tumor response to radiation.* Cancer research, 2004. **64**(15): p. 5074-5077.

640. Zhang, H., et al., *An in situ autologous tumor vaccination with combined radiation therapy and TLR9 agonist therapy.* PloS one, 2012. **7**(5): p. e38111.
641. Chakraborty, M., et al., *External beam radiation of tumors alters phenotype of tumor cells to render them susceptible to vaccine-mediated T-cell killing.* Cancer research, 2004. **64**(12): p. 4328-4337.
642. Demaria, S., et al., *Immune-mediated inhibition of metastases after treatment with local radiation and CTLA-4 blockade in a mouse model of breast cancer.* Clinical Cancer Research, 2005. **11**(2): p. 728-734.
643. Dewan, M.Z., et al., *Fractionated but not single-dose radiotherapy induces an immune-mediated abscopal effect when combined with anti–CTLA-4 antibody.* Clinical Cancer Research, 2009. **15**(17): p. 5379-5388.
644. Formenti, S.C. and S. Demaria, *Combining radiotherapy and cancer immunotherapy: a paradigm shift.* JNCI: Journal of the National Cancer Institute, 2013. **105**(4): p. 256-265.
645. Duru, N., B. Wolfson, and Q. Zhou, *Mechanisms of the alternative activation of macrophages and non-coding RNAs in the development of radiation-induced lung fibrosis.* World journal of biological chemistry, 2016. **7**(4): p. 231.
646. Pinto, A.T., et al., *Ionizing radiation modulates human macrophages towards a pro-inflammatory phenotype preserving their pro-invasive and pro-angiogenic capacities.* Scientific reports, 2016. **6**: p. 18765.
647. Crittenden, M.R., et al., *Expression of NF-κB p50 in tumor stroma limits the control of tumors by radiation therapy.* PloS one, 2012. **7**(6): p. e39295.
648. Solinas, G., et al., *Tumor-associated macrophages (TAM) as major players of the cancer-related inflammation.* Journal of leukocyte biology, 2009. **86**(5): p. 1065-1073.
649. Chanmee, T., et al., *Tumor-associated macrophages as major players in the tumor microenvironment.* Cancers, 2014. **6**(3): p. 1670-1690.
650. Sica, A., et al. *Macrophage polarization in tumour progression*. in *Seminars in cancer biology*. 2008. Elsevier.
651. Van Overmeire, E., et al., *M-CSF and GM-CSF receptor signaling differentially regulate monocyte maturation and macrophage polarization in the tumor microenvironment.* Cancer research, 2016. **76**(1): p. 35-42.
652. Pinto, A.T., et al., *Intricate Macrophage-Colorectal Cancer Cell Communication in Response to Radiation.* PloS one, 2016. **11**(8): p. e0160891.
653. Seifert, L., et al., *Radiation therapy induces macrophages to suppress T-cell responses against pancreatic tumors in mice.* Gastroenterology, 2016. **150**(7): p. 1659-1672. e5.
654. Ben-Baruch, A. *Inflammation-associated immune suppression in cancer: the roles played by cytokines, chemokines and additional mediators*. in *Seminars in cancer biology*. 2006. Elsevier.
655. Hao, N.-B., et al., *Macrophages in tumor microenvironments and the progression of tumors.* Clinical and Developmental Immunology, 2012. **2012**.
656. Kurte, M., et al., *A synthetic peptide homologous to functional domain of human IL-10 down-regulates expression of MHC class I and transporter associated with antigen processing 1/2 in human melanoma cells.* The Journal of Immunology, 2004. **173**(3): p. 1731-1737.
657. Erdman, S.E. and T. Poutahidis, *Roles for inflammation and regulatory T cells in colon cancer.* Toxicologic pathology, 2010. **38**(1): p. 76-87.

658. Schoppmann, S.F., et al., *Tumor-associated macrophages express lymphatic endothelial growth factors and are related to peritumoral lymphangiogenesis.* The American journal of pathology, 2002. **161**(3): p. 947-956.
659. Ong, S.M., et al., *Macrophages in human colorectal cancer are pro-inflammatory and prime T cells towards an anti-tumour type-1 inflammatory response.* European journal of immunology, 2012. **42**(1): p. 89-100.
660. Teng, M.W., et al., *Opposing roles for IL-23 and IL-12 in maintaining occult cancer in an equilibrium state.* Cancer research, 2012. **72**(16): p. 3987-3996.
661. Yuan, A., et al., *Opposite effects of M1 and M2 macrophage subtypes on lung cancer progression.* Scientific reports, 2015. **5**.
662. Helm, O., et al., *M1 and M2: there is no "good" and "bad"—How macrophages promote malignancy-associated features in tumorigenesis.* Oncoimmunology, 2014. **3**(7): p. e946818.
663. Wang, N., H. Liang, and K. Zen, *Molecular mechanisms that influence the macrophage m1–m2 polarization balance.* Frontiers in immunology, 2014. **5**.
664. Rodríguez, D., et al., *Tumor microenvironment profoundly modifies functional status of macrophages: peritoneal and tumor-associated macrophages are two very different subpopulations.* Cellular immunology, 2013. **283**(1): p. 51-60.
665. Nielsen, S.R. and M.C. Schmid, *Macrophages as Key Drivers of Cancer Progression and Metastasis.* Mediators of inflammation, 2017. **2017**.
666. Olmes, G., et al., *CD163+ M2c-like macrophages predominate in renal biopsies from patients with lupus nephritis.* Arthritis research & therapy, 2016. **18**(1): p. 90.
667. Liu, C., et al., *Interleukin-23A is associated with tumor growth in Helicobacter-pylori-related human gastric cancer.* Cancer cell international, 2014. **14**(1): p. 104.
668. Kortylewski, M., et al., *Regulation of the IL-23 and IL-12 balance by Stat3 signaling in the tumor microenvironment.* Cancer cell, 2009. **15**(2): p. 114-123.
669. Frischholz, B., et al., *Reduced secretion of the inflammatory cytokine IL-1β by stimulated peritoneal macrophages of radiosensitive Balb/c mice after exposure to 0.5 or 0.7 Gy of ionizing radiation.* Autoimmunity, 2013. **46**(5): p. 323-328.
670. Tsukimoto, M., et al., *0.5 Gy gamma radiation suppresses production of TNF-α through up-regulation of MKP-1 in mouse macrophage RAW264. 7 cells.* Radiation research, 2009. **171**(2): p. 219-224.
671. Rubin, P., et al., *A perpetual cascade of cytokines postirradiation leads to pulmonary fibrosis.* International Journal of Radiation Oncology* Biology* Physics, 1995. **33**(1): p. 99-109.
672. Gough, M.J., K. Young, and M. Crittenden, *The impact of the myeloid response to radiation therapy.* Clinical and Developmental Immunology, 2013. **2013**.
673. Hickson, I., et al., *Identification and characterization of a novel and specific inhibitor of the ataxia-telangiectasia mutated kinase ATM.* Cancer research, 2004. **64**(24): p. 9152-9159.
674. Lee, J.W., et al., *A novel small-molecule inhibitor targeting CREB-CBP complex possesses anti-cancer effects along with cell cycle regulation, autophagy suppression and endoplasmic reticulum stress.* PloS one, 2015. **10**(4): p. e0122628.

675. Ngiow, S.F., M.W. Teng, and M.J. Smyth, *A balance of interleukin-12 and -23 in cancer.* Trends Immunol, 2013. **34**(11): p. 548-55.
676. Aliper, A.M., et al., *A role for G- CSF and GM- CSF in nonmyeloid cancers.* Cancer medicine, 2014. **3**(4): p. 737-746.
677. Gupta, R. and L.A. Emens, *GM-CSF-secreting vaccines for solid tumors: moving forward.* Discovery medicine, 2010. **10**(50): p. 52.
678. Hong, I.-S., *Stimulatory versus suppressive effects of GM-CSF on tumor progression in multiple cancer types.* Experimental & molecular medicine, 2016. **48**(7): p. e242.
679. Nebiker, C., J. Han, and S. Castori-Eppenberger, *GM-CSF production by tumor cells is associated with improved.*
680. Uemura, Y., et al., *Effects of GM-CSF and M-CSF on tumor progression of lung cancer: roles of MEK1/ERK and AKT/PKB pathways.* International journal of molecular medicine, 2006. **18**(2): p. 365-373.
681. Chiang, C.-S., et al., *Irradiation promotes an m2 macrophage phenotype in tumor hypoxia.* Frontiers in oncology, 2012. **2**.
682. Lavin, M.F. and S. Kozlov, *ATM activation and DNA damage response.* Cell cycle, 2007. **6**(8): p. 931-942.
683. Shao, L., et al., *Deficiency of the DNA repair enzyme ATM in rheumatoid arthritis.* Journal of Experimental Medicine, 2009. **206**(6): p. 1435-1449.
684. Westbrook, A.M. and R.H. Schiestl, *Atm-deficient mice exhibit increased sensitivity to dextran sulfate sodium–induced colitis characterized by elevated DNA damage and persistent immune activation.* Cancer research, 2010. **70**(5): p. 1875-1884.
685. Petersen, A.J., S.A. Rimkus, and D.A. Wassarman, *ATM kinase inhibition in glial cells activates the innate immune response and causes neurodegeneration in Drosophila.* Proceedings of the National Academy of Sciences, 2012. **109**(11): p. E656-E664.
686. Rodier, F., et al., *Persistent DNA damage signalling triggers senescence-associated inflammatory cytokine secretion.* Nature cell biology, 2009. **11**(8): p. 973-979.
687. Pereira-Lopes, S., et al., *NBS1 is required for macrophage homeostasis and functional activity in mice.* Blood, 2015. **126**(22): p. 2502-2510.
688. Colonna, M., *DNA damage response impacts macrophage functions.* Blood, 2015. **126**(22): p. 2440-2442.
689. Mayr, B. and M. Montminy, *Transcriptional regulation by the phosphorylation-dependent factor CREB.* Nature reviews Molecular cell biology, 2001. **2**(8): p. 599-609.
690. Lonze, B.E. and D.D. Ginty, *Function and regulation of CREB family transcription factors in the nervous system.* Neuron, 2002. **35**(4): p. 605-623.
691. Gravis, D. and A. Stick, *Novel Regulation of CREB Activation by Reactive Oxygen Species in B Lymphocytes.* The FASEB Journal, 2011. **25**(1 Supplement): p. lb129-lb129.
692. Luan, B., et al., *CREB pathway links PGE2 signaling with macrophage polarization.* Proceedings of the National Academy of Sciences, 2015. **112**(51): p. 15642-15647.
693. Ma, X., T. Aoki, and S. Narumiya, *Prostaglandin E2-EP4 signaling persistently amplifies CD40-mediated induction of IL-23 p19 expression through canonical and non-canonical NF-κB pathways.* Cellular & molecular immunology, 2016. **13**(2): p. 240-250.

694. Ghosh, C.C., et al., *Gene-specific repression of proinflammatory cytokines in stimulated human macrophages by nuclear IκBα.* The Journal of Immunology, 2010. **185**(6): p. 3685-3693.
695. Karthaus, N., R. Torensma, and J. Tel, *Deciphering the message broadcast by tumor-infiltrating dendritic cells.* The American journal of pathology, 2012. **181**(3): p. 733-742.
696. Harimoto, H., et al., *Inactivation of tumor-specific CD8+ CTLs by tumor-infiltrating tolerogenic dendritic cells.* Immunology and cell biology, 2013. **91**(9): p. 545-555.
697. Krempski, J., et al., *Tumor-infiltrating programmed death receptor-1+ dendritic cells mediate immune suppression in ovarian cancer.* The Journal of Immunology, 2011. **186**(12): p. 6905-6913.
698. Watkins, S.K., et al., *FOXO3 programs tumor-associated DCs to become tolerogenic in human and murine prostate cancer.* The Journal of clinical investigation, 2011. **121**(4): p. 1361.
699. Curiel, T.J., et al., *Blockade of B7-H1 improves myeloid dendritic cell–mediated antitumor immunity.* Nature medicine, 2003. **9**(5): p. 562-567.
700. Liu, Q., et al., *Tumor-educated CD11bhighIalow regulatory dendritic cells suppress T cell response through arginase I.* The Journal of Immunology, 2009. **182**(10): p. 6207-6216.
701. Scarlett, U.K., et al., *Ovarian cancer progression is controlled by phenotypic changes in dendritic cells.* Journal of Experimental Medicine, 2012: p. jem. 20111413.
702. Janco, J.M.T., et al., *Tumor-infiltrating dendritic cells in cancer pathogenesis.* The Journal of Immunology, 2015. **194**(7): p. 2985-2991.
703. Kocián, P., et al., *Tumor-infiltrating lymphocytes and dendritic cells in human colorectal cancer: their relationship to KRAS mutational status and disease recurrence.* Human immunology, 2011. **72**(11): p. 1022-1028.
704. Kawarada, Y., et al., *NK-and CD8+ T cell-mediated eradication of established tumors by peritumoral injection of CpG-containing oligodeoxynucleotides.* The Journal of Immunology, 2001. **167**(9): p. 5247-5253.
705. Heckelsmiller, K., et al., *Peritumoral CpG DNA elicits a coordinated response of CD8 T cells and innate effectors to cure established tumors in a murine colon carcinoma model.* The Journal of Immunology, 2002. **169**(7): p. 3892-3899.
706. Nikitina, E.Y. and D.I. Gabrilovich, *Combination of γ- irradiation and dendritic cell administration induces a potent antitumor response in tumor-bearing mice: approach to treatment of advanced stage cancer.* International journal of cancer, 2001. **94**(6): p. 825-833.
707. Merrick, A., et al., *Immunosuppressive effects of radiation on human dendritic cells: reduced IL-12 production on activation and impairment of naive T-cell priming.* British journal of cancer, 2005. **92**(8): p. 1450-1458.
708. Liu, X.-D., S.-M. Ma, and S.-Z. Liu, *Effects of 0.075 Gy x-ray irradiation on the expression of IL-10 and IL-12 in mice.* Physics in medicine and biology, 2003. **48**(13): p. 2041.
709. Cao, M.-D., Z.-D. Chen, and Y. Xing, *Gamma irradiation of human dendritic cells influences proliferation and cytokine profile of T cells in autologous mixed lymphocyte reaction.* Cell biology international, 2004. **28**(3): p. 223-228.

710. Kocieda, V.P., et al., *Prostaglandin E2-induced IL-23p19 subunit is regulated by cAMP-responsive element-binding protein and C/AATT enhancer-binding protein β in bone marrow-derived dendritic cells.* Journal of Biological Chemistry, 2012. **287**(44): p. 36922-36935.
711. Timmerman, M., John M and M. Levy, Ronald, *Dendritic cell vaccines for cancer immunotherapy.* Annual review of medicine, 1999. **50**(1): p. 507-529.
712. Carl Jr, J.W. and X.-F. Bai, *IL27: its roles in the induction and inhibition of inflammation.* International journal of clinical and experimental pathology, 2008. **1**(2): p. 117.
713. Shigematsu, A., et al., *Effects of low-dose irradiation on enhancement of immunity by dendritic cells.* Journal of radiation research, 2007. **48**(1): p. 51-55.
714. Tomimatsu, N., B. Mukherjee, and S. Burma, *Distinct roles of ATR and DNA-PKcs in triggering DNA damage responses in ATM-deficient cells.* EMBO reports, 2009. **10**(6): p. 629-635.
715. McKinnon, P.J., *ATM and ataxia telangiectasia.* EMBO reports, 2004. **5**(8): p. 772-776.
716. Shi, Y., et al., *Direct regulation of CREB transcriptional activity by ATM in response to genotoxic stress.* Proc Natl Acad Sci U S A, 2004. **101**(16): p. 5898-903.
717. Bauer, M., et al., *Human monocytes are severely impaired in base and DNA double-strand break repair that renders them vulnerable to oxidative stress.* Proc Natl Acad Sci U S A, 2011. **108**(52): p. 21105-10.
718. Durackova, Z., *Some current insights into oxidative stress.* Physiol Res, 2010. **59**(4): p. 459-69.
719. Moynahan, M.E. and M. Jasin, *Mitotic homologous recombination maintains genomic stability and suppresses tumorigenesis.* Nat Rev Mol Cell Biol, 2010. **11**(3): p. 196-207.
720. Erez, N., et al., *Cancer-associated fibroblasts are activated in incipient neoplasia to orchestrate tumor-promoting inflammation in an NF-κB-dependent manner.* Cancer cell, 2010. **17**(2): p. 135-147.
721. De Monte, L., et al., *Intratumor T helper type 2 cell infiltrate correlates with cancer-associated fibroblast thymic stromal lymphopoietin production and reduced survival in pancreatic cancer.* Journal of Experimental Medicine, 2011. **208**(3): p. 469-478.
722. Gobert, M., et al., *Regulatory T cells recruited through CCL22/CCR4 are selectively activated in lymphoid infiltrates surrounding primary breast tumors and lead to an adverse clinical outcome.* Cancer research, 2009. **69**(5): p. 2000-2009.
723. Fialová, A., et al., *Dynamics of T-cell infiltration during the course of ovarian cancer: The gradual shift from a Th17 effector cell response to a predominant infiltration by regulatory T-cells.* International journal of cancer, 2013. **132**(5): p. 1070-1079.
724. Comito, G., et al., *Cancer-associated fibroblasts and M2-polarized macrophages synergize during prostate carcinoma progression.* Oncogene, 2014. **33**(19): p. 2423-2431.
725. Kitamura, H., et al., *Mouse and human lung fibroblasts regulate dendritic cell trafficking, airway inflammation, and fibrosis through integrin αvβ8–mediated activation of TGF-β.* The Journal of clinical investigation, 2011. **121**(7): p. 2863-2875.

726. Saalbach, A., et al., *Dermal fibroblasts promote the migration of dendritic cells.* Journal of Investigative Dermatology, 2010. **130**(2): p. 444-454.
727. Schirmer, C., et al., *Human fibroblasts support the expansion of IL-17–producing T cells via up-regulation of IL-23 production by dendritic cells.* Blood, 2010. **116**(10): p. 1715-1725.
728. Bell, D., et al., *In breast carcinoma tissue, immature dendritic cells reside within the tumor, whereas mature dendritic cells are located in peritumoral areas.* Journal of Experimental Medicine, 1999. **190**(10): p. 1417-1426.
729. Middel, P., et al., *Chemokine-mediated distribution of dendritic cell subsets in renal cell carcinoma.* BMC cancer, 2010. **10**(1): p. 578.
730. Peiris-Pagès, M., F. Sotgia, and M.P. Lisanti, *Chemotherapy induces the cancer-associated fibroblast phenotype, activating paracrine Hedgehog-GLI signalling in breast cancer cells.* Oncotarget, 2015. **6**(13): p. 10728.
731. Di Leonardo, A., et al., *DNA damage triggers a prolonged p53-dependent G1 arrest and long-term induction of Cip1 in normal human fibroblasts.* Genes & development, 1994. **8**(21): p. 2540-2551.
732. Papadopoulou, A. and D. Kletsas, *Human lung fibroblasts prematurely senescent after exposure to ionizing radiation enhance the growth of malignant lung epithelial cells in vitro and in vivo.* International journal of oncology, 2011. **39**(4): p. 989.
733. Brach, M., et al., *Ionizing radiation induces expression of interleukin 6 by human fibroblasts involving activation of nuclear factor-kappa B.* Journal of Biological Chemistry, 1993. **268**(12): p. 8466-8472.
734. Cohen, N., et al., *Fibroblasts drive an immunosuppressive and growth-promoting microenvironment in breast cancer via secretion of Chitinase 3-like 1.* Oncogene, 2017.
735. Hashimoto, O., et al., *Collaboration of cancer‐associated fibroblasts and tumour‐associated macrophages for neuroblastoma development.* The Journal of pathology, 2016. **240**(2): p. 211-223.
736. Bukata, S.V., et al., *PGE2 and IL‐6 production by fibroblasts in response to titanium wear debris particles is mediated through a Cox‐2 dependent pathway.* Journal of orthopaedic research, 2004. **22**(1): p. 6-12.
737. Cho, E.-A., et al., *cAMP signaling inhibits radiation-induced ATM phosphorylation leading to the augmentation of apoptosis in human lung cancer cells.* Molecular cancer, 2014. **13**(1): p. 36.
738. Sugita, K., et al., *Innate immunity mediated by epidermal keratinocytes promotes acquired immunity involving Langerhans cells and T cells in the skin.* Clinical & Experimental Immunology, 2007. **147**(1): p. 176-183.
739. He, Z., Y. Zhu, and H. Jiang, *Toll-like receptor 4 mediates lipopolysaccharide-induced collagen secretion by phosphoinositide3-kinase-Akt pathway in fibroblasts during acute lung injury.* Journal of Receptors and Signal Transduction, 2009. **29**(2): p. 119-125.
740. McGettrick, H.M., et al., *Fibroblasts from different sites may promote or inhibit recruitment of flowing lymphocytes by endothelial cells.* European journal of immunology, 2009. **39**(1): p. 113-125.
741. Pavlovic, S., et al., *Targeting prostaglandin E2 receptors as an alternative strategy to block cyclooxygenase-2-dependent extracellular matrix-induced matrix metalloproteinase-9 expression by macrophages.* Journal of Biological Chemistry, 2006. **281**(6): p. 3321-3328.

742. Schröder, N.W. and R.R. Schumann, *Single nucleotide polymorphisms of Toll-like receptors and susceptibility to infectious disease.* The Lancet infectious diseases, 2005. **5**(3): p. 156-164.
743. Pourzarandian, A., et al., *Er: YAG laser irradiation increases prostaglandin E2 production via the induction of cyclooxygenase‐2 mRNA in human gingival fibroblasts.* Journal of periodontal research, 2005. **40**(2): p. 182-186.
744. Sakurai, Y., M. Yamaguchi, and Y. Abiko, *Inhibitory effect of low‐level laser irradiation on LPS‐stimulated prostaglandin E2 production and cyclooxygenase‐2 in human gingival fibroblasts.* European journal of oral sciences, 2000. **108**(1): p. 29-34.
745. Chang, H.Y., et al., *Diversity, topographic differentiation, and positional memory in human fibroblasts.* Proceedings of the National Academy of Sciences, 2002. **99**(20): p. 12877-12882.
746. Rübe, C.E., et al., *Dose-dependent induction of transforming growth factor β (TGF-β) in the lung tissue of fibrosis-prone mice after thoracic irradiation.* International Journal of Radiation Oncology* Biology* Physics, 2000. **47**(4): p. 1033-1042.
747. Chizzolini, C., et al., *Prostaglandin E 2 synergistically with interleukin-23 favors human Th17 expansion.* Blood, 2008. **112**(9): p. 3696-3703.
748. Yao, C., et al., *Prostaglandin E2–EP4 signaling promotes immune inflammation through Th1 cell differentiation and Th17 cell expansion.* Nature medicine, 2009. **15**(6): p. 633-640.
749. Krtolica, A., et al., *Senescent fibroblasts promote epithelial cell growth and tumorigenesis: a link between cancer and aging.* Proceedings of the National Academy of Sciences, 2001. **98**(21): p. 12072-12077.
750. Sharma, P., et al., *Primary, adaptive, and acquired resistance to cancer immunotherapy.* Cell, 2017. **168**(4): p. 707-723.
751. Gotwals, P., et al., *Prospects for combining targeted and conventional cancer therapy with immunotherapy.* Nature Reviews Cancer, 2017. **17**(5): p. 286-301.
752. Petty, A.J. and Y. Yang, *Tumor-associated macrophages: implications in cancer immunotherapy.* Immunotherapy, 2017. **9**(3): p. 289-302.
753. Rosenberg, S.A., J.C. Yang, and N.P. Restifo, *Cancer immunotherapy: moving beyond current vaccines.* Nat Med, 2004. **10**(9): p. 909-15.
754. Palucka, K. and J. Banchereau, *Cancer immunotherapy via dendritic cells.* Nat Rev Cancer, 2012. **12**(4): p. 265-77.
755. Tarazona, R., et al., *Immunosenescence: limitations of natural killer cell-based cancer immunotherapy.* Cancer immunology, immunotherapy: CII, 2017. **66**(2): p. 233-245.
756. Kolb, H. and M. von Herrath, *Immunotherapy for type 1 diabetes: Why do current protocols not halt the underlying disease process?* Cell metabolism, 2017. **25**(2): p. 233-241.
757. Yeo, L. and M. Peakman, *Antigen-specific immunotherapy and influenza vaccination in type 1 diabetes: timing is everything.* Diabetologia, 2017: p. 1-5.
758. Schutt, C., H.E. Gendelman, and R.L. Mosley, *Immunotherapies for Movement Disorders: Parkinson's Disease and Amyotrophic Lateral Sclerosis*, in *Neuroimmune Pharmacology*. 2017, Springer. p. 767-797.
759. Stamou, P., et al., *Simple in vitro generation of human leukocyte antigen-G–expressing T-regulatory cells through pharmacological hypomethylation for*

adoptive cellular immunotherapy against graft-versus-host disease. Cytotherapy, 2017. **19**(4): p. 521-530.
760. Villanueva, M.T., *Immunotherapy: Macrophages steal the show.* Nature Reviews Cancer, 2017. **17**(7): p. 396-397.
761. Arlauckas, S.P., et al., *In vivo imaging reveals a tumor-associated macrophage–mediated resistance pathway in anti–PD-1 therapy.* Science translational medicine, 2017. **9**(389): p. eaal3604.
762. Gordon, S.R., et al., *PD-1 expression by tumour-associated macrophages inhibits phagocytosis and tumour immunity.* Nature, 2017. **545**(7655): p. 495-499.
763. Goodridge, H.S., et al., *Harnessing the beneficial heterologous effects of vaccination.* Nature Reviews Immunology, 2016. **16**(6): p. 392-400.
764. Levy, O. and M.G. Netea, *Innate immune memory: implications for development of pediatric immunomodulatory agents and adjuvanted vaccines.* Pediatric research, 2013. **75**(1-2): p. 184-188.
765. Liao, Y.P., et al., *Ionizing radiation affects human MART-1 melanoma antigen processing and presentation by dendritic cells.* J Immunol, 2004. **173**(4): p. 2462-9.
766. Gasse, P., et al., *IL-1 and IL-23 mediate early IL-17A production in pulmonary inflammation leading to late fibrosis.* PLoS One, 2011. **6**(8): p. e23185.
767. Wang, B.Z., et al., *Interleukin-17A antagonist attenuates radiation-induced lung injuries in mice.* Exp Lung Res, 2014. **40**(2): p. 77-85.
768. Wilson, M.S., et al., *Bleomycin and IL-1beta-mediated pulmonary fibrosis is IL-17A dependent.* J Exp Med, 2010. **207**(3): p. 535-52.
769. Harbort, C.J., et al., *Neutrophil oxidative burst activates ATM to regulate cytokine production and apoptosis.* Blood, 2015. **126**(26): p. 2842-51.
770. Insel, P.A., et al., *cAMP and Epac in the regulation of tissue fibrosis.* British Journal of Pharmacology, 2012. **166**(2): p. 447-456.
771. Kawamatawong, T., *Roles of roflumilast, a selective phosphodiesterase 4 inhibitor, in airway diseases.* Journal of thoracic disease, 2017. **9**(4): p. 1144.
772. Togo, S., et al., *PDE4 inhibitors roflumilast and rolipram augment PGE2 inhibition of TGF-{beta}1-stimulated fibroblasts.* Am J Physiol Lung Cell Mol Physiol, 2009. **296**(6): p. L959-69.