The novel roles of DNA damage repair proteins ATM and DNA-PK in the regulation of the ER stress response and IL-23 expression in dendritic cells

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Abstract

Dendritic cells (DC) are the principal, cross-priming antigen-presenting cell responsible for naïve T cell activation and differentiation. Specific programmes of cytokine secretion by DC are particularly important for the polarisation of CD4+ T-cells to discrete effector populations i.e. Th1, Th17, Tr1 etc. Our knowledge of control for some of these cytokine signals is currently limited and modulating the polarisation of defined Th-subsets remains a challenge. Improved control of Th-differentiation has considerable potential in the fields of immune disorders and infectious disease. We describe a novel immune-regulatory function of two DNA-repair proteins signal to modulate IL-23 secretion, a key signal for Th17 differentiation.

ATM and DNA-PK, are two protein kinases classically regarded for their paramount importance in repair of DNA double strand breaks. We characterised an alternate immune-regulatory function for ATM and DNA-PK for IL-23 production by DC. Therefore, constitutive expression of activated ATM and DNA-PK in DC, but not in CD14+ monocytes, related not only to their ability to repair damaged DNA, but to secrete IL-23. Increased IL-23 secretion was observed in activated human monocyte derived-DC (moDC), mouse bone marrow derived-DC and macrophages following inhibition of ATM and DNA-PK using the highly selective antagonists KU55399 and NU7441, respectively. In contrast, inhibiting ATR, a closely related single-strand DNA repair kinase, had no effect on IL-23. Interestingly, whilst inhibition of ATM only activated IL-23

secretion, inhibition of DNA-PK also led to increased IL-6, IL-1β and IL-10. IL-23 regulation through ATM/DNA-PK only occurred following toll-like receptor 4 (TLR4) activation and not with TLR1/2, 3, 5, 7/8, Dectin-1 and Dectin-1/TLR2 agonists. In support of a proposed immune-regulatory role for DNA-repair proteins, ionising radiation activated ATM and DNA-PK and consequently repressed IL-23. Radiation-dependent ATM/DNA-PK phosphorylation was prevented by KU55399 and NU7441, however only the ATM inhibitor restored IL-23 levels.

To determine the mechanism of action we tested the hypotheses that increased IL-23 resulted from

a) Altered activation of the Unfolded Protein Response (UPR) pathways,
b) Increased IL-23 was IL-1β dependent, or
c) Differential activation of NF-κB.

These were not supported by experimental evidence as

a) whilst inhibition of ATM/DNA-PK activated the UPR pathways they could not be shown to regulate IL-23
b) blocking IL-1β receptor with IL-1RA did not attenuate ATM/DNA-PK regulated IL-23.
c) Evidence for prolonged activation of NF-κB following DNA-PK inhibition was obtained, however its role remained unclear.

A role for the CREB transcription factor was established for ATM-dependent IL-23 regulation. Activated ATM phosphorylated CREB at Ser121, a residue

associated with negative CREB activity and consequently ATM inhibition enhanced CREB activity in a luciferase reporter assay. The addition of an inhibitor of the interaction of CREB with its transcriptional co-activator, CBP, abolished ATM-enhanced IL-23 response.

Control of IL-23 dependent immune responses is increasingly important in autoimmunity and cancer. This study identifies a novel immune-regulatory role for DNA-repair systems in DC resulting in the regulation of IL-23 and other Th17 associated cytokines (IL-1β, IL-6). Further studies could explore the potential of either small molecule inhibitors of ATM/DNA-PK to enhance, or stress signals (e.g. radiation) to repress IL-23 responses.

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Contents

Abstract……ii

Acknowledgements……v

Contents……vii

Figures……xiv

Tables……xx

Chapter 1 Introduction 1

1.1 Importance of dendritic cells in the immune system 2

1.1.1 Discovery of dendritic cells 2

1.1.2 Dendritic Cell Subsets 4

1.1.3 DC function 9

1.2 IL-23 regulation and its role in Th17 response 14

1.2.1 IL-23 14

1.2.2 Signalling pathways involved in IL-23 regulation 16

1.2.3 Th17 cells, a distinctive CD4 T cell subset 20

1.2.4 Regulation of Th17 and IL-17 producing cells 22

1.2.5 Significance of IL-23 in supporting Th17 cell population 25

1.2.6 Th17 response in health and disease 26

1.3 DNA repair proteins in cellular stress response 29

1.4 Toll-like receptor signalling 33

1.4.1 TLR receptors 33

1.4.2 Mammalian TLRs 34

1.4.3 DCs and TLRs 41

1.4.4 TLR signalling 43

1.5 ER stress 47

1.5.1 Three arms of ER stress response 47

1.5.2 Association between ER stress pathway and immunity 53

1.5.3 Involvement of ER stress in disease 56

1.6 Hypotheses and aims 59

1.6.1 Hypotheses 59

1.6.2 Aims and objectives 59

Chapter 2 Materials and methods 60

2.1 Primary cell isolation 60

2.1.1 Human cells 60

2.1.2 Murine cells 61

2.2 Cell culture 62

2.2.1 Media for cell culture 62

2.2.2 Primary cells 63

2.2.3 Cell lines 64

2.2.4 Freezing cell lines 64

2.3 Stimulation of dendritic cells for analysis of cytokine secretion 65

2.3.1 Human 65

2.3.2 Murine 65

2.4 Irradiation of cells 66

2.5 Cytokine secretion determination of DC supernatant 67

2.6 Measurement of TGFβ 67

2.7 Mixed lymphocyte reaction (MLR) 68

2.8 Flow cytometry 68

2.8.1 Cell purity and phenotype 69

2.8.2 Viability 69

2.8.3 Intracellular staining for phospho-ATM 70

2.8.4 SubG1 fraction assay 70

2.9 Quantitative PCR 71

2.9.1 Quantitation of mRNA levels of p19 71

2.9.2 Quantification of mRNA levels of sXBP-1 72

2.10 Western Blotting 72

2.11 Microscopy 73

2.11.1 Treatment of cells 73

2.11.2 Staining procedure 74

2.12 Statistical analysis 74

Chapter 3 Expression and importance of DNA damage repair molecules in dendritic cells 76

3.1 Introduction 76

3.2 Results 81

3.2.1 3.2.1 Cell purity and phenotype 81

3.2.2 Expression of ATM and DNA-PK in monocytes, moDC and myDC 83

3.2.3 Monocytes are more sensitive to DNA damage than DC upon radiation 85

3.2.4 ATM inhibition confers protection against apoptosis in monocytes 94

3.2.5 DNA-PK acts as the major double strand DNA break repair protein in resting monocytes and DCs 109

3.2.6 ATM or DNA-PK inhibited DC are unimpaired in their ability to stimulate T cell proliferation. 124

3.3 Discussion 127

Chapter 4 Immune regulatory role of ATM in DC 130

4.1 Introduction 130

4.2 Results 136

4.2.1 Inhibition of ATM but not ATR regulates IL-23 production in moDC 136

4.2.2 ATM inhibition induces IL-23 production in macrophages and murine BMDC 139

4.2.3 ATM regulation on IL-23 production has been observed only by TLR4 triggered moDC 141

4.2.4 Regulation of other cytokines by ATM 145

4.2.5 Ionizing radiation activates ATM and has a suppressive effect on IL-23 production by moDC, but has no effect on BMDC 146

4.2.6 Regulation of phosphorylation and transactivation activity of CREB by ATM 150

4.2.7 The effect of ATM on IL-23 is mediated CREB/CBP interaction 152

4.3 Discussion 154

Chapter 5 The role of DNA-PK in regulating IL-23 signalling in DC 160

5.1 Introduction 160

5.2 Results 167

5.2.1 Phosphorylation kinetics of DNA-PK upon LPS stimulation 167

5.2.2 DNA-PK regulates IL-23 secretion in DC 169

5.2.3 DNA-PK blockade also results in significant increase in IL-23 production macrophages and BMDC 172

5.2.4 In addition to IL-23, inhibition of DNA-PK also regulates other cytokine secretion 175

5.2.5 Involvement of different TLR signalling pathways in DNA-PK regulated IL-23 response 177

5.2.6 DNA-PK inhibited DC are unable to overcome the suppressive effect of radiation on IL-23 secretion 179

5.2.7 Consequence of blocking IL-1β receptor signalling on ATM and DNA-PK mediated IL-23 signalling 182

5.2.8 Analysis of NF-κB activation upon inhibition of ATM and DNA-PK 185

5.3 Discussion 189

Chapter 6 Investigation of the involvement of the UPR pathway in ATM or DNA-PK-regulated IL-23 response in DC 196

6.1 Introduction 196

6.2 Results 201

6.2.1 Expression of the ER stress marker Bip in ATM or DNA-PK inhibited moDC 201

6.2.2 Treatment with tunicamycin increases XBP-1s mRNA levels in DC and U937 202

6.2.3 Splicing of XBP-1 increases with ATM inhibition and synchronizes with increased IL-23 transcription in moDC and U937 204

6.2.4 XBP-1 mRNA splicing is induced upon DNA-PK inhibition and mirrors the increased IL-23 transcription in moDC and U937 206

6.2.5 IRE1 antagonist inhibits XBP1 splicing induced by ER stress inducers and in TLR4 activated DC 208

6.2.6 IRE1 inhibition has no effect on ATM or DNA-PK inhibited IL-23 regulation 210

6.2.7 TLR4 stimulation plays a suppressive role in phosphorylation of eIf2α which can be reversed by inhibition of ATM or DNA-PK 213

6.2.8 Inhibition of PERK pathway does not affect IL-23 production . 215

6.3 Discussion..... 218

Chapter 7 Final discussion 223

References 232

Figures

Figure 1.1 DC differentiation……6

Figure 1.2 The UPR……49

Figure 3.1 Purity of isolated monocytes……81

Figure 3.2 Phenotype of immature and mature DC……82

Figure 3.3 Expression of ATM and DNA-PK in DC…….. 84

Figure 3.1A Assessment of sensitivity of monocytes to IR……87

Figure 3.1B Assessment of sensitivity of monocytes to IR……88

Figure 3.1C Assessment of sensitivity of monocytes to IR……89

Figure 3.1D Assessment of sensitivity of monocytes to IR……90

Figure 3.2A Determination of apoptotic response of monocytes and DCs induced by IR……91

Figure 3.2B Determination of apoptotic response of monocytes and DCs induced by IR……92

Figure 3.2C Determination of apoptotic response of monocytes and DCs induced by IR ……93

Figure 3.2D Determination of apoptotic response of monocytes and DCs induced by IR ……94

Figure 3.3A Effect of inhibition of ATM on apoptotic response of monocytes to IR ……96

Figure 3.3B Effect of inhibition of ATM on apoptotic response of monocytes to IR……97

Figure 3.3C Effect of inhibition of ATM on apoptotic response of monocytes to IR……98

Figure 3.3D Effect of inhibition of ATM on apoptotic response of monocytes to IR……99

Figure 3.3E Effect of inhibition of ATM on apoptotic response of monocytes to IR……..100

Figure 3.3F Effect of inhibition of ATM on apoptotic response of monocytes to IR……..101

Figure 3.3G & H Effect of inhibition of ATM on apoptotic response of monocytes to IR……..102

Figure 3.4A ATM favours cell survival in monocytes, but not in DC following exposure to IR……..103

Figure 3.4B ATM favours cell survival in monocytes, but not in DC following exposure to IR……..104

Figure 3.4C ATM favours cell survival in monocytes, but not in DC following exposure to IR……..105

Figure 3.4D ATM favours cell survival in monocytes, but not in DC following exposure to IR……..106

Figure 3.4E ATM favours cell survival in monocytes, but not in DC following exposure to IR……..107

Figure 3.4F ATM favours cell survival in monocytes, but not in DC following exposure to IR……..108

Figure 3.4G & H ATM favours cell survival in monocytes, but not in DC following exposure to IR……..109

Figure 3.5A Inhibition of DNA-PK increases DNA damage and apoptosis in DC, but to a greater extent in monocytes……..111

Figure 3.5B Inhibition of DNA-PK increases DNA damage and apoptosis in DC, but to a greater extent in monocytes……112
Figure 3.5C Inhibition of DNA-PK increases DNA damage and apoptosis in DC, but to a greater extent in monocytes……113
Figure 3.5D Inhibition of DNA-PK increases DNA damage and apoptosis in DC, but to a greater extent in monocytes……114
Figure 3.5E Inhibition of DNA-PK increases DNA damage and apoptosis in DC, but to a greater extent in monocytes……115
Figure 3.5F Inhibition of DNA-PK increases DNA damage and apoptosis in DC, but to a greater extent in monocytes……116
Figure 3.5G & H Inhibition of DNA-PK increases DNA damage and apoptosis in DC, but to a greater extent in monocytes……..117
Figure 3.6A Determination of cell death upon DNA-PK inhibition by alternative method in monocytes and DC……118
Figure 3.6B Determination of cell death upon DNA-PK inhibition by alternative method in monocytes and DC……119
Figure 3.6C Determination of cell death upon DNA-PK inhibition by alternative method in monocytes and DC……120
Figure 3.6D Determination of cell death upon DNA-PK inhibition by alternative method in monocytes and DC……121
Figure 3.6E Determination of cell death upon DNA-PK inhibition by alternative method in monocytes and DC……122
Figure 3.6F Determination of cell death upon DNA-PK inhibition by alternative method in monocytes and DC……123

Figure 3.6G & H Determination of cell death upon DNA-PK inhibition by alternative method in monocytes and DC……..124

Figure 3.7 Inhibition of ATM or DNA-PK does not interfere with the ability of DCs to stimulate cell proliferation……126

Figure 4.1: Stimulation of IL-23 secretion by the ATM inhibitor KU55933-conditioned moDC……137

Figure 4.2 ATR inhibition does not affect IL-23 secretion by TLR4 triggered DC ……..138

Figure 4.3 ATM regulates IL-23 production in monocyte derives macrophages and in murine BMDC……..140

Figure 4.4A Inhibition of ATM enhances IL-23 secretion induced by TLR4 agonist only……143

Figure 4.4B IL-23 regulation by the ATM signalling pathway is restricted to TLR4 only……144

Figure 4.5 Impact of ATM inhibition on other cytokine production……146

Figure 4.6 IR-induced activation of ATM suppresses IL-23 production which can be reversed by pre-treatment with ATMi ……148

Figure 4.7 IR does not affect IL-23 secretion by ATM inhibited murine BMDC ……149

Figure 4.8 ATM mediated phosphorylation regulated CREB function…….151

Figure 4.9 ATM dependent IL-23 regulatory pathway signals through CREB……153

Figure:4.10 Proposed mechanism of regulation of IL-23 by cytoplasmic ATM through CREB……158

Figure 5.1 Activation of DNA-PK in response to LPS treatment……168

Figure 5.2 Stimulation of IL-23 secretion by DNA-PKi-conditioned moDC…170

Figure 5.3 Effect of inhibition of both ATM and DNA-PK on IL-23……..172

Figure 5.4 DNA-PK dependent IL-23 response in other cell types……..174

Figure 5.5 DNA-PK inhibition modulates IL-1β, IL-6 and IL-10 secretion along with IL-23……176

Figure 5.6A Enhanced IL-23 signalling by DNA-PK is only cooperated by TLR4 ……178

Figure 5.6B TLR4 restricted IL-23 secretion by DNA-PK inhibited DC……179

Figure 5.7 DNA-PKi blocks radiation induced activation of DNA-PK4……..181

Figure 5.8 Treatment with DNA-PKi fails to abrogate the suppressive effect of radiation on IL-23……181

Figure 5.9 Effect of recombinant IL-1β on IL-23 production……183

Figure 5.10 ATM or DNA-PK dependent IL-23 response is not mediated by IL-1β……185

Figure 5.11 Inhibition DNA-PK increases nuclear retention of NF-Kb...186-188

Figure 6.1 Expression of Bip from ATM and DNA-PK inhibited DC……201

Figure 6.2 Effect of tunicamycin on splicing of XBP-1 mRNA in moDC and U937 cell line……203

Figure 6.3 Increase in XBP-1s mRNA level and IL-23p19 transcription upon ATM inhibition in TLR4 triggered DC……205

Figure 6.4 Increase in XBP-1s mRNA level and IL-23p19 transcription upon ATM inhibition in TLR4 triggered U937 cells……..206

Figure 6.5 Regulation of LPS-dependent XBP-1 splicing and IL-23p19 gene expression by DNA-PK in DC……..207

Figure 6.6 Regulation of LPS-dependent XBP-1 splicing and IL-23p19 gene expression by DNA-PK in U937……...208

Figure 6.7 Pretreatment with IRE1i blocks TM or TG induced XBP-1s mRNA generation……..209

Figure 6.8 Effect of IRE1 inhibition on LPS-dependent splicing of XBP-1 mRNA ……...210

Figure 6.9 ATM or DNA-PK dependent IL-23 production is independent of IRE1 ……212

Figure 6.10 Regulation of phosphorylation of eIF2α in DC by ATM and DNA-PK following activation by LPS……214

Figure 6.11 Consequence of inhibiting PERK activation on IL-23 signalling mediated by ATM or DNA-PK……...217

Tables

Table 1.1 Chemokine receptor expression on DC……..11

Table 2.1 Small molecule inhibitors……66

Table 2.2 Toll-like receptor agonists……..66

Table 5.1 Effect of ATM inhibitor KU, DNA-PK inhibitor NUand PERK inhibitor (PERKi) on viability of cells……..170

Chapter 1 Introduction

Homeostasis in the immune system is vital for health and requires measured, controlled and often specific responses which are only generated and delivered at the appropriate times. The default position of the immune system in this model is "off", and even when the immune system is activated, the magnitude and duration of responses is subject to regulation to limit their extent and duration. This balance lies at the heart of healthy immunity for without good balance (ie magnitude of immune response) and direction (ie type of immunity e.g. CTL versus Ab responses [as illustrated by the inappropriate direction of IgE instead of IgG responses in asthma) the consequences for health can be profound. On the less severe end of the spectrum this includes generally mild autoimmune disorders such as psoriasis. However, because of their chronic nature, even conditions such as psoriasis (which can affect organs other than the skin) often have impacts that limit the patient's ability to fully live their life. On the other hand, impaired immune responses can be extreme and potentially life-threatening. Examples of this may include a range of immune deficiencies. For example, deficiencies in the receptor and signalling pathways for interferon (IFN)γ and upstream signal for IFN-γ, namely interleukin (IL)-12 [1]. Whilst a spectrum of disorders can exist (depending on the point of deficiency) in the case of complete IFN-γ receptor ligand-binding chain deficiency (IFNGR1 deficiency) children may die due to mycobacterial infection [2]. Indeed, murine models where components of this

key pathway were knocked out, were instrumental in demonstrating the existing of immune surveillance and reaffirming a role for the immune system in anti-cancer responses. In contrast, a particularly pronounced illustration of the loss of balance was seen recently with a life-threatening cytokine storm induced by administration of an immunomodulatory antibody, named TGN1412, specific for the T-cell co-stimulatory receptor CD28 in a phase I clinical trial setting [3]. Such marked effects are not uncommonly associated with the administration of new antibodies therapies, a subject reviewed by Frigault [4]. Understanding factors governing balance in immunity therefore has the potential to impact on a myriad of settings in health and disease. In this regard, the dendritic cell (DC) is one of the key cells at the heart of immune control as it is cited at key locations throughout the body where it is likely to encounter potential pathogens and importantly non-pathogens. The DC therefore serves to balance activation of immunity (in the case of pathogenic challenge) with ignoring or tolerising responses such as those required when encountering commensal microbial flora on the skin or mucosal surfaces.

1.1 Importance of dendritic cells in the immune system

1.1.1 Discovery of dendritic cells

Dendritic cells are central controllers of the immune system, with important roles in the initiation of innate and adaptive immunity [5]. DCs were originally

identified by Steinman and Cohn as a novel population of cells in mouse spleen on the basis of distinctive morphological criteria [6]. DC named after their unique probing, tree-like or dendritic shapes, were characterized as adherent cells that comprised up to 1.0-1.5% of the total nucleated cells of spleen. They were found in smaller numbers in Peyer's patches and lymph nodes, but was undetectable in bone marrow, thymus, liver, intestine or peritoneal cavity [6]. DC were reported to lack the expression of lymphocyte surface differentiation markers and the endocytic capacities of macrophages [7]. It was demonstrated that splenic DC had a turnover rate of 10% or more per day and new DC derived from precursors in the bone marrow and from the non-glass adherent population of spleen, probably cells from the red pulp. Dendritic cells were not found in new born mice and increased in numbers during first 3-4 weeks of age [8]. In his subsequent studies, Steinman reported the observation of cells with similar appearance as DC in small numbers in the white pulp nodules of mouse spleen. He documented the expression of H-2 and Ia alloantigens by DC and was able to isolate splenic DC with good purity, maintained them in vitro for several days and showed that they remained distinctive for several days in their cytologic and surface properties [9]. To isolate DC with greater purity, low density cell fraction was selected by floating spleen cell suspensions on dense bovine plasma albumin (BPA) column. Glass adherence then provided an adherent cell preparation (DC and immature macrophages) which eluted from glass after overnight culture. The macrophages were then selectively depleted by rosetting with IgG opsonized erythrocytes and recentrifugation on dense BPA [9]. Since then central roles

of DC in immune system have been reported by many groups. DC has been distinguished by the expression of high levels of major histocompatibility complex class II molecules and the integrin CD11c [10, 11] and by their migratory capability form non-lymphoid to lymphoid organs and superior capacity to prime T lymphocytes [12-14].

1.1.2 Dendritic Cell Subsets

A range of different subsets of dendritic cells exist. These vary according to their ontogeny, phenotype, tissue distribution and function. The work of defining DC subsets is still in progress, however, recent studies have yielded an improved understanding of these. Along with the definition of DC subsets comes an evolving nomenclature. Whilst not yet universally accepted this field is reviewed in a recent article by the group of Ginhoux and colleagues [15, 16]. A simple diagram describing human and mouse DC populations has been presented in figure 1.1.

1.1.2.1 Human DC

Human DCs are defined as cells that express high levels of MHC class II (HLA-DR) and lack lineage (Lin) markers CD3 (T cell), CD19/20 (B cell) and CD56 (natural killer cell) [17]. Human myeloid DC (mDC) are characterized as Lin^- $MHC\text{-}II^+CD11c^+$ [18-20], although in humans, it is worth bearing in mind that both monocytes and macrophages express CD11c. Human plasmacytoid DC

(pDC) are characterized as Lin-CD11c-CD4+CD45RA+CD123+ cells which have low MHC class II expression and poor T cell stimulatory ability [21]. The expression of CD303 (BDCA2) and CD304 (BDCA4) is also restricted to human pDC in peripheral blood and bone marrow [21]. These cells express high levels of TLR7 and TLR9 and produces type I interferon in response to viral infection [21].

$CD11c^+$ myeloid DC can be divided into two subgroups depending on the expression of CD1c (BDCA1) or CD141 (BDCA3) which are present in blood circulation [22]. CD1c mDC comprise the most predominant mDC subset in human blood (approximately 1% of mononuclear cells), tissue and lymphoid organs [23]. CD141 mDC represents only 0.1% of mononuclear cells [23]and have also been found in lymph node, spleen, tonsil, bone marrow [24]and non-lymphoid tissue, skin, liver and lung [25]. A discrete subset of $CD141^+$ DC has been identified in human dermis which express XCR1, CLEC9A, Necl2 and TLR3 [25]. Similar populations have also been reported in the kidney and lamina propria of humanized mice and human intestinal tissues and in the lungs of humanized immunodeficient mice reconstituted with human hematopoietic progenitors [26].

Langerhans cells (LCs) represent the major hematopoietic cells in the human epidermis and are also found in other squamous epithelia such as, bronchus, oral and genital mucous membrane [13, 27]a. Human LCs are characterized by the expression of CD45, MHC-II, high levels of the C-type lectin langerin and

the epithelial cell adhesion molecule (EpCAM) [28]. These cells also express CD1a [29], a nonpolymorphic class I MHC molecule that contributes to the capacity to present lipid antigens to T cells [30]. A modest population of

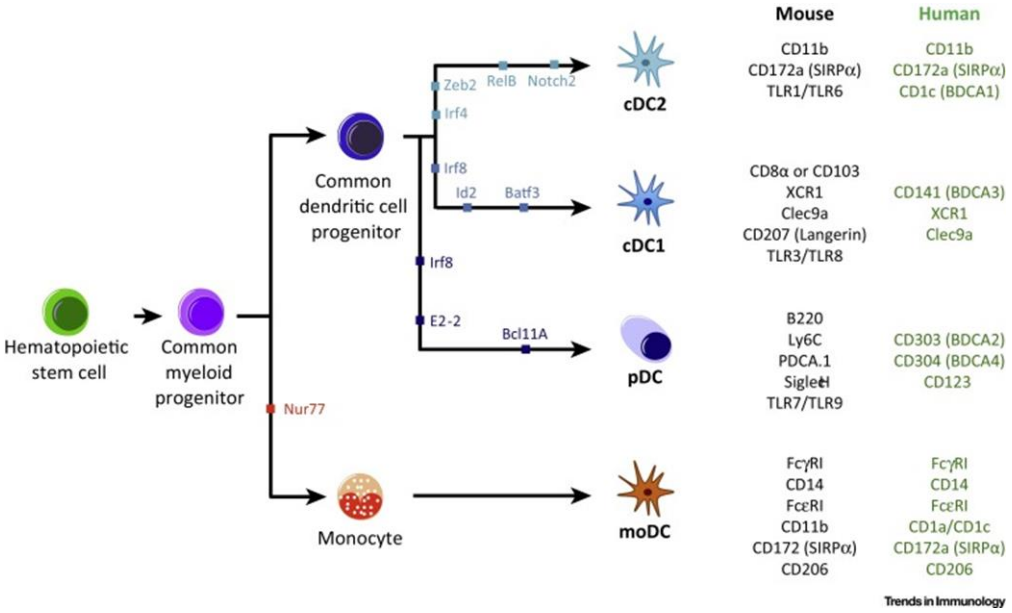


Figure 1.1 DC differentiation

DC originate from hematopoietic stem cells, which subsequently differentiate into common myeloid progenitors (CMPs). Under inflammatory conditions, CMPs into monocytes through several steps, which can further differentiate into monocytes DC (moDC). Alternatively, CMP will differentiate into a common dendritic cell progenitor (CDP) through several stages. The conventional type 1 DC (cDC1), conventional type 2 DC (cDC2), and plasmacytoid DC (pDC) subsets arise from the CDP, with the critical transcription factors shown for each lineage. Markers for each DC subset are shown on the right for mouse (black) or human (green). cDC1 in mice can be identified by expression of either CD8α in the lymphoid organs or CD103 within peripheral tissues. TLR, Toll-like receptor. Taken from [31]

$CD14^+HLA\text{-}DR^+$ cells reside in many nonlymphoid tissues that express CD11c but lack CD1c or CD141, co-stimulatory molecule and CCR7 [32, 33]. These DC populations express DC-SIGN (CD209) and the macrophage markers FXIII and CD163 [34, 35]. Although lymph node resident DCs mainly consist of CD1c+ and CD141 mDC that phenotypically resemble blood DC, migratory LCs ($MHC\text{-}II^{hi}CD11c^{int}EdCAM^+CD1a^+$), migratory dermal $CD1a^+$ DC ($EpCAM^-CD1a^+$) and dermal $CD14^+$ DC ($CD206^+$) are also constituents of lymph node DCs [24]. The most prevalent thymic DCs include $CD11c^+CD11b^-CD45RO^{low}$ cells, but lack cells resembling human $CD141^+$ mDCs and mouse $CD8^+$cDCs [36]. Another population of Lin-MHC-II+ cells, termed Slan-DC, has been identified in human blood [37]. These cells express FcγRIII (CD16) and the antigen 6-Sulpho LacNAc (SLAN) and produces large amounts of TNF-α, IL-1β and IL-12 in response to inflammation. However, this subset of DC is absent in tissues and has also been reported to represent a monocyte subset [38-41].

1.1.2.2 Mouse DC

Mouse classical DCs (cDC, refer to all DCs other than pDC) can be divided into at least two principal subsets characterized by expression of either CD8α and CD103 or CD11b [42]. Both the Shortman and Steinman groups first demonstrated the heterogeneity within the DC population and also described the existence of CD8α-expressing DC subset in murine lymphoid organs [43, 44]. An equivalent population of cDC characterized as $CD103^+CD11b^-$ cells also

exists in nonlymphoid tissues which shares its origin and function with lymphoid tissue CD8α$^+$ cDCs, but do not express CD8 [45, 46].

In the thymus, CD8α+ cDCs represent the main cDC population which are generated locally from early thymocyte progenitors [47]. In contrast, these cells constitute 20-40% of splenic and LN cDCs [47]. CD8 expression has also been detected on a subset of pDCs [48]. Tissue-migratory cDCs arrive in the lymph node in the mature state, whereas lymphoid tissue resident CD8α+ cDCs are phenotypically immature in the steady state [49]. CD103+ cDCs are present in most of the connective tissues and represent 20-30% of total cDC populations [13]. In the intestine, they are enriched in the Peyer's patches and also express CD8 on the cell surface, have low levels of MHC-II expression, and are representative of lymphoid tissue resident CD8α$^+$ cDCs [13]. CD11b$^+$ cDCs are predominantly present in lymphoid organs except for the thymus and can also be present in nonlymphoid tissue [42]. CD11b$^+$ cDCs are heterogeneous compared to CD8α and CD103 subsets and can be further subdivided based on differential expression of CD4 and the endothelial cell-selective adhesion molecule (ESAM) [50]. Although to a lesser extent in case of CD11b+ cDCs, all of these cDC subsets express Flt3, proliferate in response to Flt3 ligand (Flt3L) and are strongly reduced in Flt3L-deficient mice [13].

LCs reside in the epidermis of the skin and account for 3-5% of epidermal cells [51]. In contrast to dermal cDCs, LCs are phenotypically defined by lower MHC-II expression, intermediate CD11c levels and very high expression of the

c-type lectin langerin (CD207), which is involved in the formation of characteristic LC Birbeck granules [13]. In contrast to most DCs, the development of LCs is not dependent of Flt3 and Flt3L [15, 52]. In addition to LC, the connective tissue layer of skin, the dermis has recently been reported to host four cDC subsets: $CD103^{+}CD207^{+}$ and $CD103^{-}CD207^{+}$ cDCs, $CD207^{-}CD11b^{+}$ cDCs and cDCs negative for CD207, CD103 and CD11b [52].

1.1.3 DC function

1.1.3.1 Antigen processing and presentation

One of the most unifying features of DCs is their capacity to capture, process and present antigens on their MHC class I and II molecules, a prerequisite for T cell priming [53]. The antigens presented by MHC-I molecules are mainly derived from endogenous proteins degraded in the cytosol by the proteasome, whist MHC-II molecules present antigens that are derived from exogenous proteins degraded in the endosomal compartments [54]. DCs are specialized in ''cross-presentation'' and are able to present exogenous noncytosolic antigens on MHC-I, a critical function in immunity against viruses and intracellular bacteria [55]. Similarly, endogenous proteins that can access the endosomal compartments of DCs are efficiently presented on MHC-II molecules [54]. The presentation of exogenous depends on internalization of these antigens by one of several methods such as, pinocytosis, phagocytosis and receptor mediated endocytosis [54].

Human CD141$^+$ DCs and homologous murine CD8α$^+$ lymphoid tissue resident DCs are functionally specialized in cross-presenting exogenous antigens on MHC class I to CD8$^+$ T cells [56, 57]. Although CD1c$^+$ DCs are potent stimulators of naïve CD4$^+$ T cells, they are less efficient in cross-presenting antigen to CD8$^+$ T cells compared to CD141$^+$ DCs [27]. Likewise, murine CD11b$^+$ cDCs are inefficient in cross-presentation as compared to CD8α$^+$ cDC, but have enhance capacity of induction of CD4+ T cell response, potentially due to the expression of larger amounts of the components of the MHC class II machinery [50, 58]. The ability to cross-present has also been reported to skin derived CD103$^+$ cDCs [59]. CD8α$^+$ cDCs are also able to present glycolipid antigens in the context of CD1d and to activate and polarize iNKT cells toward Th1 or Th2 cytokine production [60].

1.1.3.2 Migration

DC are specialized for their efficient directional migration to the T cell zones within their respective lymphoid organ of residence or remote tissue draining LNs which allows optimal interactions with T lymphocytes [61]. DC migration in vivo is regulated by many different molecules, such as, small molecules, cytokines, chemokines, surface proteins, PAMPs and DAMPs (danger associated molecular patterns) [62]. Table 1.1 describes the expression of different chemokine receptors at different stages of DC maturation and their respective chemokines molecules.

Upon maturation, DC upregulate CCR7 as they migrate from the marginal zone to the T cell zone of the splenic white pulp and responds to CCL19 and CCL21 (ligands for CCR7) therein [63-65]. It has recently been shown that the mobilization of skin DC from the interstitial space to lymphatics depends on the haptotactic gradient of CCL21, but not CCL19 [66].

Table 1.1 Chemokine receptor expression on DC [67]

Cell type	Receptor	Chemokine
DC precursors	CCR2	CCL2, CCL8, CCL13
	CXCR2?	CXCL12
Immature DC	CCR1	CCL3, CCL5, CCL7
	CCR2	CCL2, CCL8, CCL13
	CCR5	CCL3, CCL4, CCL5
	CCR6	CCL20
	CXCR1	CXCL8, CXCL6
	CXCR2	CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, CXCL8
	CXCR4	CXCL12
Mature DC/Activated DC	CCR7	CCL19, CCL21
	CXCR4	CXCL12

Peripheral DC migration via afferent lymphatics is also CCR7 dependent [68]. Interestingly, in addition to the chemokine receptor switch, DC trafficking also involves autocrine desensitization by chemokine expression [69]. The chemotaxis and arrest of migrating tissue DC is facilitated by immobilized CCL21 on, or stored in, lymphatic endothelium to enter the afferent lymphatics [70]. Integrins play important roles during contact sensitization

[71], but are not essential for DC migration to LNs under physiological conditions [72].

Most of the DC mobilization signals exert their effect through production of IL-1β and TNF-α [73]. These molecules enhances DC migration by altering expression in adhesion molecules and chemokine receptors on DC as well as responsiveness to the relevant chemotactic ligands in the local microenvironment [74]. Activated integrins, such as, LFA-1, VLA-4, Mac-1 and α4β7, binds to members of the immunoglobulin superfamily, including, ICAM-1 (ligand for LFA-1 amd Mac-1), ICAM-2 (ligand for LFA-1), VCAM-1 (ligand for VLA-4 and weakly for α4β7), and MAdCAM-1 (ligand for α4β7), to aid firm arrest of rolling cells [74].

1.1.3.3 Activation of T cells

Steinman and colleagues demonstrated very early on the supreme capacity of DC to activate T cells in mixed lymphocyte reaction (MLR) *in vitro* [75]. The essential role of DC in stimulating naïve T cell responses and efficient restimulating memory T cells has been confirmed in studies of mice lacking cDC [76, 77]. DC induce de novo antigen-specific CD4+ T-helper (Th) [78, 79] and CD8+ cytotoxic T cells [80-82] in an MHC-restricted manner and can also directly induce non-antigen specific proliferation of CD8+ cytotoxic T cells in a Th-independent manner [83, 84].

Three distinct signal delivered by DC are required to induce productive, protective T cell responses, including proliferation, Th cell polarization and memory formation [85]. Signal 1 involves the interaction or TCR (T-cell receptor) with the appropriate peptide-MHC complex. Signal 2 is delivered through interactions between the co-stimulatory molecules on the surface of DC and T cell. Signal 2 is often referred to the signalling through CD28, when it engages CD80 and/or CD86. Signal 3 refers to the signals delivered by DC that determine the differentiation of T-cell into an effector cell, namely, Th1 cells, Th2 cells and cytotoxic T lymphocytes (CTLs) [86, 87].

When a T cell encounter MHC-peptide on DC in the absence of costimulatory molecules, it results in T-cell inactivation by anergy, deletion or converts into a regulatory phenotype, thus contributing to peripheral tolerance [88]. This fact is supported by the intimate interaction of steady-state T cells and DC under physiological conditions [89]. Furthermore, DCs can also actively silence T cell activation by expressing inhibitory molecules, such as, Programmed cell death 1 ligand (PD-L1) [90]. The actual Signal 2 that favours immunity (T-cell clonal expansion, differentiation into effector cells and long-termed memory formation) is therefore a fine balance of positive and negative co-stimulatory signals arising from many receptors [85]. It is also important for all the signals to come from the same DC, full T cell activation can only be directed through recognition of PAMPs/DAMPs (pathogen- or danger-associated molecular patterns) by DC, not inflammation or cytokine stimulated DC [91]. Thus the

inflammatory mediators (Signal 3) can only amplify, but not induce a T cell response.

1.2 IL-23 regulation and its role in Th17 response

1.2.1 IL-23

In the late 1990s, Kastelein, Bazan and colleagues identified and cloned the molecule p19 on the basis of homology search of the human DNA sequence database for IL-6 family of cytokines [92]. Their studies demonstrated that p19 dimerizes with IL-12p40 subunit to form a biologically active cytokine which they named IL-23 [92]. The gene encoding p19 is located on the chromosome 12 in human and on the chromosome 10 in mouse [92]. IL-23p19 shows 70% homology between human and mice [92]and has approximately 15% amino acid sequence identity with IL-12p35 [93]. Thus IL-23 and IL-12 both shares the common p40 subunit and belong to a small family of pro-inflammatory heterodimeric cytokines including IL-27 and IL-35 [94].

Human IL-23p19 is a non-glycosylated protein with a molecular weight of 18.7 kDa and 19.7kDa in mouse. It possesses the characteristic four helix bundle structure typical of all the IL-6/IL-12 family cytokines [92]. The 34.7 kDa p40 (murine 35.8 kDa) is a glycosylated type I soluble protein and is made up of three domains [95, 96]. The IL-23 receptor complex consists of IL-12Rβ1 [92]

which is also a part of the receptor complex of IL-12 [97, 98] and a novel receptor subunit named IL-23R. This unique receptor subunit was identified using a retroviral library created from an IL-23 responsive T-cell line [99]. IL-23R is a glycosylated type I membrane protein (human 70.5 kDa and mouse 79.7 kDa) and has three extracellular domains, a 37-amino acid long stalk region, a single transmembrane region and a cytoplasmic domain. It shares structural homology with IL-12Rβ2 (the other partner of the IL-12 receptor complex), specifically the N-terminal Ig-like domain and the two cytokine binding domains [99]. In humans, IL-23 receptor chains are mainly coexpressed on activated/memory T cells, T cell clones and NK cell lines, and also at low levels by monocytes, macrophages and DC populations [99]. Murine IL-23 receptor complex has been detected on activated T cells, bone marrow derived DC, LPS activated and inflammatory macrophages [99, 100]. Binding of IL-23 to signal transducing type I transmembrane β-receptors, IL-23R and IL-12Rβ1, activates noncovalently β-receptor-bound Janus kinases (JAKs) which phosphorylates several tyrosine residues within the intracellular domain of the IL-23R [99, 101]. These tyrosine residues then subsequent induce downstream signalling pathways including, signal transducers and activators of transcription (STAT) transcription factors, phosphoinisitide 3-kinase (PI3K), MAPK and NF-kB [99, 101, 102]. Signalling downstream of IL-23 depends on the activation of Tyrosine kinase (Tyk) 2(IL-12Rβ1) and Jak2 (IL-23R), which induces strong phosphorylation of STAT3 and a relatively weak activation of STAT4 [99]. By contrast, the reverse is true for IL-12 signalling induced phosphorylation of STAT4 [103, 104] and STAT3 [94].

In addition to its role in Th17 response, IL-23 plays important role in regulating innate immune responses. IL-23 controls the development of gut inflammation in T and B-cell deficient mice [105]. It has been shown that in RAG deficient mice, IL-23 but not IL-12 drives the local intestinal inflammation and IL-17 production in the intestine by anti-CD40 antibody activated cells of the innate immune system [105]. IL-23 also induces IL-17, IL-1, TNF-α and IL-6 secretion from innate immune cells [105, 106]. Genome wide association studies have revealed the association of single nucleotide polymorphisms (SNPs) within the *IL-23R* gene with Crohn's disease [107], ulcerative colitis [108], Graves' disease [109], psoriasis [110-112], RA [113, 114], ankylosing spondylitis [115, 116], Behcet' disease [117], coronary artery disease [118], Alzheimer's disease [119] and allergic rhinitis [120]. IL-23 signalling is not only important for driving inflammation, but also drives cancer development and progression [121-123]. SNPs in the IL-23R have been reported to be associated with the risk of developing multiple carcinomas, such as, gastric cancer, bladder cancer, acute myeloid leukemia, esophageal cancer or esophageal squamous cell carcinoma [124-130].

1.2.2 Signalling pathways involved in IL-23 regulation

IL-23 and IL-12 are predominantly produced by DC and phagocytic cells in response to many pathogens and Toll-like receptor agonists including LPS, CpG and PolyI:C [131]. The production of these cytokines can be further

enhanced via T cell CD40L/CD40 interactions through strong positive feedback mechanisms for DC activation [131]. Despite being closely related, the production of IL-12 and IL-23 is differentially regulated. Gram-positive bacteria preferentially induce IL-23 production over IL-12. Stimulation of moDC with *Escherichia coli* leads to significant increase in p19 expression [132]. Reports have suggested that TLR2 ligands such as peptidoglycan are much more potent inducers of IL-23 compared to TLR4 agonists such as LPS [133]. Differential expression of IL-23 have also been observed in macrophages in response to viral infection. Macrophages infected with Sendai virus produces large amount of IL-23, whereas influenza A virus infection fails to upregulate IL-23 [134]. Prostagladin E2 (PGE2) also modulates IL-23 production in mouse bone marrow derived DC [135, 136]. Similar to PGE2, other cAMP-elevating pathways, like the P2 receptors for ATP, preferentially induce IL-23 secretion [136].

Unlike IL-12, the signalling pathways regulating IL-23 production are less well characterized. Nevertheless, recently studies have been carried out to reveal the regulatory pathways controlling IL-23 production in response to various stimuli in different cellular systems. The differential expression pattern of IL-12 and IL-23 depends on the activation of PRRs engaged by different microorganisms [137].

Heat-killed Mycobacterium tuberculosis and the yeast zymosan induce significant IL-23 production in human DC, but little or no IL-12 [138]. DC

produce IL-12 after infection with *M. tuberculosis* only after IFNγ priming or costimulation with TLR7/8 ligand R848 [138]. Mycobacteria stimulate DC through at least four different PRRs including TLR2, TLR4, TLR9 and the cytoplasmic receptor NOD2 [139-142]. Studies by Gerosa F. et al showed that NOD2 and TLR2 act together to promote IL-12 and IL-23 expression in response to *M. tuberculosis* [138]. The two main PRRs that recognize zymosan are dectin-1 and TLR2. Dectin-1 ligand β-glucan together with TLR2 or TLR7 stimulation has been used to mimic cytokine production induced by zymosan [138, 143]. Dectin-1 drives maturation of DC and IL-23 response by signalling through Syk-CARD9 pathway [144]. The development of IL-23 dependent immune response during infection with *Candida albicans* has also been shown use the dectin-1 triggered Syk-CARD9 signalling pathway [144]. IL-23 secretion has been reported at significantly higher level than IL-12 in THP-1 human monocytic cell line in response to either live tachyzoites, secretory proteins or soluble antigen of *Toxoplasma gondii* RH strain [145]. T. gondii promotes IL-23 and IL-12 production in THP-1 cells via TLR2 and TLR4, respectively[146]. In this cellular model, IL-23 secretion is positively regulated by PI3K and ERK1/2 pathway and negatively regulated by p38 MAPK. By contrast, PI3K negatively regulates IL-12 production, whereas p38 MAPK and JNK pathway plays positive regulatory role in IL-12 response [146]. Consistent with this, Hepatitis B virus protein HBx- induced IL-23 expression is also dependent on the activation of ERK1/2-NFκB pathway [147]. Similarly, Butcher et al have suggested a positive role of PI3k and NF-κB in IL-23 production from human

monocytes [148]. *Francisella* driven expression of IL-23 was abrogated upon inhibition of PI3K or NF-κB [148].

Several studies have revealed the role of NF-κB transcription factor in the regulation of IL-23p19 gene expression. The work of Carmody et al. shows that p19 gene expression in dendritic cell is absolutely dependent on c-Rel which is one of the members of the NF-κB family [149]. Another group shows IκBα overexpression diminishes the expression of the p19 subunit [150]. Furthermore, the proximal κB site in the IL-23p19 promoter is indispensable to the RelA and c-Rel dependent induction of p19 transcription in mouse bone marrow derives macrophages [151]. In another study, short interfering RNA against RelA has been demonstrated to reduce the induction of the p19 gene in a human macrophage cell line [152]. Along with NF-κB, the transcription factor AP-1 plays important role in p19 gene activation in DC and macrophages in response to TLR signalling [153]. These transcription factors also promotes Il-23p19 production in IL-1β stimulated human periodontal ligament fibroblasts [154]. Celia and colleagues showed that Autophagy regulates IL-1β drive IL-23 response in a NF-κB dependent manner [155]. ATF2 which is a member of the ATF/CREB transcription factor family [156], controls IL-23p19 gene expression induced by Theiler's virus [157]. Ras-related C3 botulinum toxin substrate 1 (Rac1) negatively regulates p19 gene activation in LPS-induced human DC and macrophages [152]. Rac1 is a small GTPase involved in cytoskeletal re-organisation, cellular growth and apoptosis [158]. Rac1 inhibition augments the transcriptional activity of RelA, leading to

expression of IL-23p19, but not IL-12p40, implying differential role of RelA in regulating p19 and p40 [152]. LPS induced production of IL-23, but not IL-12, is negatively regulated by protein phosphatase 2A (PP2A) in DC [159]. It has been suggested that upon LPS stimulation PP2A interacts with the IKK complex leading to the deactivation of IKK and consequent termination of the NF-κB signalling cascade [159].

1.2.3 Th17 cells, a distinctive CD4 T cell subset

In 1986, the work of Mosmann and colleagues [160] revealed the existence of two distinct types of CD4+ T helper cell clones, Th1 and Th2 that had distinct profile of cytokine production. For long time, IL-12 dependent Th1 responses were assumed to be essential for the induction of autoimmunity, based on studies using neutralizing antibody specific for IL-12p40 or IL-12p40 deficient mice [161-164]. The thought of the presence of another subset of T cells raised from the observation that mice that lack IFN-γ and IFN-γ-receptor as well as mice deficient in other molecules involved in Th1 differentiation such as IL-12p35, IL-12Rβ2 and IL-18, were not protected from EAE, but rather develop more severe pathology [165-169]. On the contrary, IL-12p40 deficient mice did not develop the disease [167, 170]. The discovery that IL-12p40 is a shared component of IL-12 and IL-23 [92] led to the understanding that the latter cytokine might contribute to this disparity. Cua et al. at the DNAX Research Institute showed that IL-23, but not IL-12 was essential for the development of EAE by using IL-23p19 knockout mice and comparing them

with IL-12p40-deficient mice [100]. In the subsequent study, they demonstrated that IL-23 promotes a subset of IL-17-producing T cells and that these are capable to induce neurological disease [171]. Two reports in 2005 provided convincing evidence that IL-17 producing T cells named Th17 cells are completely separate and early lineage of CD4+ Th cells produced directly from naïve CD4 T cells [172, 173].

Th17 cells are defined by their unique molecular and functional signature [174]. Th17 cells are characterized by their capacity to produce IL-17A, IL-17F, IL-21, IL-22 and CCL20 [175-177]. Retinoic acid-related (RAR) orphan receptor γ thymus (RORγt), encoded by the gene *RORc*, was the first transcription factor identified to be expressed in Th17 cells [178]. In addition to RORγt, other transcription factors such as RORα, aryl hydrocarbon receptor (AHR), and interferon regulatory factor (IRF4) control the generation of this T cell population [178-182]. Th17 cells have an increased cell surface expression of dipeptidyl peptidase IV, CD26 which is a multifunctional ectoenzyme regulating multiple aspects of T cell activation and function [183]. It has been shown that the higher the expression of CD26, the larger the amount of IL-17A produced by T cells [183]. Th17 cell are distinguished from other T cell subsets by the high expression of the inducible costimulator (ICOS), IL-23R and CCR6 [184, 185].

The observation of IL-17A and IFN-γ producing double positive cells in vivo suggests that Th17 cells could at least partially function like Th1 cells (a

phenomenon referred to as ‘’T cell plasticity’’) [186]. These converted Th17 cells are termed as non-classical Th1 cells which can be distinguished from classical Th1 cell through the expression of the lectin-like receptor CD161 [187]. Th17 precursors are characterized by CD161, IL-23R and CCR6 expression, but do not express IL-17A either at the mRNA or protein level [187]. Upon exposure to IL-1β and IL-23, these cells mature into IL-17A producing Th17 cells [187]. On the other hand, Th17 cells convert into Th17/Th1 phenotype in the presence of IL-12 and these cells coexpress RORγt, T-bet, CCR6, CXCR3, CD161 and IL-23R [187]. Recently, the transcription factors Runx1 or Runx3, together with T-bet, have been shown very important in the generation of IFN-γ producing Th17 cells [188].

1.2.4 Regulation of Th17 and IL-17 producing cells

Harrington et al. and Park et al. described that IL-23 promoted generation of Th17 cells when IL-4 and IFN-γ were blocked and were independent of transcription factors specific for Th1 or Th2 lineages, T-bet, STAT1, STAT4 or STAT6 [172, 173]. It has now been established that TGF-β, IL-1β and IL-6 are essential to initiate Th17 cell development whereas IL-21 and IL-23 contribute to the expansion and long-term survival of Th17 cells [189].

Although the discovery of Th17 cells originated from the identification of IL-23 dependent IL-17 producing T cells, soon it was realized that IL-23 alone cannot potentiates the differentiation of Th17 cells from naïve CD4+ T cell precursors

[171, 190]. Three separate studies in 2006 revealed that the addition a combination of TGF-β and IL-6 during initial stage of T cell recognition of cognate antigen drives Th17 cell differentiation [190-192]. IL-6 plays an essential role in this process by activating STAT3 [193], which in turn activates several Th17 lineage specific genes, such as, *RORc, IL17A and IL23R [194]*. Bettelli et al. reported that STAT3 negatively regulates the generation of Treg cells by inhibiting TGF-β induced FOXP3 expression [190]. IL-21, an IL-2 family member, also induces IL-17 from Naïve T cells (by suppressing TGF-β induced FOXP3 expression) together with TGF-β even in the absence of IL-6 [195]. A study with *IL-1R1*$^{-/-}$ mice showed that these mice failed to develop antigen specific Th17 cells and were resistant to EAE describing a critical role of IL-1β in Th17 differentiation [106]. IL-6 induces the expression of IL-1R by Th17 cells and signalling through IL-1R activates the transcription factor IRF4, which in turn reinforces RORγt expression [196].

The role of TGF-β is complex in the generation of Th17 population. Whereas some studies show TGF-β is required for in vitro Th17 cell development [197-199], others describe TGF-β as a dispensable factor and a cocktail of IL-6. IL-1β and IL-23 is sufficient to drive differentiation of this T cell subset [200-202]. There are also discrepancies in cytokine requirement between human and mouse models of Th17 development [174]. A recent study has delineated the role of TGF-β3 in promoting pathogenic Th17 cell development by upregulating IL-23R expression [203].

In addition to Th17, γδT cells are an important innate source of IL-17 during infectious diseases and autoimmune inflammation [204-210]. Other innate IL-17 producing cells include NK cells,DC3+ invariant NKT (iNKT) cells, lymphoid tissue inducer (LTi)-like cells and myeloid cells [211]. These cells can express RORγt and produce IL-17 independent of IL-6 stimulation [211]. In CD4+CD8+ double positive thymocytes, RORγt is first expressed as a crucial survival factor [212]. RORγt is repressed when αβTCR thymocytes mature and go through the TCR selection process [212]. On the contrary, γδT cells and iNKT cells retain the expression of RORγt and preferentially develop into IL-17 producing cells as these cell exit thymus without undergoing TCR selection [213, 214]. RORγt mediated programme can be induced in innate cells like CD3-LTi and NK cells through other STAT3 activator cytokines such as IL-21 and IL-23 [211]. The transcription factor AHR is also expressed by γδT cells, LTi cells and NK cells [215, 216]. It has been suggested that AHR coordinate with RORδt to inhibit TGF-β induced FOXP3 activation and leads to maximal amount of IL-17 and IL-22 production [217].

IL-23 also has important role in stimulating IL-17 production from innate immune cells [211]. Many of these innate IL-17 producing cells have constitutive expression of IL-1R and IL-23R, both of which are also essential components of Th17 differentiation programme [211]. IL-1β or IL-23 can induce rapid IL-17 production from γδT cells [204, 215], whereas addition of both cytokines promotes optimal IL-17 secretion [204]. In vitro stimulation of human NKT cells with IL-23 induced the secretion of IL-17 [218]. Similarly, LTi

cells treated with IL-23 ex vivo produced IL-23 [219, 220]. Furthermore, similar results were observed when iNKT cells obtained from the drainig lynph nodes of type II collagen immunized mice were cultured with IL-23 [221]. Exogenous IL-23 and IL-6 potentiates IL-17 production from conventional NK cells activated in vivo with the intracellular parasite *T. gondii* [222].

1.2.5 Significance of IL-23 in supporting Th17 cell population

Several studies establishes strong relationship between IL-23 and Th17 by showing that IL-23 induces production IL-17 from activated T cells and that T cells expanded in the presence of IL-23 are able to transfer EAE and CIA [100, 171-173, 223, 224]. The involvement of IL-23 in the initial differentiation of Th17 cells has become less obvious with the fact that IL-23R is not expressed on naïve T cells and that a cocktail of IL-6, IL-21 and TFG-β is required for the development of Th17 cells [174]. However, IL-23 is essential for the development of mature and long term Th17 response [174]. Consistent with this, IL-23p19 deficiency results in decreased number of Th17 cells and IL-23 is required for prolonged in vitro culture of Th17 cells [174].

McGeachy et al. showed that absence of IL-23 signalling arrests the activation of Th17 cells at an early developmental stage and these cells fail to induce encephalitogenicity in EAE [225]. IL-23 promotes GM-CSF and IFN-γ expression in Th17 cells and the pathogenicity of these cytokines has been confirmed in EAE [202, 226-228]. IL-23 drives sustained Th17 response by conferring

resistance to the inhibitory effect of IL-27 and IL-2, probably by downregulating the IL-27R in mature Th17 cells [229, 230]. In the absence of TGF-β, pathogenic Th17 cells driven by IL-23 also have reduced expression of AHR, C-MAF and IL-10, compared to TGF-β induces Th17 populations [203, 231]. The upregulation of IL-23R is an important factor in IL-23 induced differentiation and stabilization of pathogenic Th17 cells [99, 231]. IL-6 and IL-21 induces the expression of IL-23R which depends on STAT3 [232]. However, RORγt mice have been reported to have reduced level of IL-23R, depicting the dependence of IL-23R on RORγt [233]. IL-6/IL-21, together with IL-1β and TGF-β are essential elements for the initiation of Th17 development and induced activation of RORγt and STAT3, which in turn might promote IL-23R expression and IL-23 responsiveness [174].

1.2.6 Th17 response in health and disease

Under physiological conditions, the major site of IL-17 producing CD4+ T cells is the lamina propria of the small intestine, but these cells can be readily induced at other mucosal sites following infection or vaccination [178, 234-236]. Thus IL-17 immunity is most effective against a variety of mainly extracellular bacteria and fungi in the gastrointestinal track, the airways and the skin [237].

IL-17 is a pleiotropic cytokine and signals through the IL-17 receptor (IL-17R), composed of IL-17RA and IL-17RC subunits [238]. IL-17R is widely expressed on hematopoietic and nonhematopoietic cells, such as endothelial and

epithelial cells [238]. IL-17A promotes granulopoiesis by triggering the increased expression of G-CSF and the transmembrane form of stem cell factor in bone marrow stroma [239]. In addition to granulopoiesis, IL-17 induces expression of GM-CSF, TNF-α, IL-6, IL-8, IL-1β and multiple chamokines (CXCL1, CXCL10) and chemokine receptors that act as powerful chemoattractants for granulocytes [240]. Th17 cells has important contribution to immunity against Klebsiella pneumonia [241] and many other bacteria including *Citrobacter rodentium*, *Staphylococcus aureus*, *Bordetella pertussis*, *Salmonella and Shigella sp*., *Bacteriodes fragilis* and *Borrelia burgdoferi* [242-245]. IL-17 is also requires for induction of Th1 response against some intracellular pathogens such as *Francisella Tularensis* and *Mycobacterium tuberculosis* [246, 247]. The roles of Th17 cells in antifungal immunity are well establishes. Th17 cells provide protection against mucocutaneous candidiasis by stimulating expression of dectin-1 and dectin-2 on DC and macrophages [144]. IL-17 has also been shown to regulate systemic fungal immunity by controlling the development function of NK cells [248]. The production of many antibacterial peptides and proteins is synergistically induced by IL-22 and IL-17 [175]. IL-22 has important role in tissue protection against invasion by *Toxoplasma gondii* and other gram negative bacteria [249]. IL-21 controls CD8-driven responses and promotes B cell responses and memory formation [250].

The process of providing protection against infectious pathogens is often involved rapid recruitment and activation of granulocytes and macrophages

that are the sources of tissue damaging reactive oxygen species [251]. In the presence of prolong cytokine stimuli, Th17 cells recruit inflammatory myeloid cells that are capable causing severe local tissue damage [196, 252]. The association of IL-17 has been associated with various human autoimmune conditions such as multiple sclerosis, rheumatoid arthritis and psoriasis [253]. In animal model of adjuvant induced arthritis, IL-17R deficiency [254] or treatment with IL-17R agonist [255] resists the development of disease pathology. Similarly, IL-17 deficient animals have delayed onset of EAE with reduce severity [256]. Consistent with this, treatment with IL-17 neutralizing antibody in mice immunized with a myelin antigen blocks chemokine expression and the subsequent onset of EAE [171, 257].

The association of Th17 cells and IL-17A have been described in various human tumours [121, 258-261]. The effects of Th17 cells in cancer immunity are dictated on a number of factors including the type of cancer, the source of IL-17 (IL-17 derived from Th17 cells or exogenously administered), the functional characteristics of the cells and/or exposure to therapeutic intervention [184]. Martin-Orozco and colleagues have demonstrated IL-17 promotes tumour rejection in the presence of lymphocytes, whereas favours tumour growth and angiogenesis in the absence of them [262]. High frequency of Th17 cell infiltration into the tumour vasculature strongly correlates with poor prognosis in patients with colon or pancreatic cancer [263, 264]. Inflammatory Th17 cells and associated cytokines mediate tumour growth by driving angiogenesis [265]. Indeed, IL-17A transfection results in

rapid growth and vascularization of tumours compared to wild-type mice [266]. However, increased numbers of Th17 cells have been associated with improved survival in ovarian cancer patients [267-271]. The role of IL-17 in tumour immunosurveillance has been supported in a study which describes that IL-17 deficiency increases tumour susceptibility in mice by reducing leukocyte infiltration and compromising CD8+ effector T cell differentiation [272]. Thus the role of Th17 subset in cancer is controversial. One possible explanation for this disparity might be the differential functions of these cells in different settings. Short acute inflammation associated with anti-tumour response might be beneficial to fight cancer, whereas prolonged inflammation induced by Th17 may favours certain types of tumours [273].

1.3 DNA repair proteins in cellular stress response

The most important protein kinases in the DNA damage response (DDR) signalling pathway are ataxia-telangiectasia mutated (ATM), ATM- and Rad3-Related (ATR) and DNA-dependent protein kinase (DNA-PK) [274]. These large serine/threonine kinases belong to the members of the phosphatidylinositol-3-kinase-like kinase family (PIKKs) [275]. ATM and ATR phosphorylated hundreds of proteins especially at ser/Thr-Glu motifs in response to DNA damage, whilst DNA-PK seems to regulate a small number of targets [276, 277]. Mechanism of DNA damage-induced activation of ATM and DNA-PK has been discussed in chapters 4 and 5, respectively. In addition to playing essential role in maintaining genome integrity, recently these proteins have

been found to be associated with other cellular stress pathways, such as, oxidative stress, hypoxia and ER stress [278-280].

Whereas the primary responses of ATM and DNA-PK are towards DSBs, ATR mainly function as a transducer of signal emerging from UV damage and collapsed replication forks. Nonetheless, it also responds to DSBs later and with slower kinetics[281]. In the DDR, activation of ATR involves recruitment of ATR to replication protein A (RPA) coated single strand DNA (ssDNA) breaks via interaction with ATR-interacting protein (ATRIP). The ATM and ATR kinases phosphorylates multitude of substrates in response to DNA damage[282]. The general assumption is that, while ATM is rapidly activated in response to DSB formation and subsequently phosphorylates numerous substrates; ATR is switched on independently and continues phosphorylation of some of these substrates. However, recent findings suggest ATM dependent activation of ATR in response to DSBs during the S and G2 phases of the cell cycle that are not part of the replication process. Thus, in addition to have functional redundancy, ATM may act upstream of ATR in some phases of the cell cycle[281].

Several key features of the A-T disorder, such as cerebellar degeneration and ataxia, rule out ATM functions that are not dependent on checkpoint activation upon DNA damage [283]. Oxidative stress could be a potential reason for the degenerative process considering the high rates of metabolism and high oxygen concentration in neuronal cells [278]. Abnormal control of reactive oxygen species (ROS) has been found in A-T due to lower levels and function of

antioxidant systems in A-T cells compared to normal cells [284]. The fibroblasts from A-T patients are more sensitive to oxidative stress than healthy fibroblasts and increased levels of protein [284] and lipid damage are correlated with increased levels of ROS produced in ATM- deficient mice under physiological conditions [285]. ATM-deficient mice showed progressive bone marrow failure because of defective haematopoietic stem cell functions that was associated with deterioration in the redox balance [286]. Several laboratories have shown that treatment with the antioxidants catalase and N-acetyl cysteine (NAC) improved oxidative stress in ATM-deficient cells by neutralizing ROS production [287, 288]. Thus ATM has been suggested as a redox sensor along with its role as a DNA damage sensor [289]. Recent findings have described a novel mechanism of ATM activation by direct oxidation [289, 290]. In contrast to the formation of ATM monomers and requirement of free DNA ends and MRN for ATM activation by DSBs, oxidizes ATM form active dimer in which the two monomers are covalently linked by intermolecular disulphide bonds [289]. In the absence of MRN and DNA, ATM activity on p53 phosphorylation has been demonstrated with the addition of hydrogen peroxide (H_2O_2) [289]. When primary human fibroblasts were treated with low concentrations of H_2O_2, activated ATM phosphorylated p53 and CHK2, but not histone H2AX, which is a hallmark of DSB induction [289]. Moreover, the heterochromatin protein Kap1 which was phosphorylated by ATM after DSB formation remained unphosphorylated following H_2O_2 exposure. These observations indicate to the different substrate specificity of ATM under conditions of DNA damage and oxidation [289]. However, it is very difficult to separate ATM activation through

oxidation from that by DNA damage, as oxidative stress and ROS formation usually led to DNA damage. Thus, often ATM is exposed to both DNA damage and oxidation at the same time. On the other hand, DNA binding by MRN is disrupted by oxidative stress, which in turn inhibits activation of ATM by MRN and DNA, suggesting the fact that under high ROS concentrations, oxidation may be the main operative pathway for ATM activation[278, 283].

Transient periods of low oxygen tension or hypoxia are common in all solid tumours and often followed by cycles of reoxygenation [291]. Hypoxia is strongly associated with development of tumour, malignancy, metastasis and resistance to potential therapy [291]. Under hypoxic condition, ATM undergoes autophosphorylation in an MRN-independent manner and in the absence of DNA damage. Once stimulated, hypoxia induced ATM appears to act in a very similar fashion to damage activated ATM, with regard to the phosphorylation of downstream targets. Kap1 and DNA-PKcs also found to be phosphorylated in response to hypoxia in an ATM dependent manner, whereas there was no sign of autophosphorylation of DNA-PKcs [279]. The cellular localization ATM is another critical difference between DNA damage and hypoxia induced activation of ATM. In response to DNA damage, ATM has been found to be retained at the sites of DNA breaks forming large nuclear foci, whereas in response to hypoxia, ATM remains defused throughout the nucleus [279]. The cellular localization pattern is similar to that seen in response to high salt and chlorine, pointing to the fact that there may be a similar mechanism of activation for non DNA damaging stresses [292]. In addition ATM directly

regulates the transcription factor complex HIF-1 which composed of a heterodimer of HIF-1α and HIF-1β subunits, and regulates metabolism, mitochondrial function, and angiogenesis under hypoxia [293, 294]. REDD1 is one of the target genes of HIF-1. The protein from this gene binds to 14-3-3 proteins and detaches these proteins from TSC2, allowing TSC2 to inhibit mTORC1 [295]. ATM phosphorylates and stabilizes HIF-1α under hypoxic conditions, which promotes mTORC1 inhibition and growth suppression [293].

1.4 Toll-like receptor signalling

1.4.1 TLR receptors

One of the most intriguing capacities of the immune system is to sense the presence of microbial infection and defeat the invader without destroying self-tissues. The reliable detecting of pathogens is not a trivial task considering their enormous molecular heterogeneity and rapid mutation rates. To deal with this problem, multicellular organisms have developed several distinct recognition strategies [296, 297]. In vertebrate animals, the immune system can be broadly divided into innate and adaptive immunity. The adaptive immune response is characterized by specificity and memory and relies on highly diverse antigen receptors- the T- and B-cell receptor generated through DNA rearrangement. The specific receptors generated by clonal selection and expansion account for the ability of the host to respond to a broad range of potential antigens [296, 298]. The innate immune system was previously

regarded as a nonspecific immune response with the main roles being to engulf and destroy foreign substances and to present antigens to activate the cells functioning in adaptive immunity. However, the cells of innate immunity possess significant specificity and are capable of discriminating between self and foreign pathogens [299-302]. This discrimination capacity relies on germline-encoded pattern-recognition receptors (PRRs) that have evolved to detect conserved molecular patterns of microbial structure referred to as pathogen-associated molecular patterns (PAMPs) [303, 304]. There are three main reasons for PAMPs being the suitable targets for innate immune recognition. First, they are invariant among entire classes of pathogens. Second, they are unique to microorganisms and not produced by the host, which allows distinction between self and non-self. Third, they have crucial roles in microbial survival, thus limiting the possibility of immune evasion through adaptive evolution of these molecules [305].

1.4.2 Mammalian TLRs

The Toll receptors (TLRs) are the best characterized PRRs that are evolutionary conserved between insects and humans [306]. Toll was first identified as a transmembrane protein in Drosophila which plays a central role in the establishment of embryonic patterns [307]. Later on, Toll signalling pathway was shown to play key role in antifungal immunity in this organism [308]. In mammals, the homologous family of Toll receptors are referred to as TLRs [309]. It is possible that most mammalian species possess 10 to 15 TLRs,

although the number of genes may vary between different species [297]. TLRs are type I transmembrane glycoproteins with an extracellular leucine rich repeat (LRR) domain that is responsible for PAMP recognition; and an intracellular Toll-interleukin 1 (IL-1) receptor (TIR) domain mediating downstream signalling [307, 309, 310]. To date, the number of functional TLRs that have been identified in human and mice are 10 and 12, respectively. TLR11, TLR12 and TLR13 have been lost from the human genome and mouse TLR10 is non-functional due to a retrovirus insertion.TLR1-TLR9 are conserved in both species [311]. The distinctive function of each TLR in terms of PAMP recognition and immune responses have been confirmed by using mice deficient in respective TLR [312]. Studies of the crystal structure of several TLR extracellular domains have provided the evidence that a number of PAMPs serves as ligand for TLRs [313]. Bacterial PAMPs are mainly the cell wall constituents, such as lipopolysaccharides, cell-wall lipoproteins, peptidoglycan and lipoteichoic acids. One of the cell-wall components of fungus, namely β-glucan, serves as an important fungal PAMP. As the viruses use the host cell to synthesize all their components, the viral nucleic acids are the main targets for innate immune recognition [305]. Immune system discriminated between self and viral nucleic acid depending on the structural features and specific chemical modifications that are unique to viral DNA and RNA. The cellular localization of viral nucleic acids is also important in maintaining tolerance to host molecules [314]. PAMP recognition by TLRs takes place in various cellular compartments, such as the plasma membrane, endosomes, lysosomes and endolysosomes [312].

The first characterized mammalian Toll was TLR4, which is expressed by a variety of cell types [309]. It is primarily present in the cells of the immune system, including DC and macrophages [309]. TLR4 provokes response to bacterial lipopolysaccharide (LPS), a basic structural component of the outer layer of Gram-negative bacteria that can induce septic shock [312, 315]. The hydrophobic domain of LPS, known as lipid A, is responsible for biological activity of LPS [304]. TLR4 was discovered by position cloning of the *lps* gene in the C3H/HeJ mouse strain which is hyporesponsive to LPS [316, 317]. C3H/HeJ mouse have a mis-sense point mutation within the fragment of *Tlr4* gene encoding the TIR domain, which converts a highly conserved proline to histidine. This mutation abrupt downstream signalling cascade and is responsible for LPS unresponsiveness in this mouse strain [316, 317]. The role of TLR4 in LPS signalling was also confirmed in *Tlr4* knockout mice [318]. Several accessory molecules are involved in the recognition process of LPS by TLR4. One of these proteins is called MD-2 which lacks transmembrane section and forms a complex on the cell surface in association with the extracellular domain of TLR4. This complex functions as the main LPS binding moiety [319, 320]. It has been shown that five of the six lipid chains of LPS bind the hydrophobic domain of MD-2 and the remaining lipid chain binds TLR4. In addition, the phosphate groups interact with the positively charged residues of TLR4. This multimeric receptor is composed of two copies of TLR4-MD2-LPS complex which induces downstream intracellular signalling pathway by recruiting adaptor molecules [311]. Two additional proteins, such as LBP

(LPS-binding protein) and CD14 have been reported to be involved in LPS recognition [320]. LBP is a soluble plasma protein that transfers LPS monomers to CD14 [321]. CD14 is can either be secreted into serum or present as glycosylphosphatidylinositol-linked, leucine-rich repeat-containing protein on the surface of macrophages [322]. CD14 is a high affinity LPS receptor that binds LBP and transports LPS-LBP to the TLR4-MD2 complex [311]. Other than LPS, TLR4 is involved in recognition of several other ligands, such as the fusion protein of respiratory syncytial virus, *Streptococcus pneumoniae* pneumolysin, mouse mammary tumour virus envelope proteins and the plant-derived cytostatic drug paclitaxel [312].

TLR2 mediates cellular response to a broad range of PAMPs derived from bacteria, viruses, fungi and parasites [312]. These include bacterial lipoproteins [140, 323, 324], peptidoglycan and lipoteichoic acid from Gram-positive bacteria [325, 326], mycobacterial cell-wall lipoarabinomannan [327, 328], fungal zymosan [329], glycosylphosphatidylinositol lipid from *Trypanosoma Cruzi* [330], the hemagglutinin protein from measles virus [311], and a phenol-soluble modulin secreted by *Staphylococcus epidermidis* [331]. TLR2 also binds to atypical LPS of *Leptospira interrogans* [332] and *Porphyromonas gingivitis* [333], both of which have different structure than Gram-negative LPS. TLR2 normally form heterodimers with either TLR1 or TLR6, which determines the ligand specificity and downstream signal transduction emanating from TLR2. Precisely, triacylated lipopeptides from Gram-negative bacteria and mycoplasma are recognized by the TLR2-TLR1

heterodimer, whereas the TLR2-TLR6 homodimer binds to diacylated lipopeptides from Gram-positive bacteria and mycoplasma [311]. The different lipid binding pockets of TLR1 and TLR6 play role in discriminating the structures of lipoproteins by these heterodimers [334, 335]. Both TLR2-TLR1 and TLR2-TLR6 complexes possess an m-shaped structure. In case of recognition of Pam_3CSK_4 (a triacylated lipopeptide) by TLR2-TLR1-ligand complex, two of the three lipid chains bind TLR2, whereas the third chain interacts with the hydrophobic region of TLR1. However, TLR6 does not have the hydrophobic channel, which restricts binding capacity of the TLR2-TLR6 heterodimer to triacylated lipopeptides. TLR2 also acts in cooperation with other cell surface coreceptors that help in PAMP recognition. These include dectin-1, a C-type lectin that binds fungal β-glucan and promotes its internalization, and CD36, which acts in conjunction with the TLR2-TLR6 complex to assist in sensing some of the TLR2 agonists [336]. It has not been demonstrate yet whether heterodimerization of TLR2 is stimulated by appropriate ligands or takes place prior to ligand interaction. Interestingly, many cell types constitutively express TLR1 and TLR6, but TLR2 expression is regulated and appears to be limited to antigen-presenting cells and endothelial cells [337].

TLR3 was first identified as a receptor for a synthetic analogue of double-stranded RNA (dsRNA), polyinosinic-polycitydylic acid (poly IC), which imitates viral infection [311]. Most viruses produce dsRNA at some point of their life cycle. Structural studies of the human TLR3 extracellular domain bound to

dsRNA has provided insights into the ligand recognition mechanism [338, 339]. The large horseshoe like structure of the ectodomain of TLR3 provides large surface area that facilitates dsRNA binding. The dsRNA interacts with the lateral side of the convex surface of the ectodomain of TLR3 via two different sites at the N and C termini. This allows TLR3 to form a stable homodimer through the C-terminal region. TLR3 plays essential roles in antiviral immunity by inducing production of type I interferon and inflammatory cytokines in response to viral infection. Deficiency in TLR3 is associated with susceptibility to herpes simplex virus type I (HSV-1) infection in humans [340] and lethal infection with murine cytomegalovirus in mice [341]. The ligands for TLR3 include the genomic RNA of reoviruses, dsRNA intermediates produced during the replication of single-stranded (ssRNA) viruses, such as, encephalomyocarditis virus, respiratory suncytial virus and Weat Nile virus, and certain small interfering RNAs [312, 342].

TLR5 recognizes flagellin which is a conserved protein component of bacterial flagella, polymeric rod like structure involved in bacterial mobility [304]. Unlike most other PAMPs, this TLR ligand is a protein and does not possess any posttranslational modification that would make its unique to host cellular proteins. However, the N and C-termini of flagellin are highly conserved, most probably due to their roles in the formation of a hydrophobic core of flagella. Any variation in these regions results in significant structural impairment [343]. TLR5 is highly expressed by CD11c+CD11b+ lamina propria DC in the small intestine. Lamina propria DC plays unique roles in inducing the differentiation of naïve T cells into TH17 cells and TH1 cells and naïve B cells

into plasma cells in response to flagellin [344]. TLR11, structurally similar to TLR5, is expressed in high levels in the kidney and bladder of mouse. TLR11 is involved in the recognition of uropathogenic bacteria in mice [345]. It also binds to the the profiling-like molecule produced by *Taxoplasma gondii* [346].

TLR7 was originally identified to recognize imidazoquinoline derivatives such as imiquimod and resiquimod (R848) and guanine analogues, for example, loxorbine. The ligands for TLR7 include ssRNA of RNA viruses such as influenza A virus, vesicular stomatis virus and human immunodeficiency virus [312, 347]. It also binds to synthetic poly (U) RNA and some small interfering RNAs [348]. The high expression of TLR7 in pDC is responsible for the production of large amount s of type I interferon by this DC subset in response to RNA viruses [312]. In addition, TLR7 on conventional DC stimulates type I interferon secretion from bacterial RNA species such as group B *Streptococcus* [349].

TLR8 is phylogenetically closely related to TLR7 and recognizes R484 and viral ssRNA. Responses against these agonists are impaired in TLR7 deficient mice, but not in TLR8 deficient mice. The highest expression of TLR8 is found in monocytes among various tissues and its expression is upregulated following bacterial infection [311].

Unmethylated 2'-deoxyribo(cytidine-phosphate-guanosine) (CpG) present in bacterial DNA and certain oligonucleotides can active murine and human

lymphocytes, whereas eukaryotic DNA and methylated oligonucleotides cannot [350]. Unmethylated CpG motifs are recognized by TLR9 and more frequently present in bacteria and viruses than in mammalian cells. Synthetic CpG oligodeoxynucleotides serve as TLR9 ligands and directly stimulates B cells, DCs and macrophages and drives the production of TH1-like cytokines such as IL-12 and IL-18 [304, 312]. The essential role of TLR9 in CpG DNA recognition has been confirmed using *Tlr9* knockout mice. Signalling induced by CpG DNA requires its internalization into late endosomal or lysosomal compartments. The sequence motifs flanking CpG dinucleotides for optimal response in mouse versus human are slightly different [296]. TLR9 in pDC serves as a sensor of DNA virus infection such as murine cytomegalovirus, HSV-1 and HSV-2 [312, 347]. TLR9 also directly recognizes the insoluble crystal hemozoin which is produced by *Plasmodium falciparum* after digestion of host haemoglobin [351].

1.4.3 DCs and TLRs

Among different types of PRRs expressed by DC, TLRs play critical roles in DC function and lead to the generation of different effector T cell subsets [352]. In both human and mouse, distinct DC populations express distinct sets of TLR and respond to certain types of pathogens. In mice, all splenic DC subsets express TLR1, TLR2, TLR4, TLR6, TLR8 and TLR9 [353]. Mouse CD8α+ DC do not express TLR5 and TLR7 and fail to respond to TLR7 agonists [353, 354]. Moreover, mouse pDC lack the expression of TLR3. The expression and

responsiveness of certain TLRs also vary in freshly isolated DC versus in vitro differentiated DC. Although there is low level of TLR4 expression on splenic DC, freshly isolated murine DC populations do not activate in response to LPS [355]. On the contrary, in the presence of GM-CSF and IL-4, bone marrow derived CD11c+CD11b+ DC have high expression of TLR4 and respond vigorously to LPS [355]. CD11c+ human myeloid DCs express TLRs 1, 2, 3, 5, 6 and 8, whereas freshly isolated human pDC express TLR7 and TLR9 [297]. Some studies have reported that TLR7 is exclusively expressed by pDC [356, 357], whereas others have found TLR7 expression on both pDC and myeloid DC [358, 359]. Human blood monocytes express TLR1, TLR2, TLR4 and TLR5, but gradually lose their receptors during differentiation into immature DC in the presence of GM-CSF and IL-4 and instead acquire TLR3 expression [360]. Interestingly, unlike human fibroblasts, which have cell surface expression of TLR3, human myeloid DC and in vitro differentiated immature DC express TLR3 in the intracellular compartments [361]. pDC from both human and mouse lack TLR3, but yet very efficient in detecting viral infection by recognizing viral genome through TLR9 and TLR7 [362, 363]. Studies using inhibitors of endosomal pathways have revealed that TLRs 3, 7, 8 and 9 require an acidic environment for endosomal activation, whereas TLR1, TLR2, TLR4, TLR5 and TLR6 do not require endosomal maturation and are expressed on the DC cell surface [297, 361].

1.4.4 TLR signalling

Signal transduction pathways activated by TLRs result in the differential patterns of gene expression that function in host defence, including inflammatory cytokines, chemokines, MHC and co-stimulatory molecules [296]. These differences can be explained by the existence of TIR domain containing adaptor molecules which are recruited by specific TLRs and activate distinct signalling pathways. These adaptors include MyD88, TIRAP, TRIF and TRAM [311]. MyD88, the first identified member of this family, is essential for all TLR-mediated signalling pathways except TLR3, and activates NFkB and MAPKs to produce inflammatory cytokines [312]. However, TLR3 and TLR4 induce type I interferon and inflammatory cytokines by activating the transcription factors of IRF3 and NFkB through TRIF [311]. TIRAP is involved in recruiting MyD88 to TLR2 and TRAM is a specific adaptor in TLR4-mediated TRIF-dependent pathway [311].

Signal emanating from TLR4 activates both the MyD88 and TRIF-dependent pathways involving all four adaptors. TLR4 activates MyD88-dependent pathway earlier than TRIF-dependent pathway by initially recruiting TIRAP at the plasma membrane which then recruits MyD88 to induce the initial activation of NFkB and MAPK [364]. TLR4 is then subjected to dynamin-dependent endocytosis and forms complex with TRAM and TRIF in the endosome. This results in IRF3 activation as well as late-phase activation of NFkB and MAPK [365-367]. Interestingly, in contrast to other TLRs, TLR4

induced inflammatory cytokine production is dependent on the activation of both the MyD88- and TRIF-dependent pathways. Although it remains a mystery why activation of either pathway alone is not sufficient for triggering inflammatory cytokine production via TLR4, the synergistic activation of both pathways might explain why LPS is a very strong immunostimulator and sufficient to cause endotoxic shock [311].

1.4.4.1 The MyD88-dependent pathway

After the stimulation of TLRs by their cognate PAMPs, MyD88 is recruited to the cytoplasmic TIR domain, where it recruits the IL-1 receptor-associated kinases IRAK4, IRAK1. IRAK2 IRAK-M. At first, IRAK4 is activated which plays a vital role in the activation of NFkB and MAPK downstream of MyD88 [312]. The subsequent activation of IRAK1 and IRAK2 is necessary for robust activation of NFkB and MAPK [368]. Upon binding of MyD88, IRAK4 mediates phosphorylation of a key residue or residues in the kinase activation loop of IRAK1 leading the induction of kinase activity of IRAK1 [298]. Autophosphorylation of its N-terminal residues by activated IRAK1 facilitates binding of TRAF6 to this complex [298]. The resulting IRAK1-TRAF6 complex is then detaches from the receptor and interacts with a preformed complex of TAK1, TAB1 and TAB2 or TAB3 at the plasma membrane [298]. TRAF6 is an E3 ubiquitin ligase that catalyses the synthesis of lysine63 (K63)-linked polyubiquitin chain on target proteins. It interacts with the ubiquitin – conjugating enzyme 13 (UBC13) through its RING-finger domain and this

UBC13-TRAF6 complex promotes TAK1 activation through the assembly of a K63-linked polyubiquitin chain. The K63-linked polyubiquitin chain bind to the novel zing finger-type ubiquitin-binding domain of TAB2 and TAB3 which are the two regulatory subunits of the kinase TAK1 complex. The K63-linked polyubiquitin chains also bind to the ubiquitin-binding domain of NEMO, a regulatory subunit of IKK complex regulating NFkB activation. Thus interaction of TAK1 and IKK complex might be mediated by the K63-linked polyubiquitin chain which allows TAK1 to phosphorylate IKKβ through its close proximity to the IKK complex. This results in the subsequent phosphorylation and degradation of IkB proteins leading to NFkB activation [369]. TAK1 activation also results in activation of MAPKs such as ERK1, ERK2 p38 and JNK by prompting phosphorylation of MAPKs rather than ubiquitination. This leads to activation of a number of transcription factors, such as AP-1 as well as influencing translation [311].

1.4.4.2 The TRIF-dependent pathway

The TRIF dependent pathway results in the activation of both IRF3 and NFkB [342]. Like MyD88-dependent pathaway, activation of NFkB by TRIF involves recruitment of TRAF6 and activation of TAK1 most probably through ubiquitination-dependent mechanism [311]. The C-terminal region of TRIF contains a RIP homotypic interaction motif that is responsible for recruiting the adaptor RIP1. After stimulation of TLR3 agonists, RIP1 is exposed to K63-linked polyubiquitination which is required for NFkB activation [311]. The

adaptor TRADD is involved in RIP1 activation downstream of TLR3 signalling. This has been confirmed in studies where TRADD-deficient cells exhibit impaired RIP1 ubiquitination with associated loss of NFkB activation [370, 371]. TLR3 mediated RIP1 ubiquitination and NFkB activation is dependent on Pellino-1 which is a member of the Pellino family of RING-like domain-containing E3 ubiquitin ligases [372]. Thus TRIF forms a multiprotein signalling complex with TRAF6, TRADD, Pellino-1 and RIP to activate TAK1, which subsequently activates the NFkB and MAPK pathways [311]. TRIF-dependent pathway also leads to activation of IRF3 and interferon-β transcription. Two noncanonical IKKs TBK1 and IKKi (IKKε) catalyse IRF3 phosphorylation and induce its nuclear translocation [373]. TRAF3 is involved in the activation of TBK1-IKKi by TRIF. TRAF3 plays an important role in interferon-β induction by different nucleic acid sensing TLRs as TRAF3 deficiency diminishes Interferon production by TLR3, as well as by TLR7 and TLR9 [374, 375]. In contrast to its role in promoting IRF3 activation, TRAF3 inhibits MyD88-dependent pathway. Following interaction with the MyD88 signalling complex during TLR4 signalling, TRAF3 undergoes K48-linked ubiquitination and degradation via cIAP1 and cIAP2, which are only present in the MyD88 signalling complex. Degradation of TRAF3 results in cytoplasmic translocation o the membrane proximal signalling complex leading to TAK1 activation [376]. NRDP1, a RING containing E3 ligase has also been reported to differentially regulate the MyD88- and TRIF-dependent pathways. It negatively regulates activation of the MyD88-dependent pathway by interacting with and degrading MyD88. At

the same time, it stimulates TBK1 activation through K63-linked ubiquitination by UBC13 [377].

1.5 ER stress

1.5.1 Three arms of ER stress response

The endoplasmic reticulum (ER) is a dynamic tubular network that is involved in several metabolic processes, such as gluconeogenesis and lipid synthesis. It is also the major site for calcium storage in the cell and takes part in the biogenesis of autophagosomes and peroxisomes[378]. Most secreted and transmembrane proteins fold and mature in the lumen of the ER and then are transferred to other compartments in the endomembrane system, presented on the cell surface or secreted extracellularly[379]. Integration of multiple environmental and cellular signals is required to constantly adjust the folding efficiency of the ER and to maintain the quality of cell surface and secreted proteins with high fidelity. Cell ensures adaptation to fluctuations in protein folding requirements through the action of signal transduction pathways that have sensors facing the ER lumen and effectors that convey the message to compartments of the cell[380]. A pioneering study in mammalian cells provided the first clue for the existence of a homeostatic mechanism that overcomes perturbations in protein folding at the ER. This study demonstrated that pharmacological and genetic manipulations that increased the load of unfolded protein in ER selectively upregulated the expression of

several ER-resident chaperones[381]. This intracellular signal transduction feedback loop that controls gene expression under conditions of ER stress is known as unfolded protein response (UPR)[378].

Sensing the imbalance between the amount of unfolded protein in the ER and the capacity of the cellular machinery that deals this load, UPR sets three main responses in action[382, 383]. The first two of these responses function to resolve the stress. First, there is a transient adaptation that decreases the load of proteins entering the ER by lowering protein synthesis and translocation into the ER. Second, a long term adaptation ensues in transcriptional activation of UPR target genes that increase ER abundance to satisfy needs by mediating expansion of the ER membrane and populating the expanded organelle space with newly synthesized protein folding machinery. The third mechanism eliminates damaged cells by apoptosis, when cells fail to re-establish homeostasis but continue to experience prolonged and unmitigated ER stress[380, 384]. This intensive and complex cellular response is mediated by three different classes of ER stress transducers. Each class represents a distinct arm of the UPR which is operated by protein kinase RNA (PKR)-like ER kinase (PERK), inositol-requiring enzyme-1 (IRE1) or activating transcription factor 6 (ATF6)[380]. The IRE1 branch represents the most conserved and sole UPR pathway in lower eukaryotes[385]. An ER chaperone immunoglobulin-binding protein (Bip) (also known as glucose-regulated protein, GRP78) interacts with the ER luminal domain of PERK, IRE1 and ATF6. It has been proposed that Bip dissociates from these molecules upon

accumulation of unfolded proteins, leading to their activation[386]. Overexpression of Bip markedly inhibits PERK and IRE1 activation and represses UPR, whilst reduction in Bip levels triggers UPR[387, 388]. However, in addition to dissociation of Bip, other cellular factors modulate activation of the ER stress sensors. Deletion of Bip binding site in the ER luminal domain of yeast IRE1 does not lead to constitutive activation of this mutant. Moreover, accumulation of unfolded proteins in the ER regulates activation of this mutant[389, 390].

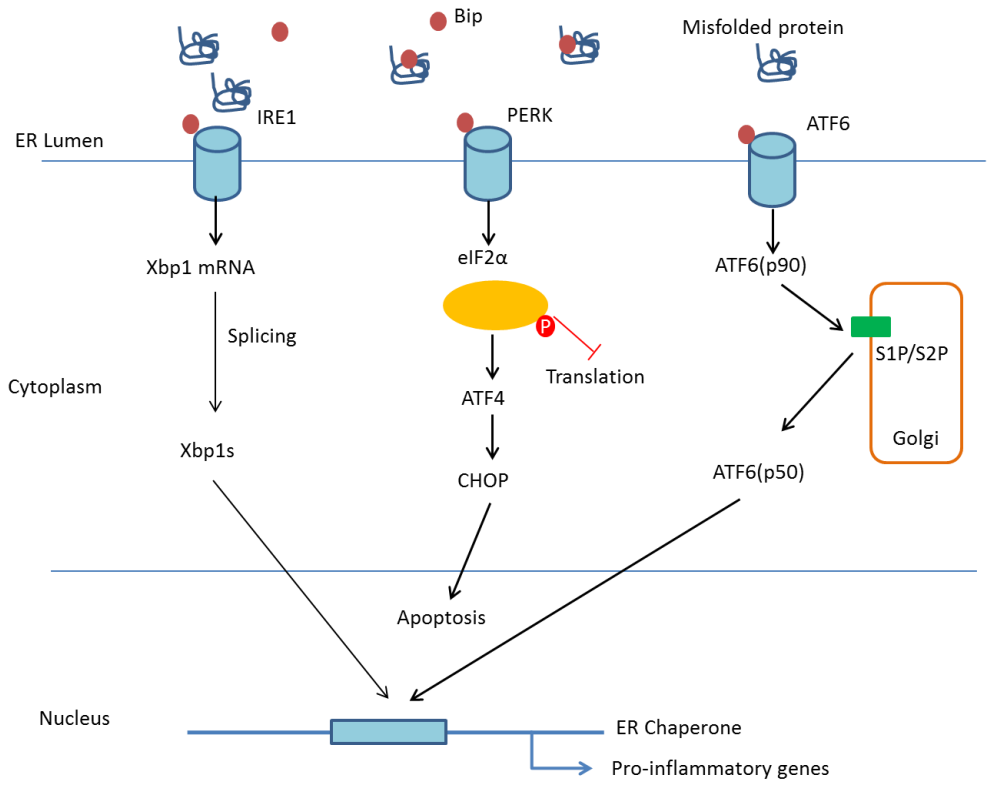


Figure 1.2 The UPR

The unfolded protein response stress sensors, inositol-requiring protein 1 α (IRE1α), protein kinase RNA-like endoplasmic reticulum (ER) kinase (PERK) and activating transcription factor 6 (ATF6), transduce messages regarding the folding status of the ER to the cytosol and nucleus to restore protein-folding capacity. The RNase activity of IRE1αprocesses the mRNA encoding unspliced X box-binding protein 1 (XBP-1) to produce an active transcription factor,

spliced XBP-1 (XBP-1s). Upon activation, PERK phosphorylates the initiation factor eukaryotic translation initiator factor 2α (eIF2α) to shut down global protein synthesis, but favours the translation ATF4mRNA, leading to activation of the transcription factor, C/EBP homologous protein (CHOP) which induces apoptosis. Under ER stress, ATF6 is transported to the Golgi apparatus, where it is cleaved by site 1 protease (S1P) and S2P. The transcription factors emerged from ER upregulate expression of genes encoding ER chaperone and proinflammatory proteins.

The initial characterization of ER stress pathway was done in Saccharomyces cerevisiae which utilizes a linear pathway controlled mainly by one stress sensor, Ire1 and a downstream transcription factor, Hac1[380]. IRE1 is a type I ER-resident transmembrane protein which contains a novel luminal domain and a cytoplasmic domain with protein kinase activity[391, 392]. Under ER stress conditions, IRE1 oligomerizes in the plane of the membrane which allows trans-autophosphorylation of adjacent kinase domains. Oligomerization can be triggered by dissociation of Bip or by direct binding of unfolded polypeptide chain to the luminal domain of IRE1 which forms a shared central groove resembling the peptide binding domains of major histocompatibility complexes (MHCs)[393, 394]. Possibly through comformational change[379], this trans-autophosphorylation activates the cytosolic RNase domain that causes precise endonucleolytic cleavage (26-nucleotide-long intron) of the mRNA encoding the transcription factor X box-binding protein 1 (XBP1). This causes a shift in the coding reading frame of XBP1 mRNA [395-397] resulting in the expression of an active and stable transcription factor termed, spliced XBP1 (XBP1s). The activated XBP1s then

translocate to the nucleus and acts as a potent activator of UPR target genes to control ER-associated degradation (ERAD), protein folding and protein flux into the ER [398, 399]. XBP1s also regulate phospholipid synthesis, which is required for the expansion of the ER membrane under ER stress[383].

PERK is also a type I transmembrane protein located in the ER membrane which is phylogenetically related to IRE1 and similar in structure and function[387]. Like IRE1, PERK also contains cytoplasmic protein kinase domain that undergoes activating trans-autophosphorylation through oligomerization in ER stressed cells. Activated PERK phosphorylates the α-subunit eukaryotic translation initiation factor-2 (eIF2α) at Ser-51, indirectly inhibiting eIF2 and inhibiting mRNA translation. Lower levels of translation initiation result in global reduction in the load of newly synthesized proteins which in turn reduces the entry of proteins into the already stressed ER lumen[400]. However, limited activity of eIF2 preferentially favours the translation of some mRNAs containing short open reading frames in their 5'-untranslated regions. One of these mRNAs encodes a transcription factor ATF4 that drives the expression of several UPR genes , such as, transcription factor C/EBP homologous protein (CHOP) and growth arrest and DNA damage-inducible 34 (GADD34). The transcription factor CHOP controls expression of genes involved in apoptosis[379]. Phosphorylation of eIF2α can also lead to activation of NFκB [401, 402]. In order to ensure cell survival, the level of phosphorylated eIF2α is tightly regulated. Activated PERK is dephosphorylated within minutes of restoring ER homeostasis[387, 403]. GADD34 and CReP (constitutive repressor of eIF2α phosphorylation) are two

of the genes that trigger dephosphorylation of eIF2α by encoding the substrate targeting subunits of two phosphatase complexes [403-405]. In contrast to GADD34, CReP expression is constitutive and functions in baseline eIF2α dephosphorylation[403].

ATF6 is initially synthesized as an inactive precursor, bound to the ER membrane by a transmembrane portion and bears a stress sensing region that resides in the ER lumen[406]. Upon onset of ER stress, it is packaged into transport vesicles and is transported from the ER to the Golgi apparatus[407]. There it is processed by two Golgi-resident proteases, S1P and S2P (site-1 and site-2 protease), that consecutively removes the luminal domain and the transmembrane segment, respectively[406, 408]. The released N-terminal cytosolic portion, ATF6(N), then enters into the nucleus to activate gene expression. Many of these target genes encode ER resident proteins that are involved in protein folding, such as Bip (a chaperone of the heat shock protein HSP70 family), protein disulphide isomerase and glucose-regulated protein 94 (GRP94; a chaperone of the Hsp90 family) [379]. Binding of Bip in the luminal domain of ATF6 is proposed to mask its Golgi localization signal. Under accumulation of unfolded proteins, Bip release may contribute to its activation [409]. ATF6 is present as a monomer and as oligomers, probably due to the presence of intra- and intermolecular disulphide bonds at its ER luminal domain. Under ER stress, reduced ATF6 monomers may only move to the Golgi for subsequent processing and activation [410].

1.5.2 Association between ER stress pathway and immunity

The protein folding machinery in the ER is mainly challenged in specialized secretory cells such as immune cells due to their high demand of protein synthesis, which creates a constant source of stress [378]. The involvement of UPR in immune system was first identified in B-cell lymphopoiesis, where XBP1 was shown crucial for the development of terminally differentiated antibody secreting plasma cells [411, 412]. UPR is primed when a B-cell matures into a plasma cell, as there is an associated expansion of the ER and secretory apparatus to comply with the extremely increased secretory function of the plasma cell [413, 414]. Later on, XBP1 was reported to be highly expressed by myeloma cells which are the malignant equivalent of plasma cells [415]. Although, *Xbp1* deficient mice developed normally with normal numbers of B and T cells and many B-cell functions remained unaltered, they failed to mount antibody responses to T-cell-independent and T-cell-dependent antigens. B cells from these mice did not express plasma cell marker (CD138) and plasma cells were absent in the secondary lymphoid organs [411]. Furthermore, IRE1 deficient B-cell does not progress developmentally beyond the pro-B-cell stage [416]. Cleavage of ATF6 occurs in stimulated B cells reflecting its involvement in terminal B-cell differentiation [417]. However, PERK pathway does not seem to be involved in this process, as PERK deficiency does not affect plasma-cell differentiation [416, 418]. Intact UPR is also essential for the survival and development of dendritic cells. Deficiency in XBP-1 markedly reduces the number of conventional DC and

plasmacytoid DC (pDC) due to an increased sensitivity to apoptosis during differentiation and also following stimulation with TLR ligands [419]. Basal level of XBP-1 splicing in freshly isolated DC suggests that activation of this vital arm of UPR allows for optimal DC function. The lacking of XBP-1 results in more profound reduction in pDC compared with conventional DC. pDC has a distinct spherical plasmacytoid morphology which resembles that of a plasma cell. Similar to plasma cell, multiple cytoplasmic extensions and an expanded ER develop in pDC in response to bacterial or infection, with accompanying production of large amount of IFN-α. This explains the requirement of vigorous UPR in this cell type [419]. In the absence of ER stress, constitutive activation of the UPR sensor IRE1 and its target, XBP1 in DC has been reported in another study. Absence of XBP1 in CD11c+ cells results in defective phenotype, ER homeostasis and antigen-presenting ability of CD8α+ conventional DC, whist the closely related CD11b+ conventional DC remains unaffected [420]. In addition to *Xbp1* mRNA, the RNase domain of IRE1 can also regulate the cleavage of additional mRNA species in a process termed 'regulated IRE1-dependent decay' (RIDD) [421-423]. In CD8α+ DC, the ER homeostasis is dysregulated by the loss of XBP1 transcriptional activity, whereas the RIDD pathway is responsible for controlling their phenotype and antigen cross-presentation by regulating mRNAs that encode integrins and components of the MHC class I machinery [420]. XBP1 is also an indispensable factor and plays highly selective role in the development of mouse eosinophils. XBP1 becomes vigorously and selectively activated during eosinophil differentiation and requires for the survival of mature eosinophil in

a cell-intrinsic way. Hematopoietic deletion of XBP1 results in downregulation of genes encoding proteins which control protein folding in ER and balance of secretory pathways. After the commitment of the progenitor cells to the eosinophil lineage, defective basal ER protein folding capacity prevents terminal maturation by downregulating expression of genes encoding the major lineage determinant GATA-1 and key eosinophil granule proteins [424]. Recent evidences have suggested crosstalk between the elements of UPR signalling pathway with that of the innate immune system. CREB-hepatocyte (CREBH) is an ER membrane resident transcription factor that is structurally related to ATF6 [424]. CREBH also undergoes ER stress regulated intramembrane proteolysis, releasing an N-terminal segment that moves to nucleus [425]. Unlike ATF6, it does not activate the expression of genes that increase the capacity of the secretory pathway, instead, links ER stress in the liver to the secretion of C-reactive protein and serum amyloid P-component, which are both linked with innate immune responses. Moreover, pro-inflammatory cytokines, IL-6 and IL-1β can induce activation of CREBH and UPR [425]. In mice and *Caenorhabditis elegans*, XBP1 deficiency abolishes the ability of these animals to clear bacterial pathogens [426, 427]. Certain TLRS, such as, TLR2 and TLR4 specifically induces *Xbp1* mRNA splicing to trigger production of inflammatory mediators, including IL-6 [428]. Surprisingly, TLR stimulation suppresses ATF6 and PERK signalling but particularly promotes XBP1 dependent IL-6 mRNA upregulation without activating classical UPR [428, 429]. In fact, some evidence suggests that activation of XBP1 splicing by TLRs is not dependent on protein misfolding [428]. However, this process is

dependent on IRE1 and mediated through a specific signalling pathway involving MYD88, TIRAP, TRAF6 and NADPH oxidase 2 (NOX2) [428]. XBP1 promotes the polyIC induced inflammatory immune responses in DC and enhances the production of IFNβ and other inflammatory cytokines [430]. UPR has also been implicated in the regulation synergistic IFNβ induction and TLR stimulation in macrophages through XBP-1. Binding of XBP-1 to a *cis*-acting enhancer element increases recruitment of CBP and IRF3 to *ifnb1* promoter which in turn augments IFNβ production [431]. Further study has demonstrated that phosphorylation and nuclear translocation of IRF3 are triggered by different ER stress inducers even in the absence of exogenous TLR stimulation [432].

1.5.3 Involvement of ER stress in disease

Although co-operation between ER stress signal and those emanating from TLRs may be appropriate for optimal response against infection, it may result in pathologic inflammatory responses. Abnormalities of ER stress have been implicated in a number of human diseases, such as, neurodegenerative diseases, bipolar disease, myositis, cardiovascular diseases, gut inflammation and type I diabetes [433-435]. Dysregulated UPR has also been reported to induce autoimmunity. Various models suggest four possible mechanisms through which aberrant UPR contribute to autoimmunity: misfolded proteins may be recognized by autoreactive immune cells; autoimmunity may be provoked through release of neo-autoantigens and UPR-related autoantigens

from dying cells that fail to resolve ER stress; normal mechanisms of immune tolerance may be perturbed in cells with defective UPR giving rise to autoimmunity; upregulation of ERAD associated proteins may confer resistance to UPR mediated apoptosis and survival benefit to autoreactive cells [436].

Involvement of UPR has been shown in human spondyloarthritis using transgenic mice engineered to overexpress HLA-B27 and human β-2 microglobulin that recapitulate many features of this disorder [437]. Even under physiologic conditions, HLA-B27 is prone to misfolding which prolongs Bip binding and this seems to restrict to HLA-B27 among other MHC class I molecules [438, 439]. Macrophages derived from the bone marrow of HLA-B27 transgenic rats showed increased levels of Bip and *Xbp1s* mRNA compared to wild type rats when stimulated with IFNα, IFNβ or TNF [440]. Aberrantly folded HLA-B27 molecules expressed on the cell surface have been postulated to induce an autoimmune response [441, 442]. Overexpression of Bip has been seen in the synovium in patients with RA that selectively stimulates synovial T cells compared to control T cells. 80% patients with RA were positive for autoantibodies against Bip compared to only 7% patients with other rheumatic conditions, a study that was also confirmed in collagen induced arthritis model of mouse[443, 444]. Stress proteins may elicit immune responses by functioning as immune adjuvants themselves [445]. The development of inflammatory colitis, such as inflammatory bowel disease can be attributed to the defective UPR in intestinal epithelial cells. IRE1β is known

to be expressed by intestinal epithelial cells [446]. IRE1β deficient mice show higher expression of Bip which is indicative of higher basal level of ER stress in these cells [446]. Furthermore, when treated with dextran sodium sulfate, these mice develop intestinal inflammation 3-5 days sooner than control mice [446]. In EAE, a role of UPR has been defined. Intracerebral expression of IFNγ before induction of EAE protects mice from the disease [447]. IFNγ induces UPR by activating PERK in oligodendrocytes which may indirectly confer disease protection in mice. The protection is lost in *Perk* deficient mice [447]. ER stress may have a role in the pathogenesis of atherosclerosis. CHOP dependent activation of p38, ERK1, ERK2, JNK1 and JNK2 is associated with elevated IL-6 but not TNF expression in atherosclerosis lesions [448]. Oxidized phosphorylcholine which is another important factor of atherosclerotic lesions, promotes the ATF4 and XBP1 arms of UPR, as well as IL-6 and IL-8 secretion in aortic endothelial cells [449].

1.6 Hypotheses and aims

1.6.1 Hypotheses

1. Dendritic cells are more resistant to DNA damage than monocytes because they express activated DNA repair proteins.
2. The ATM kinase pathway, but not ATR kinase, regulates IL-23 in DC. This activity is shared by another DNA double strand breakrepair protein, DNA-PK.
3. The regulation of IL-23 by ATM and DNA-PK occurs through changes in the ER-stress pathways.

1.6.2 Aims and objectives

The aim of this study was to identify the underlying signalling pathways through which IL-23 response is regulated in DC by ATM and DNA-PK. We intended to provide a better understanding of the roles of ATM and DNA-PK in DC function. This study was conducted with a view to establish a way of manipulating IL-23 dependent responses in inflammation, autoimmunity and cancer.

Chapter 2 Materials and methods

2.1 Primary cell isolation

2.1.1 Human cells

2.1.1.1 Isolation of cells from blood

Ethical approval was obtained from Nottingham University Ethics Committee to take blood from healthy donors. A written informed consent form was signed by each donor before phlebotomy. Donors with significant medical problems or currently on immunosuppressive medication or with anaemia were asked not to volunteer, although no specific screening were performed or any data recorded. Buffy coats were purchased from National Blood Service, Sheffield.

Heparinised whole blood or buffy coats were diluted with phosphate buffered saline (PBS) (Gibco, Life Technologies) as needed, layered carefully 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, counted and washed once in cold MACS buffer (PBS + 1% Foetal calf serum (Sigma), 2ml 0.5M EDTA pH-8.0 (Sigma)) 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 second followed by the addition of 2ml 10xPBS (Sigma) and PBS.

2.1.1.2 Myeloid dendritic cells

CD1c+myDC were isolated from fresh peripheral blood using the Miltenyi CD1c isolation kit. After washing in cold MACS buffer, 300µl MACS buffer, and 100µl each of Fc blocking reagent, CD19 microbeads and CD-1c-biotin were added per 10^8 cells and incubated at 4^0C for 15 minutes. Cells were then washed in MACS buffer and CD19+ cells were depleted by using an LD column (Mlitenyi). The negative fraction was washed in MACS buffer and incubated at 4^0C for 15 minutes with 400µl MACS buffer and 100µl anti-biotin microbeads (Miltenyi) per 10^8 cells. Cells were again washed in MACS buffer and CD1c+ cells were positively selected through an MS column (Miltenyi). Cell suspension was then eluted onto a second MS column to increase purity.

2.1.1.3 Monocytes

The negative fraction from CD1c+ isolation was subsequently used for monocyte isolation, otherwise cell fraction after first MACS wash was directly used. Depending on the cell number, 50-100µl CD14 microbeads (Miltenyi) and 200-400µl MACS buffer were added to the re-suspended cell pellet and incubated at 4^0C for 15 minutes. After washing, CD14+ cells were positively selected using an LS column (Miltenyi).

2.1.2 Murine cells

2.1.2.1 Ethical approval and animal sacrifice

Ethical approval to use mice was obtained from Nottingham University Biomedical Services Unit. Healthy C57BL/6 mice (6-8 weeks old) were

sacrificed using approved Schedule 1 methods by individuals approved by the Home Office.

2.1.2.2 **Cell isolation**

After sacrificed, the whole mouse was soaked in 70% ethanol. Then the femurs and tibias were dissected out and soaked for 30 seconds in 70% ethanol before transferring into murine DC medium. The bones were cut at both ends to flush out the bone marrow into a petri dish containing murine DC medium using a 20ml syringe filled with medium and a 25g needle. After collecting bone marrow from all the bones, the medium was transferred from the petri dish into a 50ml falcon tube and cells were separated by pipetting. The cell suspension was then centrifuged at 180xg for 5 minutes, supernatant discarded and the cells resuspended in murine DC medium. Cells were counted and viability was checked with trypan blue staining using a haemocytometer.

2.2 Cell culture

2.2.1 Media for cell culture

The medium for human DC was RPMI 1640, 10% (v/v) foetal calf serum (FCS) and 1% sodium pyruvate (all from Sigma). The composition of Murine DC medium was same as for human DC with the addition of 1/100 Penicillin/Streptomycin (Sigma). T cell medium was RPMI 1640 with 10% (v/v) FCS, 1% sodium pyruvate, 1% HEPES buffer, 1% L-glutamine, 1% non-essential amino acids and 20µM 2-mercaptoethanlo (all from Sigma). The medium for

U937 cell line was RPMI 1640 (Sigma) with 10% (v/v) heat inactivated FCS (Gibco). For freezing cells, 40% cell culture medium, 50% FCS and 10% DMSO (Sigma) was used.

2.2.2 **Primary cells**

2.2.2.1 **Human**

2.2.2.1.1 **Myeloid DC**

myDC were cultured in DC medium with 1000U/ml rhGM-CSF (Peprotech). MyDC were plated as 10^4 cells / well in tissues culture plate immediately after isolation and rested for 2 hours at 37^0 before treating the cells.

2.2.2.1.2 Monocyte derived DC

For the generation of moDC, freshly isolated monocytes were cultured in DC medium with 1000u/ml rhGM-CSF and 1000U/ml rhIL-4 (Immunotools) at a concentration of 0.5-1.0x10^6 cells/ ml in 75 or 175cm^2 flasks. On day 3, 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.

2.2.2.2 **Murine**

2.2.2.2.1 **Bone marrow derived DC**

Murine bone marrow derived cells were cultured at 1x10^6 cells/ml in murine DC medium with recombinant murine GM-CSF (10ng/ml) and recombinant murine IL-4 (10ng/ml) (both R&D Systems) in 75 of 175cm^2 tissue culture flasks. On day 3, medium and all floating cells were gently decanted and an equal volume of medium supplemented with rmGM-CSF and rmIL-4 was added carefully to the remaining plastic adhering monocytes. The cells were

cultured for further 4 days and then the immature BMDC were harvested, washed and plated in tissue culture plates for experiments.

2.2.3 **Cell lines**

2.2.3.1 **U937**

Frozen vials of U937 cells were defrosted from liquid nitrogen stores by warming up in the water bath at 37^0C. As soon as the cell suspension was defrosted, it was transferred to a falcon tube and warmed U937 medium was added dropwise whilst mixing the cell suspension. The cells were then centrifuged at 180xg for 5 minutes and resuspended in fresh U937 medium for culturing in T25 flask. Once the cell density reached the maximum as judged by eye, the cell suspension was aspirated and pelleted in a falcon tube. Then the cells were passaged at 1:3 for routine culture. All cell lines were routinely tested negative for the presence of mycoplasma.

2.2.4 **Freezing cell lines**

After harvesting from flasks, cells were washed once in complete medium, counted and resuspended at 0.5-1.0x10^7 cell /ml in freezing medium. Cells were then quickly aliquoted as 1ml per tube and placed in a cryopreservation vessel filled with isopropanol. Cells were then cooled to -80^0C 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.

2.3 Stimulation of dendritic cells for analysis of cytokine secretion

2.3.1 Human

myDC or moDC were plated in 96-well tissue culture plates at $1x10^4$ or $5x10^4$ cells /well respectively in triplicates in 50 or 100µl DC medium as appropriate and treated with one drug or combination of drugs and toll-like receptor agonist in a final volume of 200µl. When comparing myDC and moDC, similar numbers of cells were plated for both cell types. After resting for 2 hours (myDC) or overnight (moDC), cells were treated with small molecule inhibitors (Table 2.1) followed by stimulation with single or dual TLR agonists (Table 2.2), 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 -20^0C for subsequent cytokine secretion analysis by ELISA.

2.3.2 Murine

BMDC were plated and stimulated as for human DC but at $5x10^4$ cells/well and with 10ng/ml rmGM-CSF and rmIL-4 for all experiments.

Table 2.1 Small molecule inhibitors

Drug	Target	Source	Stock con[c]	Solvent	Working con[c]
KU55399	ATM	Tocris	10mM	DMSO	10µM
NU7331	DNA-PK	Tocris	5mM	DMSO	3µM
PERKi	PERK	Toronto Research Chemicals	10mM	DMSO	1µM
IRE1i	IRE1		10mM	DMSO	10µM
NU6027	ATR	Tocris	10mM	DMSO	10µM
C-Ci (Napthol AS-E)	CREB-CBP	Merck Millipore	10mM	DMSO	10µM
IL-1Ra	IL-1 receptor antagonist	R&D	10µg/ml	PBS with 0.1% BSA	50ng-100ng
Tunicamycin	ER stress inducer	Sigma	1mg/ml	Solution	1µg/ml-10µg/ml
Thapsigargin	ER stress inducer	Sigma	1mM	Solution	0.3µM

Table 2.2 Toll-like receptor agonists

TLR agonist	TLR	Source	Stock con[c]	Optimised working con[c]
Pam cys	TLR1/2	Invivogen	1mg/ml	10µg/ml
Poly I:C	TLR3	Sigma	5mg/ml	20µg/ml
Ultrapure LPS (S. Minnesota)	TLR4	Invivogen	5mg/ml	500ng/ml
Flagellin	TLR5		100mg/ml	1mg/ml
R848	TLR7/8	Invivogen	1mg/ml	2.5µg/ml
Curdlan	Dectin-1		2mg/ml	20µg/ml
Zymosan	Dectin-1/TLR2		2mg/ml	20µg/ml

2.4 Irradiation of cells

DC were irradiated after treating with small molecule inhibitors in triplicate in 96 well tissue culture plates prior to activation with TLR agonist. For western

blot analysis, cells were irradiated in 12-well plates. Radiation (6Gy of 195kVp X-rays, 0.87Gy/min, 0.5mm Cu filter, 48.4cm FSD) was delivered using a Gulmay Xstrahl cabinet irradiation facility. Cell morphology was examined by phase-contrast microscopy (X40) following radiation and after a further 24-48hours culture and viability was determined by AnnexinV/PI assay. Viability was unimpaired at the doses used.

2.5 Cytokine secretion determination of DC supernatant

DC supernatants were analysed for cytokine production using commercial ELISA kits for human IL-23 (eBioscience), IL-12p70 (BD Biosciences), IL-6 (ImmunoTools), IL-10, IL-1β, IL-27 and murine IL-12p70 (all R&D Systems). All ELISAs were performed as per manufacturers' instructions using the reagents supplied with the kits. The assay sensitivities for human IL-23, IL-12p40, IL-6, IL-10, IL-1β and IL-27 were 15pg/ml, 3.9pg/ml, 3.9pg/ml, 15.6pg/ml, 1.9pg/ml and 78.1pg/ml respectively. Absorbance was measured at 450nm using a spectrophotometer and data calculated using GraphPad Prism software.

2.6 Measurement of TGFβ

Detection of active TGFβ secreted by DC was kindly undertaken by Jo Porte, Respiratory Medicine, University of Nottingham by the TMLC co-culture assay. TMLC cells were harvested by trypsinization and resuspended in serum-free DMEM at $5X10^5$ cells/ml. The medium was removed from the experimental cells and equal volume (100µl) of TMLC cells and medium containing experimental stimulant was added. Cells were incubated at 37^0C overnight

and washed in PBS before lysis in reporter lysis buffer (Promega, Hampshire, UK). Cells were agitated with a pipette and centrifuged at 1500xg for 5min at 4^0C. The supernatant was added to luciferease assay buffer (Promega, Hampshire, UK) and the luminescence measured using a MicroLumatPlus microplate laminator (EG&G, Berthold, Hertfordshire, UK). TGFβ was quantified by comparing values obtained under experimental conditions to readings obtained from a standard curve derived from increasing concentrations of active TGFβ1.

2.7 Mixed lymphocyte reaction (MLR)

DC ($1x10^6$ cells) were treated with inhibitors and activated with LPS in 12-well tissue culture plate for 24 h, harvested, re-counted and re-plated in sextuplet in 96-well round bottom tissue culture plates at 5 consecutive 1:3 serial dilutions starting with 10^4 DC/well. The responder PBMC populations (both autologous and allogeneic) were either isolated from fresh blood on the day or recovered from liquid nitrogen and added at a density of $1x10^5$/well. Cell proliferation during the last 18 h of 5-day or 3-day culture was determined by uptake of 0.5 µCi methyl-[^{3}H] thymidine (Amersham, UK). Cells were then harvested onto filter plates and radioactivity was determined in a liquid Scintillation counter.

2.8 Flow cytometry

Flow cytometry buffer was prepared with PBS, 1% FCS and 0.1% sodium azide (Sigma). All FACS samples were acquired on 9-parameter MACSQuant® Flow

Cytometer and analysed using FlowJo software. If appropriate, cells were fixed using 2% formaldehyde and stored in the dark at 4^0C until acquisition.

2.8.1 Cell purity and phenotype

MyDC, monocytes and naïve T cells were washed in cold FACS buffer in flow cytometry buffer tubes at 1000rpm for 5minutes, supernatant decanted and cells resuspended by vortexing. Antibodies were added to the resuspended cells and incubated on ice in the dark for 30 minutes. Cells were then resuspended in 500µl buffer and acquired. Mouse anti-human HLA-DR IgG2a FITC (clone: AC122), CD86 IgG1 Vioblue (clone: FM95), Cd80 IgG1 APC (clone: 2D10) where all from Miltenyi Biotec, CD40 IgG1 FITC (Clone 5C3), CD83 IgG1 PE (Clone HB15e) were purchased from BD Biosciences. Isotypes used mouse anti-humanIgG2a FITC (cloneS43.10), IgG1 vioblue and APC (clone IS5-21F5 were purchased from Miltenyi Biotec, IgG1 FITC and PE (Clone MOPC-21 both) from BD Biosciences.

2.8.2 Viability

Cells were washed twice with cold PBS and then resuspended in 1x binding buffer at a concentration of $1x10^6$ cells/ml. 100 µl of the solution ($1x10^5$ cells) were transferred to a 5ml FACS tube. Then FITC-labelled Annexin V (5 µl) and Propidium Iodide (PI) (5 µl) 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 400 µl of 1x binding buffer and acquired within 1h.

2.8.3 **Intracellular staining for phospho-ATM**

Cells were harvested with 2% cold methanol, washed (1000 rpm for 5 minutes) and incubated for 15 minutes at RT in 500µl of 2% formaldehyde. Then 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 and then stained with primary antibody in 0.2% saponin (in FACS buffer) for 1h at RT. Nonspecific binding was blocked with 500 µl 5% FCS in FACS buffer for 15 minutes and then stained with secondary antibody in 0.2% saponin (in FACS buffer) for 30 minutes on ice. The cells were washed after 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) (dilution 1:50).

2.8.4 **SubG1 fraction assay**

Cells were suspended at $1x10^6$ cells/ml in 1ml of PBS in 5ml FACS tubes, centrifuged at 200g for 5minutes at room temperature and the PBS supernatant removed by aspiration. The cell pellet was gently resuspended in 1ml of fluorochrome solution with caution to avoid nuclei fragmentation and separation of apoptotic bodies from apoptotic nuclei. Fluorochrome solution was 0.1% sodium citrate (wt/v), 0.1% Triton X-100 (v/v), 50mg/l PI in deionized/distilled water. Fluorochrome solution was stoed in the dark at 4^0C

for several months. The tubes were then place in the dark at 4^0C for at least 1h and no longer than 24h before flow cytometry analysis.

2.9 Quantitative PCR

DC ($1x10^6$ cells) were plated in DC medium with GM-CSF in 12-well plates. Cells were pretreated with KU55399 or NU7331 for 1h and/or activated with LPS 500ng/ml + IFNγ 50ng/ml. At 2h, 6h, 9h, 12h and 20h, cells were harvested into cold PBS, RNA was isolated using Nucleospin RNA ll Extraction kit (Macherey-Nagel, Düren, Germany) and cDNA was prepared (GoScript Reverse Transcription System, Promega).

2.9.1 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/95^0C of 1 cycle, then 50 cycles of 15s/95^0C and 60s/60^0C. 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 = $2^{-\delta\delta CT}$. Amplification efficiency was 90-110%.

2.9.2 **Quantification of mRNA levels of sXBP-1**

RNA isolation and cDNA preparation were performed as described in the previous section. qPCR was carried out using KAPA Biosystem (Woburn, MA) with KAPA SYBER® FAST Universal 2xqPCR Master Mix and on Stratgene MX3005P. Primers were obtained from Eurofins MWG Operon (Ebersberg, Germany). Primers and probes were used at 10pmol/µl. The method of quantification was same as that described for IL23p19 where the house keeping gene was HPRT.

Note that HPRT was retained as a house keeping gene for sXBP1 qPCR in order to permit direct comparison of data with the laboratory of Dr Jane Goodall and Prof Hill Gaston (University of Cambridge).

2.10 **Western Blotting**

moDC were plated in 12-well tissue culture plate at $1x10^6$ cells in 1ml with GM-CSF. After resting, cells were treated with the inhibitor of interest for 1 hour then simulated with TLR4 agonist. 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 -20^0C 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 (w/v) in PBS-0.1% Tween-20 for 1h, probed with primary antibodies

overnight at 4^0C. 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, rabbit anti-human CREB phosphoSer133 from Abcam, mouse anti-human CREB phosphoSer121 from Cell Signalling and mouse anti-human β (Sigma, 1/10,000). Infrared-dye conjugated secondary antibodies: donkey anti-rabbit-800 and donkey anti-mouse-600 (dilution 1/15,000) 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.

2.11 Microscopy

2.11.1 Treatment of cells

moDC were seeded on coverslips in 6-well tissue culture plate at a concentration of $0.5x10^6$ cells/ml with well containing 400µL of medium. The cells were left untreated of treated with drug and/TLR agonist in a final volume of 500µL. Cells were rested overnight to allow them attach to the

coverslip so that the cells were not washed away during the staining procedure. The harvesting points were 2h, 4h and 6h post activation with LPS.

2.11.2 **Staining procedure**

The medium was removed carefully without disturbing the cell attached to the coverslip. Then the wells were washed with 2ml of ice cold PBS and incubated on ice for 5 min with 1ml of 100% cold methanol. After that, 2 ml of cold 0.5% saponin (in flow cytometry buffer) was added to each well and incubated for 2h on ice and/or in the cold room. Then the cells were stained overnight with 1 ml of anti p65 NFkb antibody (Santa Cruz Biotechnology, Inc.) diluted as 1:500 in 0.2% saponin (FACS buffer). On next day, each well was wash with 1ml of 0.2% saponin for three times (5-10 minutes wash each time). Cells were then stained with 1 ml of secondary antibody (anti mouse alexafluor-488) at a concentration of 1:1,000 in 0.2% saponin. The plates were then covered with foil and incubated for 2h on the shaker at 4^{o}C. At the end of the incubation period, the coverslips were taken off carefully from the wells and mounted on microscopic slide using mounting medium. The edges were then sealed with nail polish to prevent drying of the cells.

2.12 **Statistical analysis**

Statistical analysis was performed using Graphpad Prism® software and Microsoft® Excel (*:0.05>p value>0.01,**:0.01>p value>0.001,***:p value<0.001). Non-parametric paired t-test was done for data from one experiment for comparing biological triplicates between different treatments.

Data from multiple donors containing two sets of treatments were tested by non-parametric unpaired t-test. Overall significance for a set of experiment with multiple conditions of triplicate measurements was tested using ordinary one-way ANOVA performing multiple comparisons. Data from multiple donors for comparing effect between treatments were tested with one-way ANOVA where one variable applied or two-way ANOVA if two variables applied for individual group against all other groups. 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 Expression and importance of DNA damage repair molecules in dendritic cells

3.1 Introduction

The innate immune system of mammalian cells identifies external dangers by the engagement of highly conserved different classes of pattern recognition receptors (PRR), which are expressed by macrophages and DC. Following triggered by danger signals, DC produce cytokine linking innate and adaptive immune responses and manipulate T cell responses [450-452]. Cells harbour the DNA repair pathways to compensate the damage of the genome caused by various exogenous and endogenous factors. In addition to the canonical roles of maintaining genomic stability, the proteins involved in these signalling pathways are recently being reported to be involved in other intracellular stress responses and in induction of immune responses [453-456].

The integrity of eukaryotic genome is under constant mutational threat because of exposure to various internal and external agents that cause damage to DNA. These agents include oxygen free radicals, UV light, ionizing radiation, and different chemical agents, the chemotherapeutic agents that cause DNA damage [457]. These factors introduce a wide variety of genetic alteration, such as deletions, translocations, and chromosome loss, which in turn result in cell death [282]. So for the prevention of undue cell death and/or neoplasia, it is essential to maintain the genomic stability. Among all the different types of DNA lesions, DNA double strand breaks pose a tremendous treat to genome integrity and cell viability, if left unrepaired or

incorrectly repair. Thus correspondingly, there are multiple evolutionary conserved pathways that allow cells to detect, respond to and correct chromosomal alterations. The two major repair pathways for DSBs are error-prone non-homologous end-joining (NHEJ) and high-fidelity homologous recombination (HR) [458]. In mammalian cells, NHEJ repairs most of the ionizing radiation induced DSBs which occurs predominantly in the G0 and G1 phases of the cell cycle by tethering DSB ends directly or after limited processing [459, 460]. On the other hand, DSBs induced by stalled replication fork are repaired by HR and occurs during late S and G2 phases, as HR requires a sister chromatid as a template for repair [460]. In conjunction with these pathways, mammalian cells also maintain a DNA damage response pathway which senses DNA damage and initiate a wide spread signalling cascade that involves DNA repair, activation of cell cycle checkpoints, and extensive modulation of gene expression and many metabolic pathways. This broad, powerful signalling network begins with rapid accumulation of a large group of proteins as sensors at DSB sites and continues with the activation of several protein kinases with partially redundant functions. These transducer proteins relay the signal to numerous downstream effectors, which are mainly key molecules in the downstream DDR branches. However, the overall cellular responses induced by DSBs go far beyond repair[282].

The fundamental transducer of DSB is the serine- threonine kinase ataxia telangiectasia(A-T) mutated (ATM), which becomes activated upon DSB formation and triggers phosphorylation of multiple substrates [277]. This

protein belongs to a conserved family of phosphoinositide 3-kinase-like protein kinases (PIKKs), that also two other major DSB sensors: the subunit of the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) and ataxia telangiectasia and Rad3 related (ATR) [275]. These three proteins exhibit close functional relationship, having distinct and redundant functions. There is recent evidence suggesting the broad capacity of ATM as a regulator of other cellular processes, such as oxidative stress, ER stress [278] and non DDR aspects, among that mitochondrial homeostasis[458, 461]. DNA-PK is also a DNA-activated serine/threonine protein kinase which is abundantly expressed in almost all mammalian cells. It has critical roles in DNA-damage repair pathway, including NHEJ and HR. However, high levels of DNA-PK in human cells mirrors the involvement of this protein in different cellular signalling pathways besides DNA repair. Recently DNA-PK has been reported to be involved in metabolic gene regulation in response to insulin stimulation and in the regulation of homeostasis of cell proliferation[462]. Our studies have shown that inhibiting ATM and/or DNA-PK with highly selective antagonists increased secretion of IL-23 in human monocyte derived dendritic cells. Although none of these DNA damage sensors have previously been described to play a role in regulating the IL-23/TH17 axis, there are some indirect evidences linking ATM kinase to immune activation. In a colitis model of mouse, ATM deficiency resulted in unrepaired DNA damage and persistent immune activation[453]. ATM activation was also shown to induce IFN-stimulated and IFN-α and –γ genes in T cells after etoposide-induced DNA damage[454]. Thus understanding the intracellular mechanisms linking these

molecules in the context of immune regulation may have important therapeutic implications in terms of cancer and autoimmune diseases.

The key players in the immune system are monocytes and DC [463]. Monocytes are derived from progenitor cells in the bone marrow. Upon maturation, they are released into the bloodstream, circulate in the blood and enter the tissue to differentiate into macrophages and myeloid dendritic cells [464]. DC present antigenic peptides on their surface MHC molecules thus induce an effective immune response by stimulating differentiation of T cells. In the process of engulfing invading pathogens, DC produce a burst of reactive oxygen species (ROS) as a defence mechanism against infections [465]. These cells can also play roles in the pathogenesis of chronic inflammatory diseases by activating neutrophils and leading to chronic ROS production [466]. The cytokines and chemokines produced by excessive and chronic monocyte/DC activation together with a continuous production of high level of ROS may cause severe damage to cellular DNA and normal tissues leading to severe autoimmune conditions such as RA, IBD, psoriasis, atherosclerosis and neurodegenerative diseases [467]. DNA lesions, such as, 8-oxo-guanine and thymidine glycol, induced by ROS can lead to genomic instability, mutations and eventually cancer [468]. Thus there must be a check point system to control excessive activation and ROS generation by the mononuclear phagocyte system following infection and inflammation to avoid adverse immunological reaction and damage to normal tissue.

It has been shown that human macrophages and DC show high level of resistance to DNA damage when exposed to ROS inducing agents. These cell display very low level of DNA damage after exposure to genotoxic stress and are very efficient in repairing single strand break (SSB) and double strand break (DSB) ensuing cell survival [469]. In contrast, the cells they are derived from, the monocytes, are highly sensitive to the genomic threats imposed by those ROS producing treatments displaying high level of DSBs, activation of DDR pathway and apoptosis. This extreme sensitivity of monocytes can be described by their lack of expression of three important players (XRCC1, lig IIIα and PARP-1) in base excision repair (BER) and DNA-PKcs [469]. This DNA repair defect in monocytes thus functions as a self-regulatory mechanism to maintain homeostasis by preferably killing the precursor cells. This in turn controls the adverse immunological consequences by keeping cytokine and ROS producing mature cells in check [469].

In this chapter, we aimed to compare the sensitivity of monocytes with DC derived from them to ionizing radiation to re-establish the resistance of DC to IR. The role of DNA repair molecules in monocytes and DC was studied by inhibiting ATM and DNA-PK to establish their importance in these cells. Furthermore the effect of inhibition of these DNA damage repair proteins on classical DC function was investigated. These assays would provide valuable insight in future implication of ATMi and DNA-PKi for immunological therapies.

3.2 Results

3.2.1 3.2.1 Cell purity and phenotype

The purity of isolated monocytes were determined by flow cytometric analysis of CD14 expression (Figure 3.1) and routinely exceeded 95%. Further experiments confirmed the phenotype of the DCs upon maturation. Maturation was determined by examining expression of CD80, CD86, CD40, CD83 and HLA-DR at 24 hours post-activation. CD80, CD83 and CD86 expression was low on immature DCs and elevated in mature DCs (Figure 3.2). Similarly, expression of HLA-DR low in immature DC and high level of expression observed after maturation (Figure 3.2). CD40 expression was similar in both immature and mature DC (Figure 3.2).

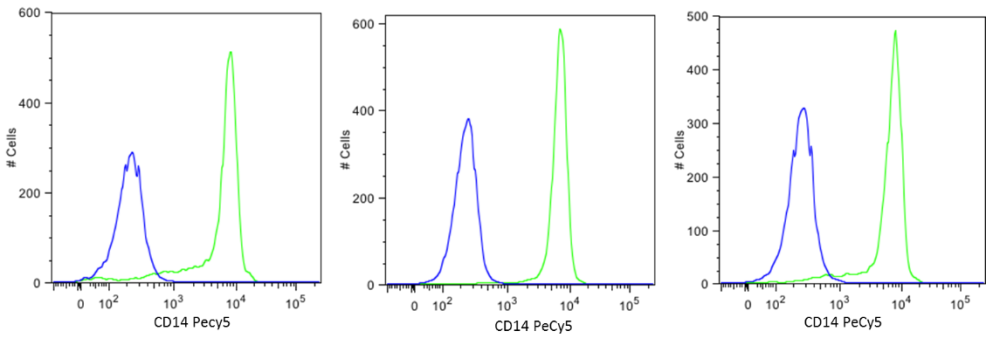


Figure 3.1 Purity of isolated monocytes

After isolation, the purity of monocytes was determined with CD14 PECy5 antibody (green line) with matched isotype control (blue line) and acquired by flow cytometry. Shown is the typical staining pattern obtained from three representative donors.

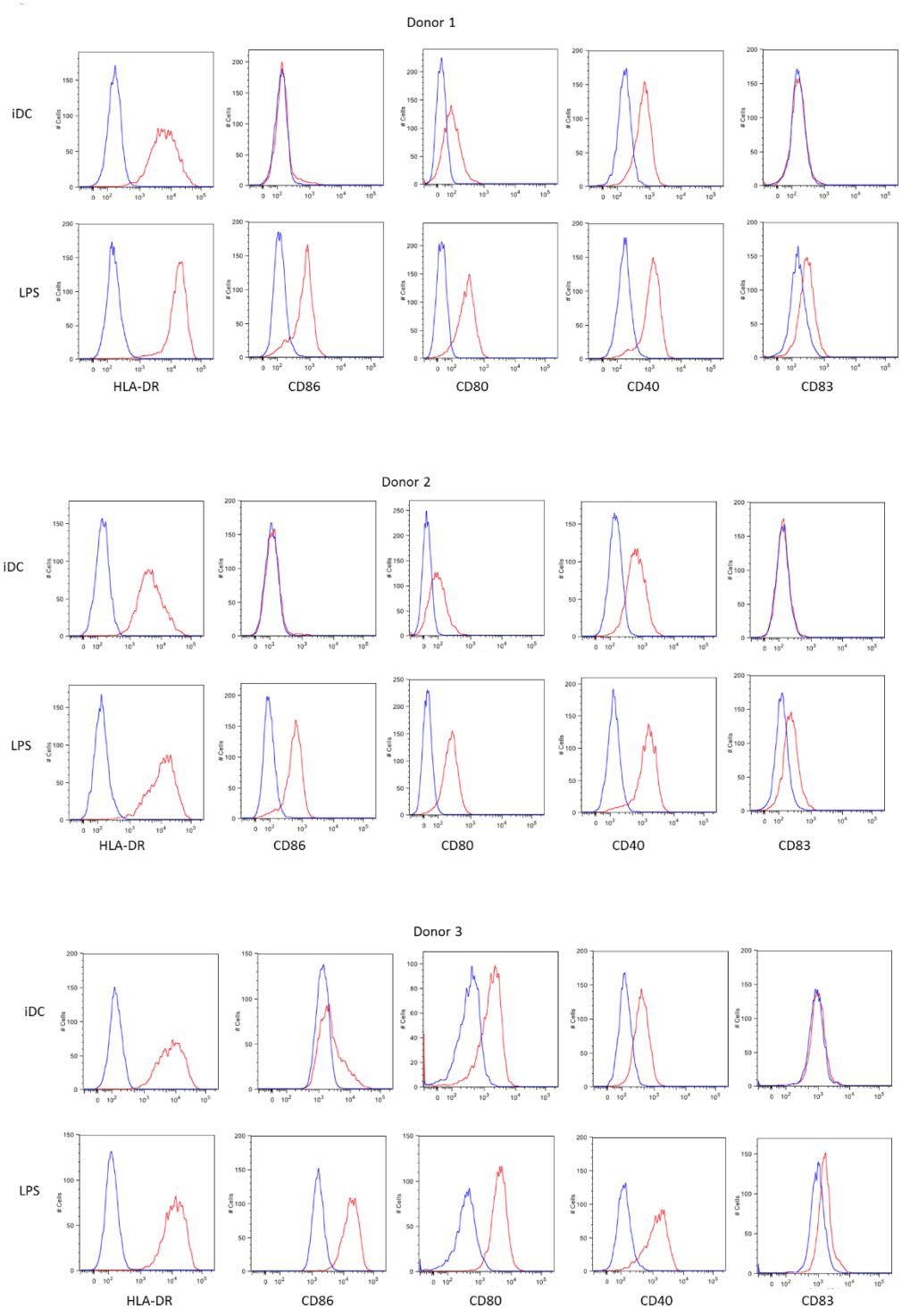


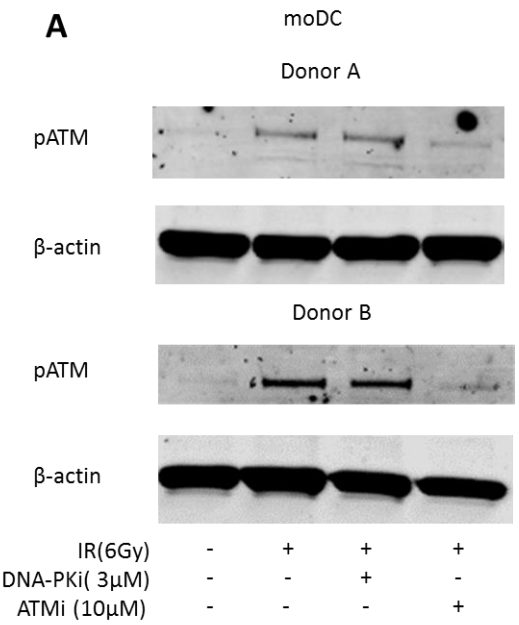
Figure 3.2 Phenotype of immature and mature DC

Immature DC and DC activated with LPS for 24 hr from 3 donors have been presented. Cells were stained with antibodies (red lines) and matched isotype

controls (blue lines) and acquired by flow-cytometry. Shown is typical staining pattern achieved compared to isotype control.

3.2.2 Expression of ATM and DNA-PK in monocytes, moDC and myDC

As DC have been shown to have basal level of activated ATM and DNA-PK [469], we aimed to compare the levels of phospho-ATM and phospho-DNA-PK in resting monocytes, moDC and CD1c+ myDC, and also in response to IR. moDC showed a low basal level of activated ATM which was increased following exposure to IR (Figure 3.3). As expected, phosphorylation of ATM was abolished by treatment with ATMi, but not DNA-PKi (Figure 3.3 A). There was no evidence of a phospho-ATM band in extracts of monocytes or isolated CD1c+ myDC. In contrast, both moDC and CD1c+ myDC had activated DNA-PK in their resting state and activation of DNA-PK was increased following IR treatment. Treatment with DNA-PKi, but not with ATMi, abrogated IR-induced activation of DNA-PK in both cell types (Figure 3.3B).



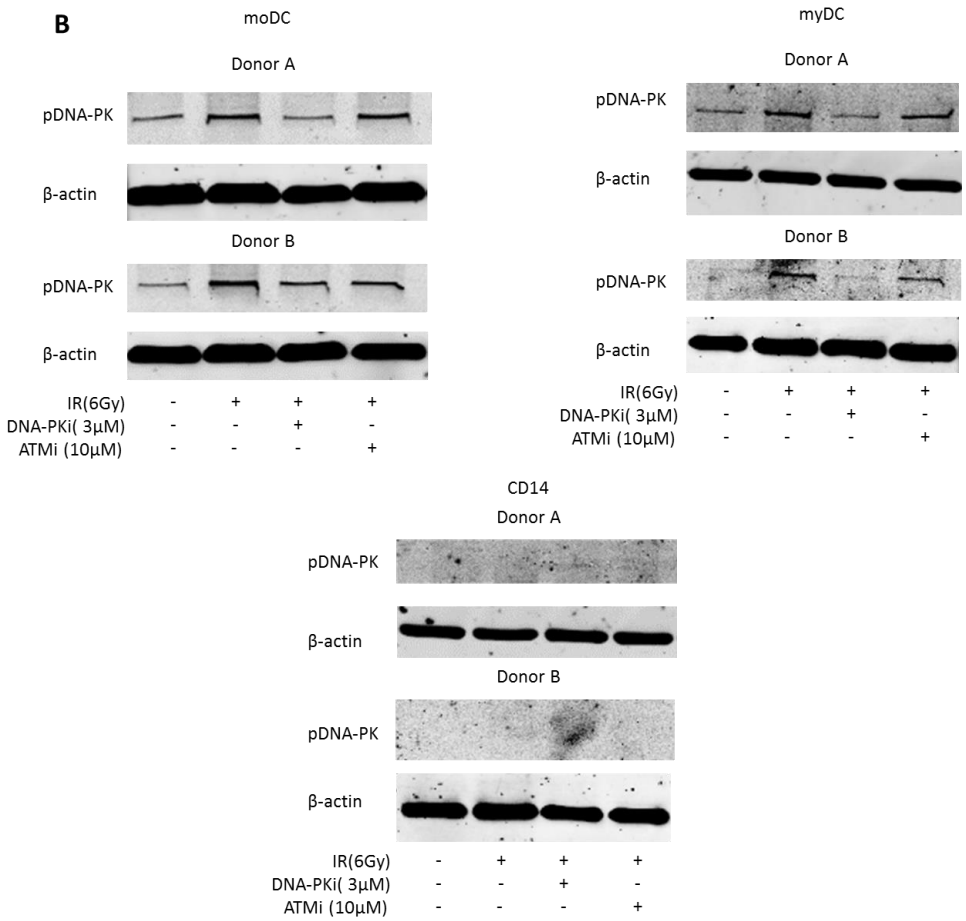


Figure 3.3 Expression of ATM and DNA-PK in DC

Western blots of phospho-ATM (Ser1981) in resting moDC and at 1 hr post IR (6Gy) in the presence or absence of ATMi/DNA-PKi for two donors in Figure 3.3 A and in CD14, moDC and myDC for phospho-DNA-PKcs (Ser 2056) in Figure 3.3 B have been presented. Cells were treated with either ATMi or DNA-PKi at the indicated concentrations for 1 hr prior to IR and then harvested at 1 hr post IR. B-actin was used as a loading control.

3.2.3 Monocytes are more sensitive to DNA damage than DC upon radiation

Human monocytes were isolated from peripheral blood of healthy donors and differentiated into DC by treatment with the cytokines IL-4 and GM-CSF. Maturation process was confirmed by checking the surface markers regularly. All these cells are non-proliferating, that is, monocytes and DC derived from them do not proliferate during the differentiation procedure and throughout the experiment. To compare the sensitivity of monocytes with DC, the cells were exposed to 6Gy radiation and apoptosis was measured using two different techniques over a period of 72 hr. In sub-G1 fraction assay, a fluorogenic compound, PI is used that binds stoichiometrically to nucleic acids. So fluorescence emission is proportional to the DNA content of the cell and when analyzed by a flow cytometer, apoptotic cell show a broad hypodiploid peak which can be easily distinguished from the narrow peak of cells with normal diploid DNA in the red fluorescence.

Even in the absence of radiation, monocytes showed a higher level of cell death than DC (Figure 3.1 and 3.2) which indicated the better survival potential of DC in prolonged in vitro cultures. When exposed to IR, monocytes exhibited a greater level of cell death than moDC. Whilst cell death was typically 40-50% for monocytes, at 48hr DC showed approximately less than 20% death. However by 72hr the proportion of subG1 fraction was similar for both cell types. DC were resistance to the genotoxic effect of IR as shown in figure 3.1D, whereas for monocytes that was not the case.

The mechanism of cell death was further investigated using annexin V/PI double staining method. One of the earlier events in apoptosis is the translocation of membrane phosphatidylserine (PS) from the inner to the outer surface of the plasma membrane without concomitant loss of membrane integrity. This change in plasma membrane can be detected by fluorochrome labelled Annexin-V, which has high affinity for PS in the presence of $Ca^{2+.}$ As the cell membrane is still intact at this stage, nuclear dye PI cannot enter the cells and early apoptosis can be discriminated from necrosis. Monocytes were found to be more prone to cell death following irradiation than DC (Figure 3.2). There was insignificant effect of IR on DC between the control group and irradiated group in terms of cell survival. Although for two donors (Figures 3.2 B & C lower panel) survival decreased with time in DC, there was no significant reduction in viability by IR compared to non-irradiated group (Figure 3.2D). Upon IR treatment in monocytes, more than 50% of the cells were dead by 24hr. However, in 2 out of 3 donors showed higher percentage of live cells at 72 hr post radiation (Figures 3.2 A & B upper panel). This might happened due to experimental error or the dead cells might become undetectable at the later time point. Overall, there was higher rate of cell death in monocytes compared to DC induced by IR (Figure 3.2 D).

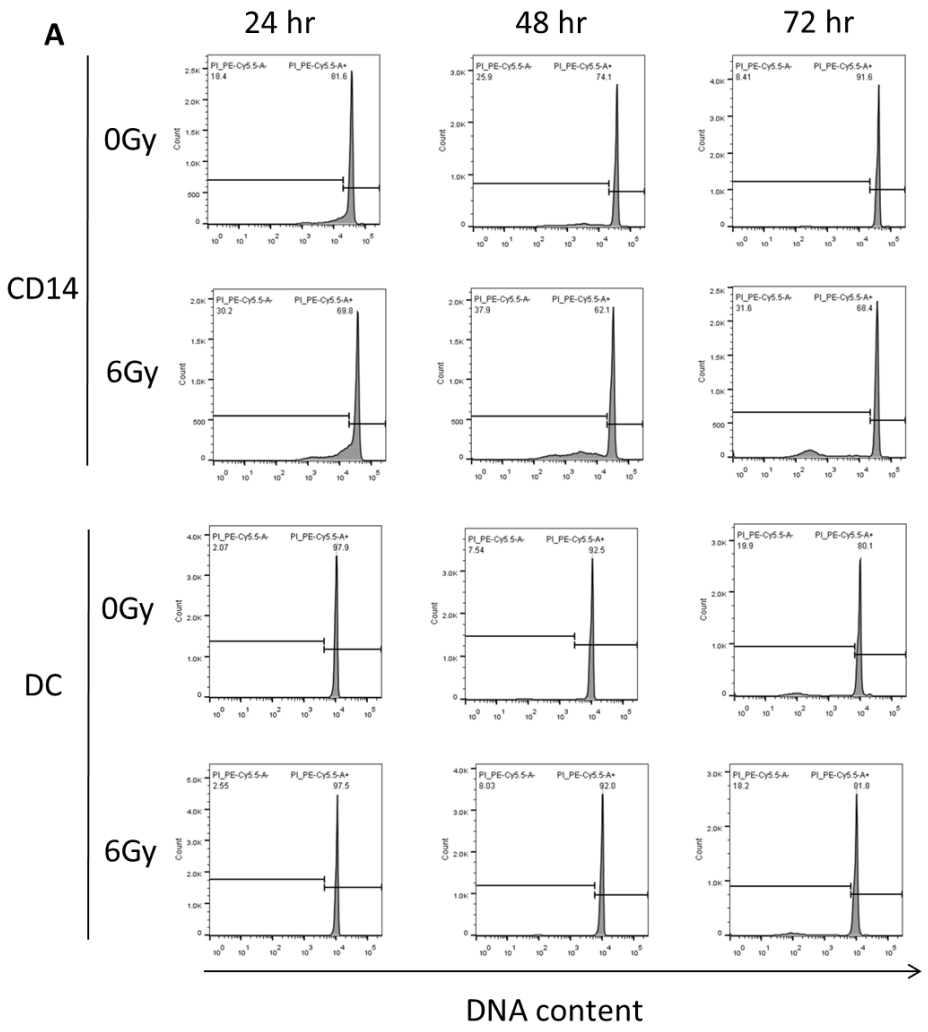


Figure 3.1A Assessment of sensitivity of monocytes to IR.

Purified monocytes (CD14+ cells) and DC derived from them were left untreated or exposed to IR (6Gy). Cells were incubated with PI to stain DNA and fluorescence was detected by flow cytometry. Cell death was determined by measuring the subG1 fraction assay at 24hr, 48hr and72 hr. This figure shows data from one donor with histogram showing the percentages of induced subG1 fraction and normal intact DNA in monocytes and DCs with or without IR.

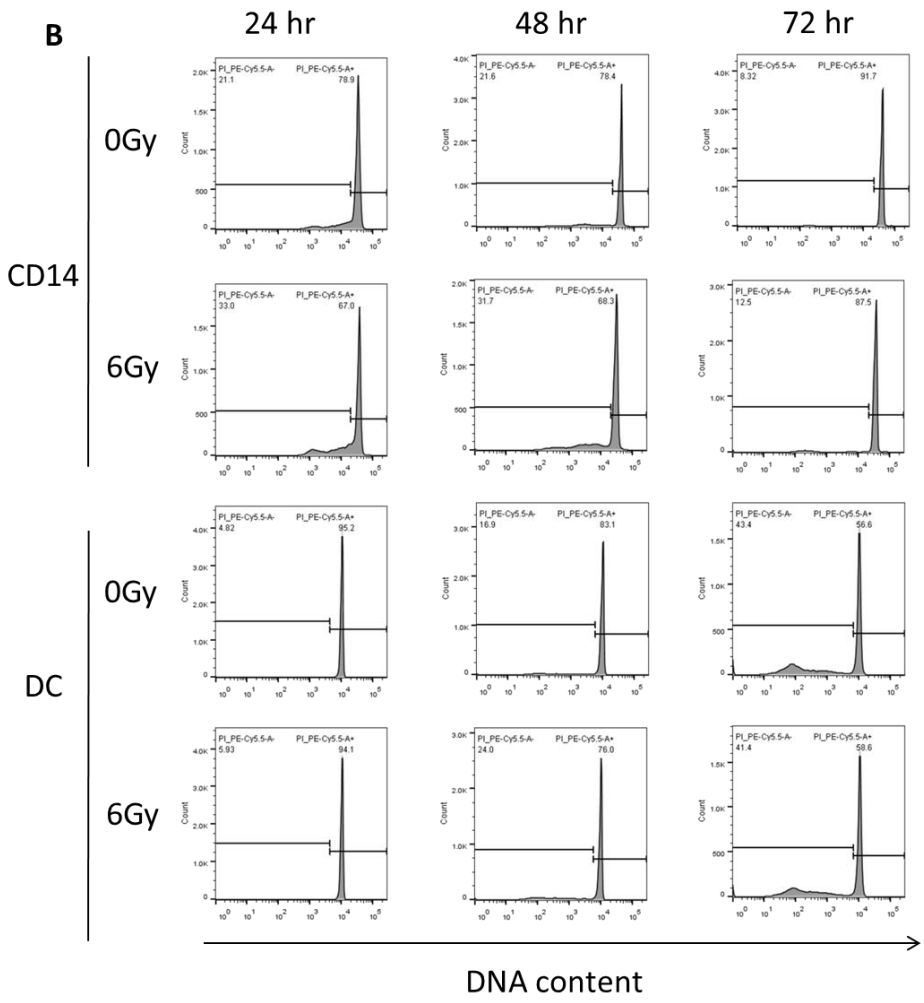


Figure 3.1B Assessment of sensitivity of monocytes to IR.

Purified monocytes (CD14+ cells) and DC derived from them were left untreated or exposed to IR (6Gy). Cells were incubated with PI to stain DNA and fluorescence was detected by flow cytometry. Cell death was determined by measuring the subG1 fraction assay at 24hr, 48hr and72 hr. This figure shows data from one donor with histogram showing the percentages of induced subG1 fraction and normal intact DNA in monocytes and DCs with or without IR.

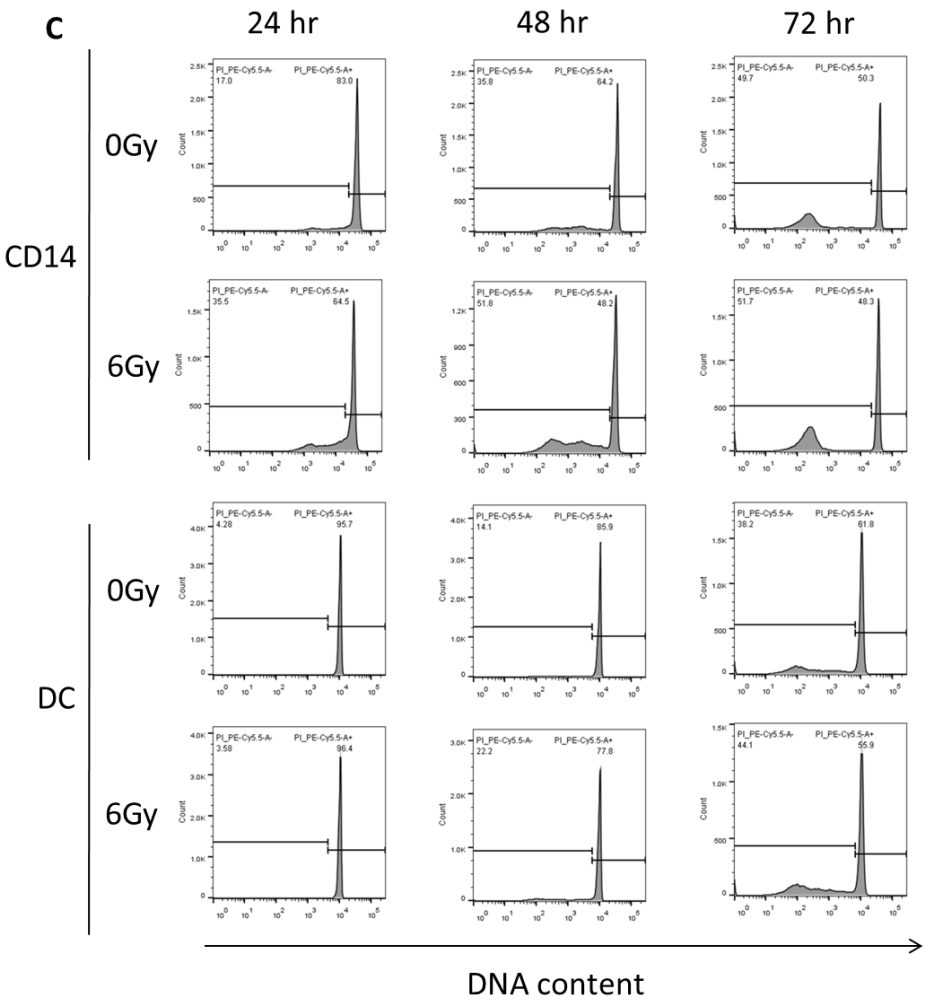


Figure 3.1C Assessment of sensitivity of monocytes to IR.

Purified monocytes (CD14+ cells) and DC derived from them were left untreated or exposed to IR (6Gy). Cells were incubated with PI to stain DNA and fluorescence was detected by flow cytometry. Cell death was determined by measuring the subG1 fraction assay at 24hr, 48hr and72 hr. This figure shows data from one donor with histogram showing the percentages of induced subG1 fraction and normal intact DNA in monocytes and DCs with or without IR.

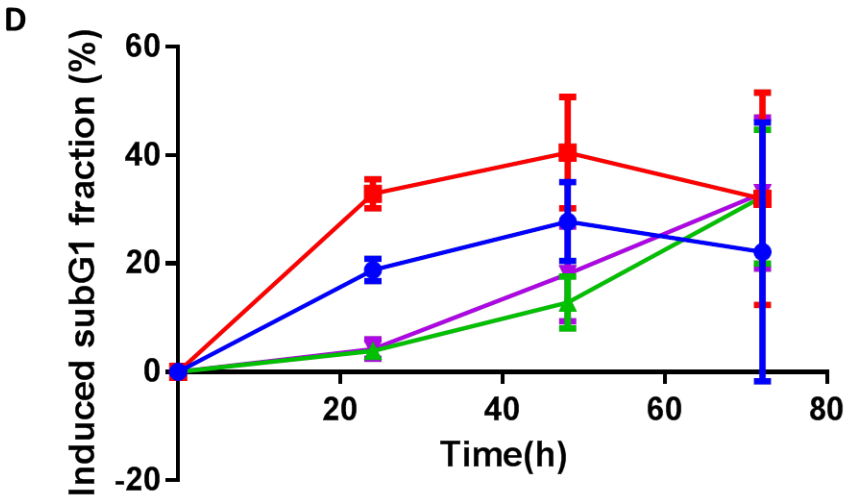


Figure 3.1D Assessment of sensitivity of monocytes and DCs to IR.

Figure D provides an overview of the data from all 3 the above donors summarizing the time dependence of cell death of monocytes and DCs after irradiation with 6Gy, blue line: untreated CD14, red line: CD14 exposed to IR, green line: untreated DC, violet line: DC exposed to IR. Data represent mean±SD.

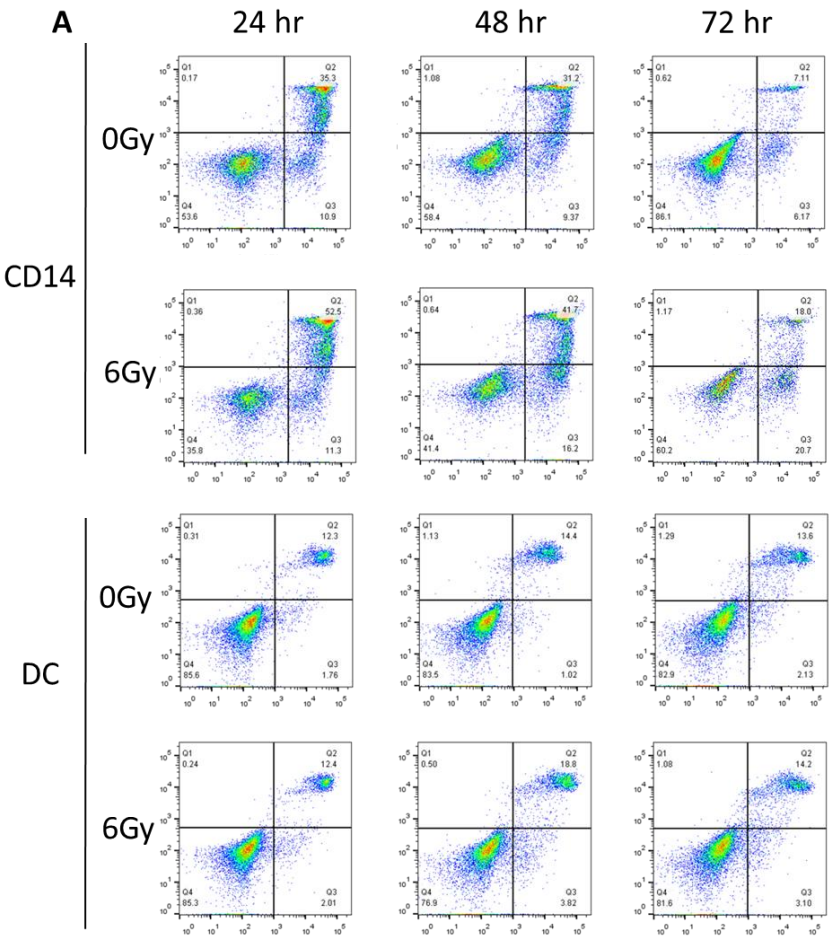


Figure 3.2A Determination of apoptotic response of monocytes and DCs induced by IR

Monocytes and DC were treated as described in the previous section. Frequency of induced apoptosis was determined by annexin V/PI double staining for 24hr, 48 hr and 72 hr. One have been represented where in each plot the population on the bottom right hand corner shows the proportion of early apoptotic cells and on the upper right hand corner is the percentage of late apoptotic cells.

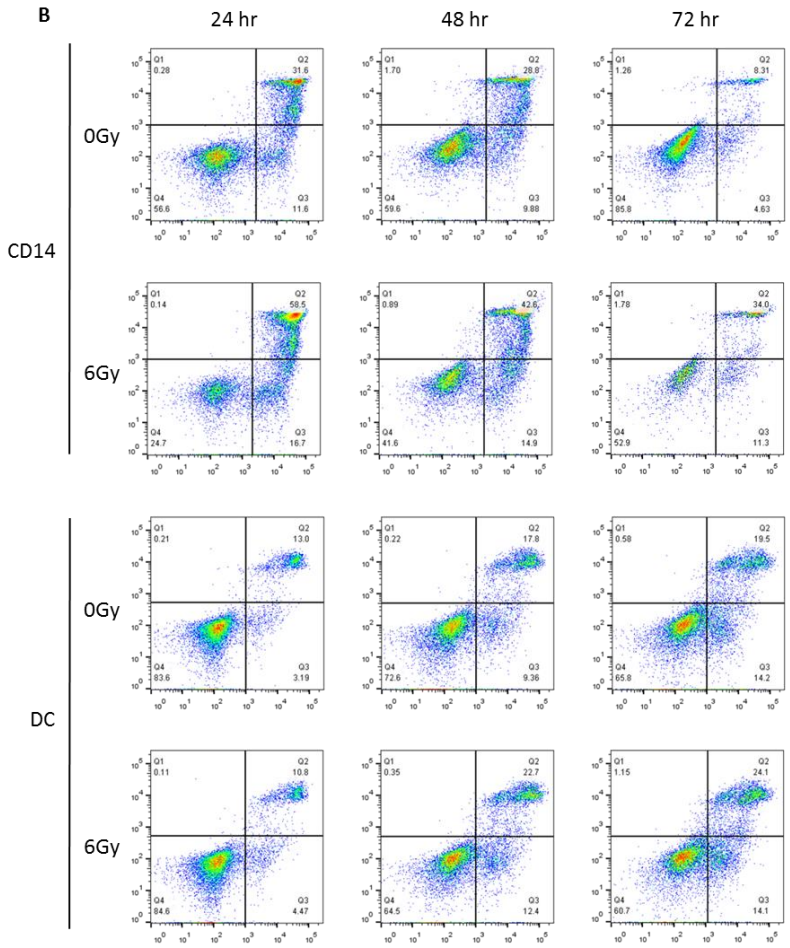


Figure 3.2B Determination of apoptotic response of monocytes and DCs induced by IR

Monocytes and DC were treated as described in the previous section. Frequency of induced apoptosis was determined by annexin V/PI double staining for 24hr, 48 hr and 72 hr. One have been represented where in each plot the population on the bottom right hand corner shows the proportion of early apoptotic cells and on the upper right hand corner is the percentage of late apoptotic cells.

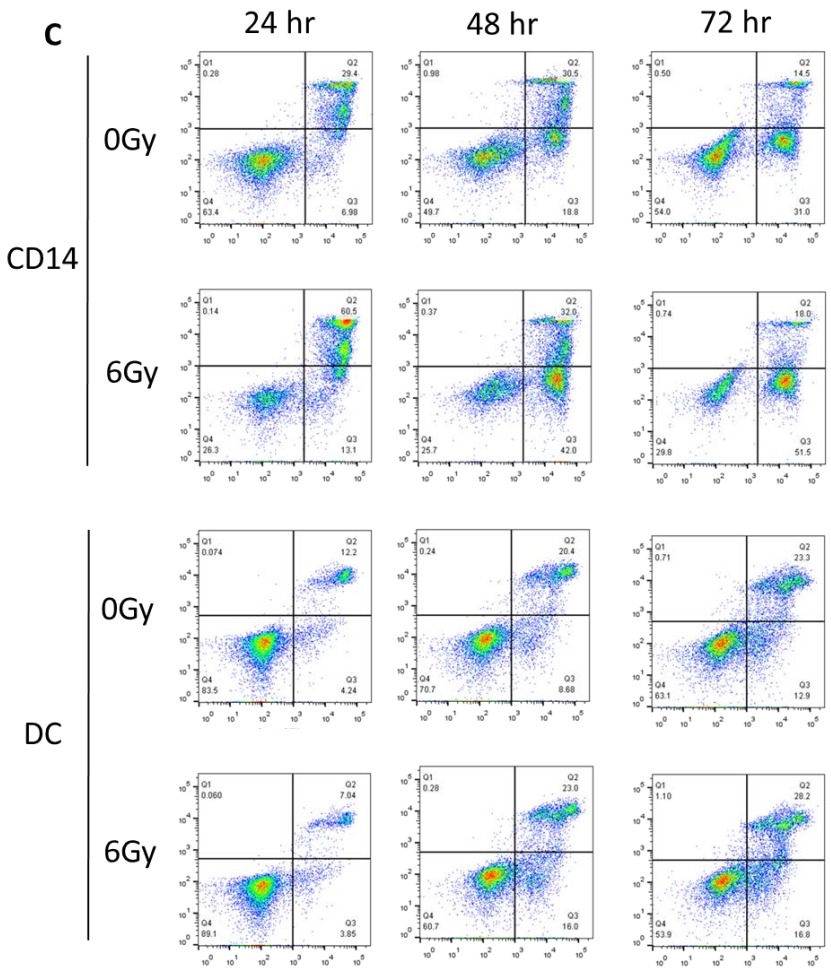


Figure 3.2C Determination of apoptotic response of monocytes and DCs induced by IR

Monocytes and DC were treated as described in the previous section. Frequency of induced apoptosis was determined by annexin V/PI double staining for 24hr, 48 hr and 72 hr. One have been represented where in each plot the population on the bottom right hand corner shows the proportion of early apoptotic cells and on the upper right hand corner is the percentage of late apoptotic cells.

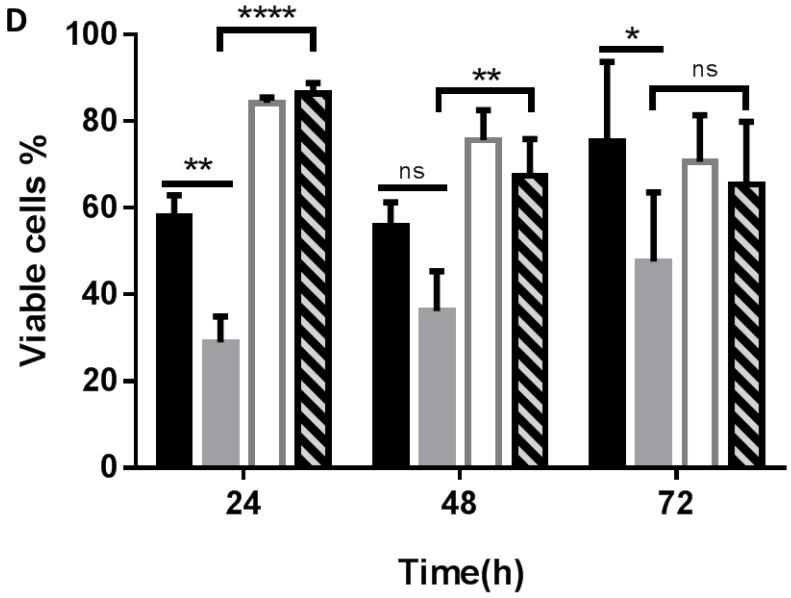


Figure 3.2D Determination of apoptotic response of monocytes and DCs induced by IR

The summary figure D, represents the frequency of cell survival (double negative cells) induced by IR for all three donors done as mean±SD. Black bar: untreated CD14, grey bar: CD14 exposed to IR, clear bar: untreated DC, hashed bar: DC exposed to IR. For D, the overall significance between groups was tested by two way ANOVA.

3.2.4 ATM inhibition confers protection against apoptosis in monocytes

ATM plays a central role in DDR pathway by initiating and coordinating DNA damage signalling that either causes cell cycle arrest or leads to apoptosis in case of unrepairable damage [274]. Although this protein only functions in DSB repair in proliferating cells, ATM has also been reported to become activated in resting cells exposed to genomic stress [469]. So we next determined the effect of blockade of ATM activation in monocytes and DC

following exposure to IR. In DC, inhibition of ATM activation on IR exposure demonstrated minimal or no additional effect in the formation of subG1 peak compared to the untreated group. In all the three donors done, there was a modest 10% increase in cell death in DC when pre-treated with ATMi (Figure 3.3 D, E, F). The same was observed for in Annexin V/PI uptake assay in DC (Figure 3.4 D, E, F) and overall the effect was non-significant (Figure 3.4 H). Interestingly in monocytes, ATM blockade appeared to provide survival benefit when exposed to IR. One of the donors had increased cell survival at three time points studied as demonstrated by both subG1 assay (Figure 3.3 C) and annexin V/PI double staining (Figure 3.4 C). For the other two donors (3.3 A &B), the effect was observed upto 48 hr which was also reflected in the summary figure (3.4 G) as significant increase in viable cells at 48 hr.

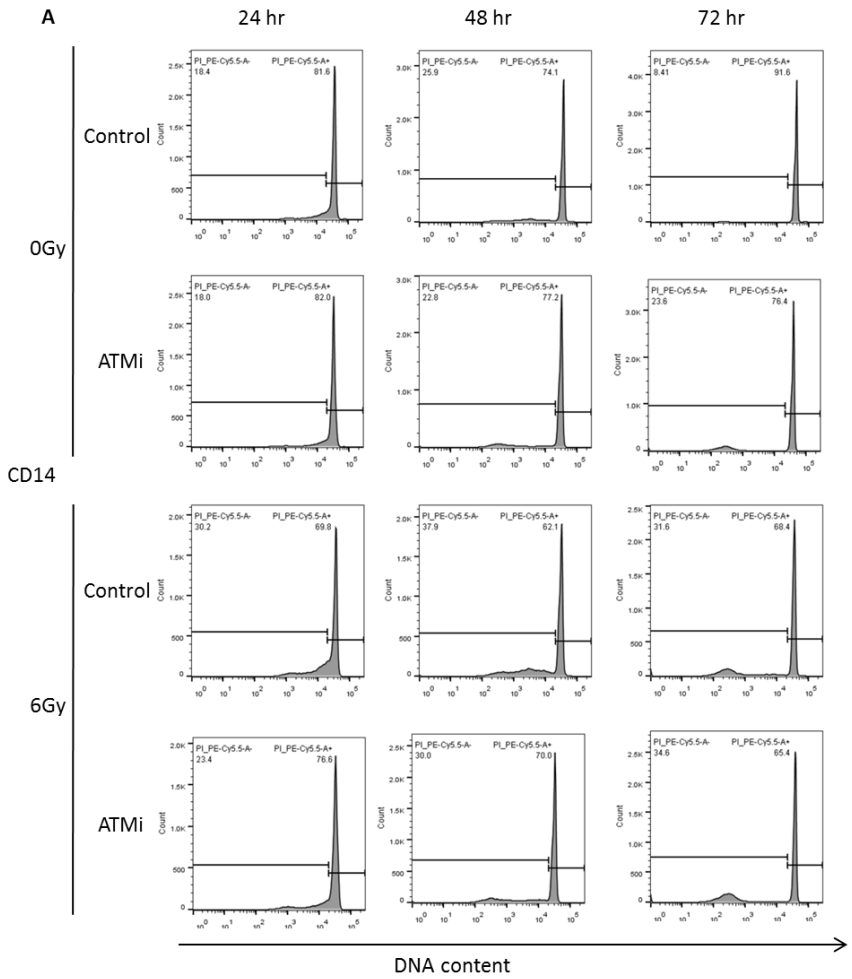


Figure 3.3A Effect of inhibition of ATM on apoptotic response of monocytes to IR

Cells were exposed to 6Gy IR after preincubation with 10µM ATMi inhibitor for 1 hr. The control groups were left untreated or only treated with ATMi but not exposed to IR. Similarly as described previously the amount of cell death as measured by the DNA fragmentation of dead cells was determined by PI staining over 72 hr. Monocyte from single donor was assessed for the effect of ATM inhibition on apoptosis induced by IR.

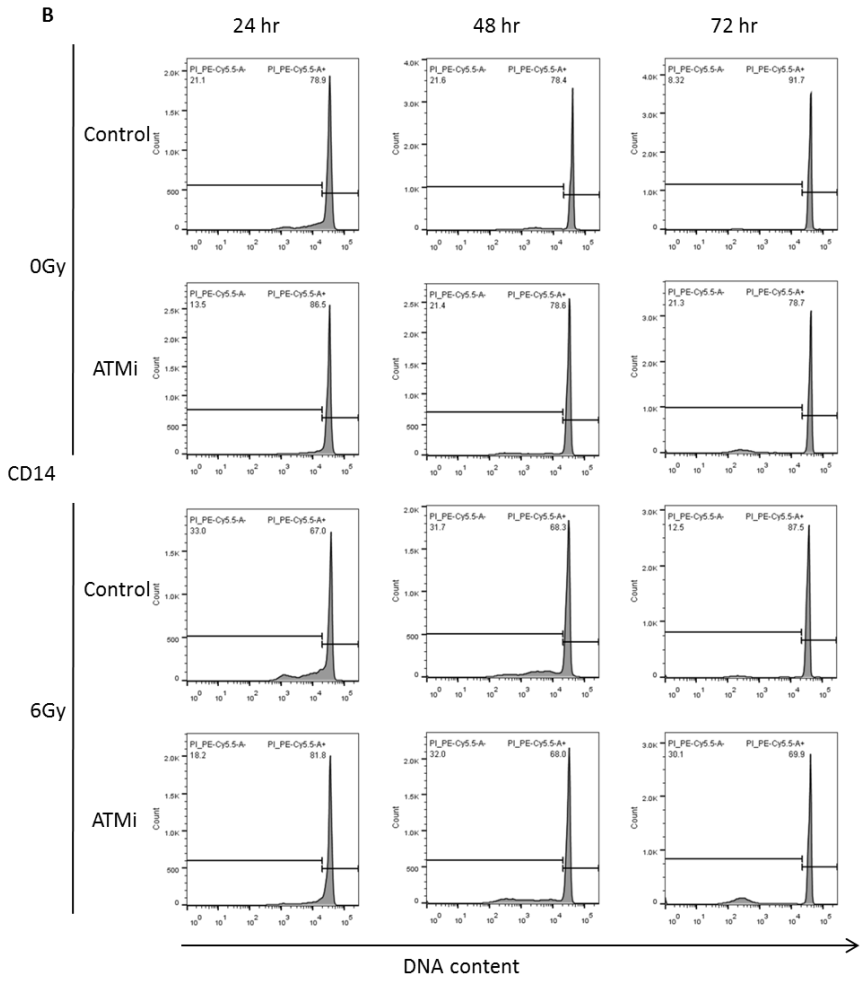


Figure 3.3B Effect of inhibition of ATM on apoptotic response of monocytes to IR

Cells were exposed to 6Gy IR after preincubation with 10µM ATMi inhibitor for 1 hr. The control groups were left untreated or only treated with ATMi but not exposed to IR. Similarly as described previously the amount of cell death as measured by the DNA fragmentation of dead cells was determined by PI staining over 72 hr. Monocyte from single donor was assessed for the effect of ATM inhibition on apoptosis induced by IR.

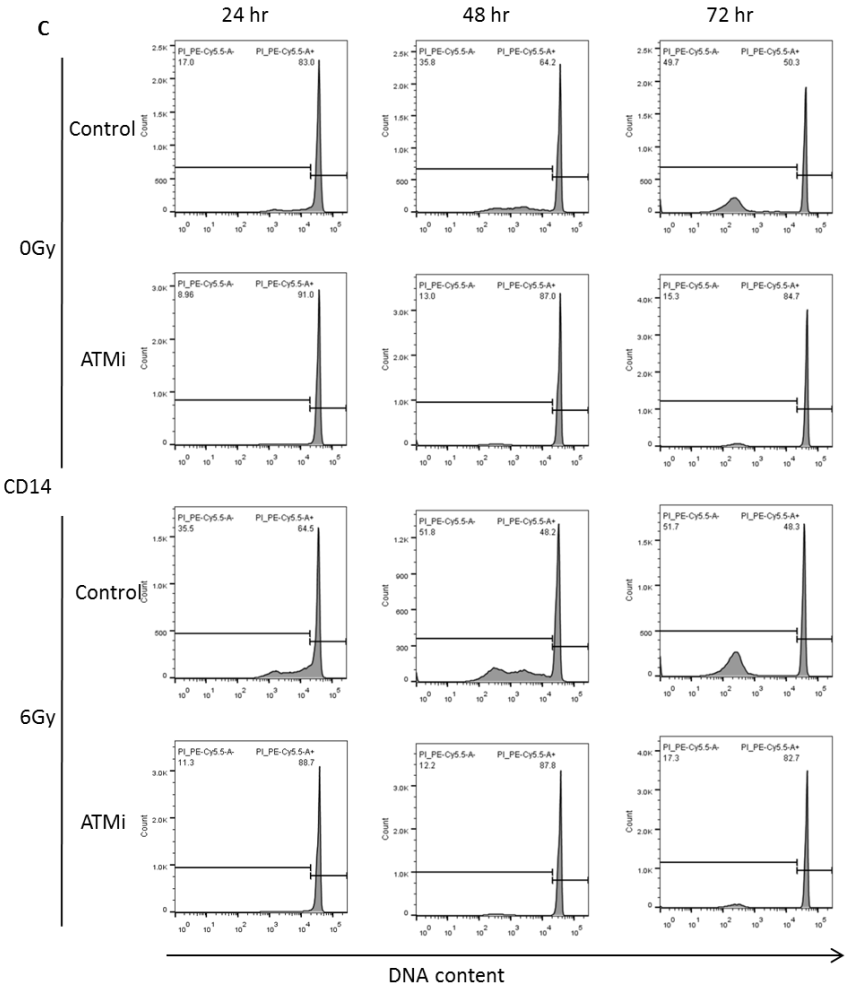


Figure 3.3C Effect of inhibition of ATM on apoptotic response of monocytes to IR

Cells were exposed to 6Gy IR after preincubation with 10µM ATMi inhibitor for 1 hr. The control groups were left untreated or only treated with ATMi but not exposed to IR. Similarly as described previously the amount of cell death as measured by the DNA fragmentation of dead cells was determined by PI staining over 72 hr. Monocyte from single donor was assessed for the effect of ATM inhibition on apoptosis induced by IR.

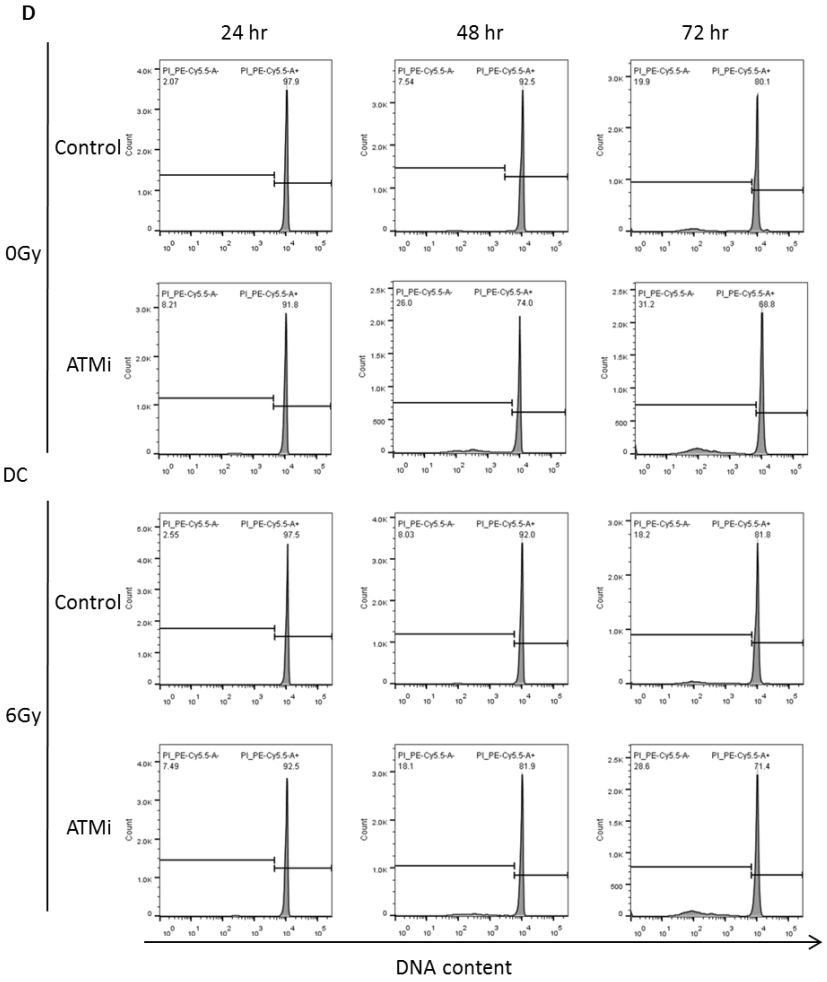


Figure 3.3D Effect of inhibition of ATM on apoptotic response of DC to IR

Cells were exposed to 6Gy IR after preincubation with 10µM ATMi inhibitor for 1 hr. The control groups were left untreated or only treated with ATMi but not exposed to IR. Similarly as described previously the amount of cell death as measured by the DNA fragmentation of dead cells was determined by PI staining over 72 hr. DC from single donor was assessed for the effect of ATM inhibition on apoptosis induced by IR.

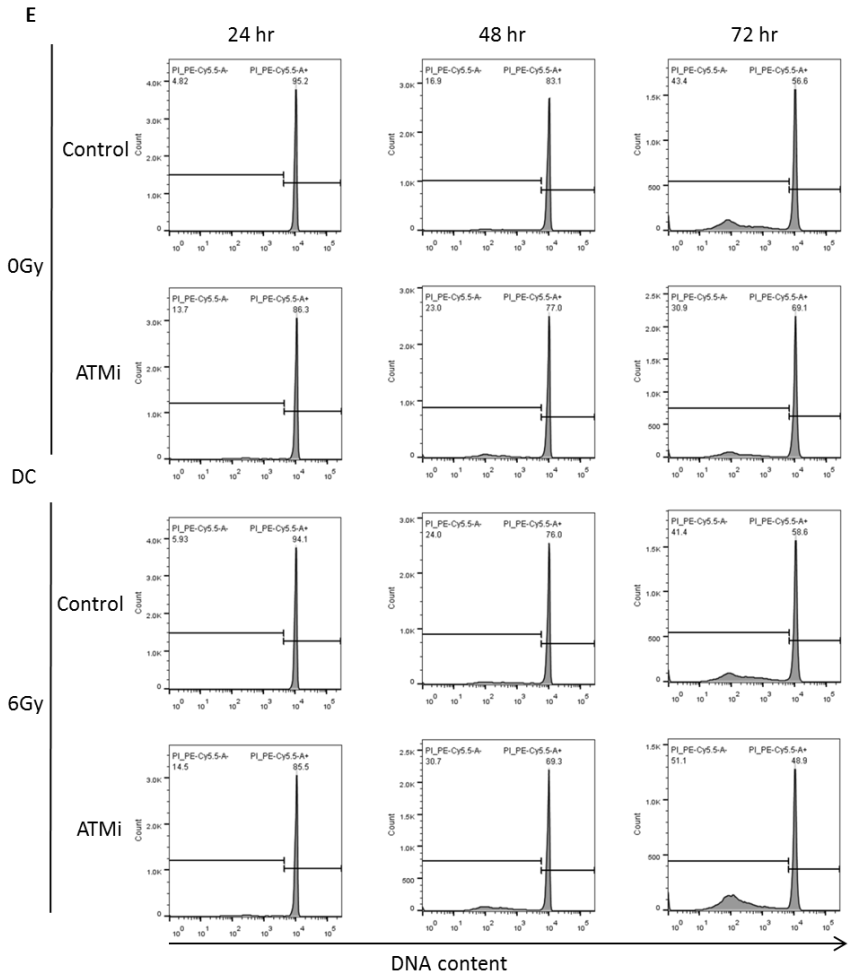


Figure 3.3E Effect of inhibition of ATM on apoptotic response of DC to IR

Cells were exposed to 6Gy IR after preincubation with 10µM ATMi inhibitor for 1 hr. The control groups were left untreated or only treated with ATMi but not exposed to IR. Similarly as described previously the amount of cell death as measured by the DNA fragmentation of dead cells was determined by PI staining over 72 hr. DC from single donor was assessed for the effect of ATM inhibition on apoptosis induced by IR.

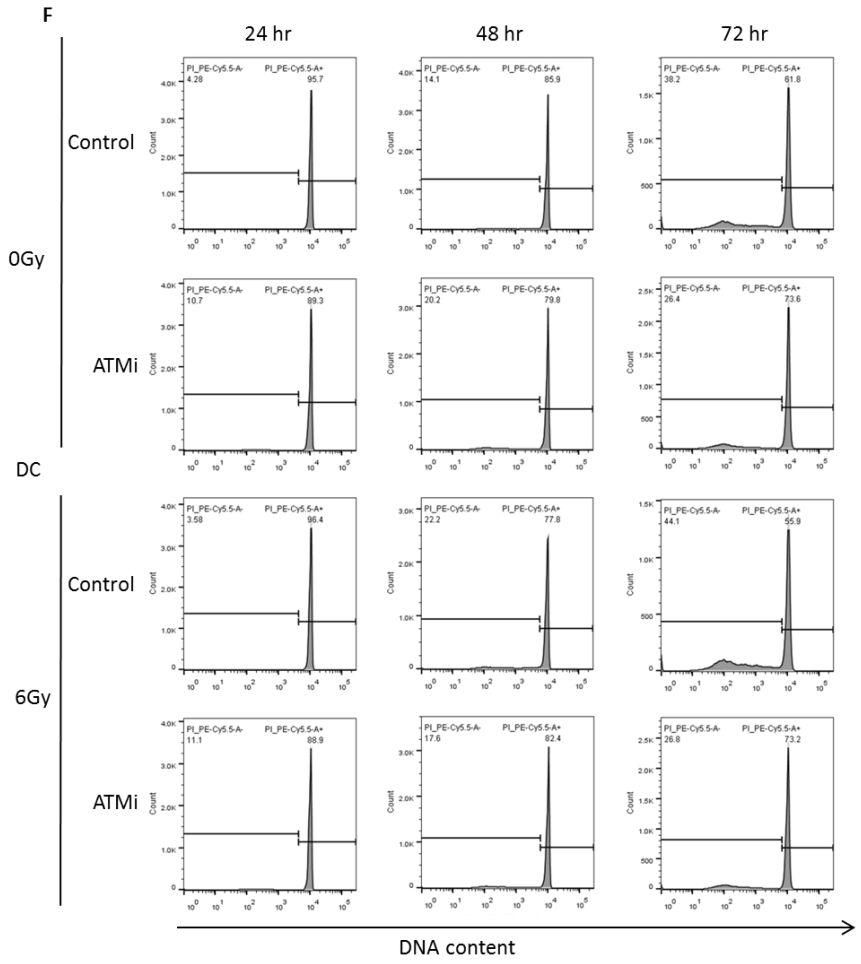


Figure 3.3F Effect of inhibition of ATM on apoptotic response of DC to IR

Cells were exposed to 6Gy IR after preincubation with 10µM ATMi inhibitor for 1 hr. The control groups were left untreated or only treated with ATMi but not exposed to IR. Similarly as described previously the amount of cell death as measured by the DNA fragmentation of dead cells was determined by PI staining over 72 hr. DC from single donor was assessed for the effect of ATM inhibition on apoptosis induced by IR.

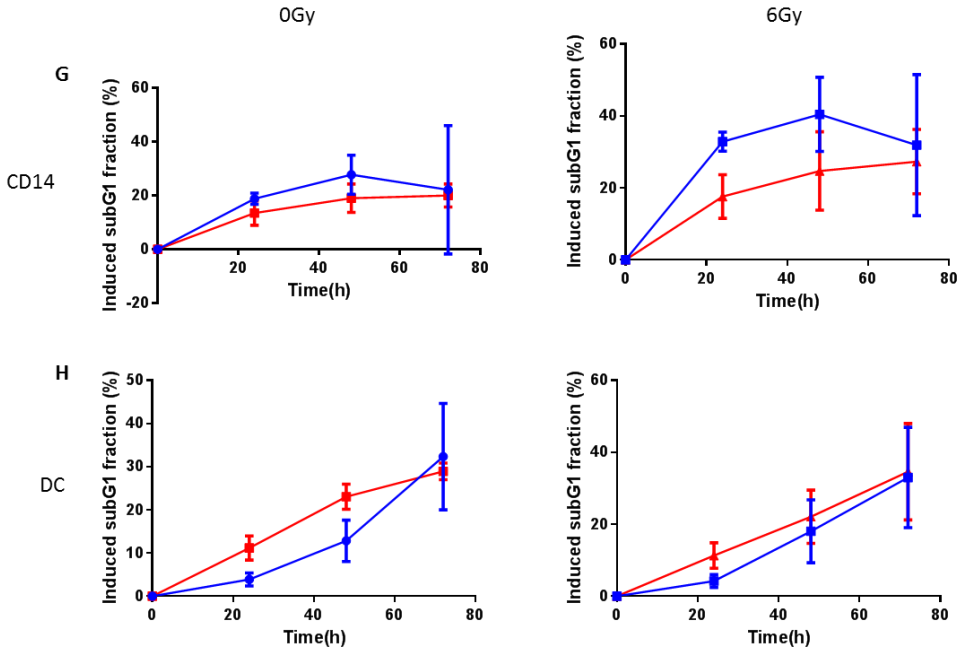


Figure 3.3G & H Effect of inhibition of ATM on apoptotic response of monocytes and DCs to IR

The overall conclusion have been drawn in figure G (monocytes) and H (DC) with the left panel showing ATM inhibition effect without IR and the right panel with IR. Blue line represent either untreated CD14 or DC and red line represents either CD14 or DC pretreated with ATMi. Error bar indicates SD of three independent experiments.

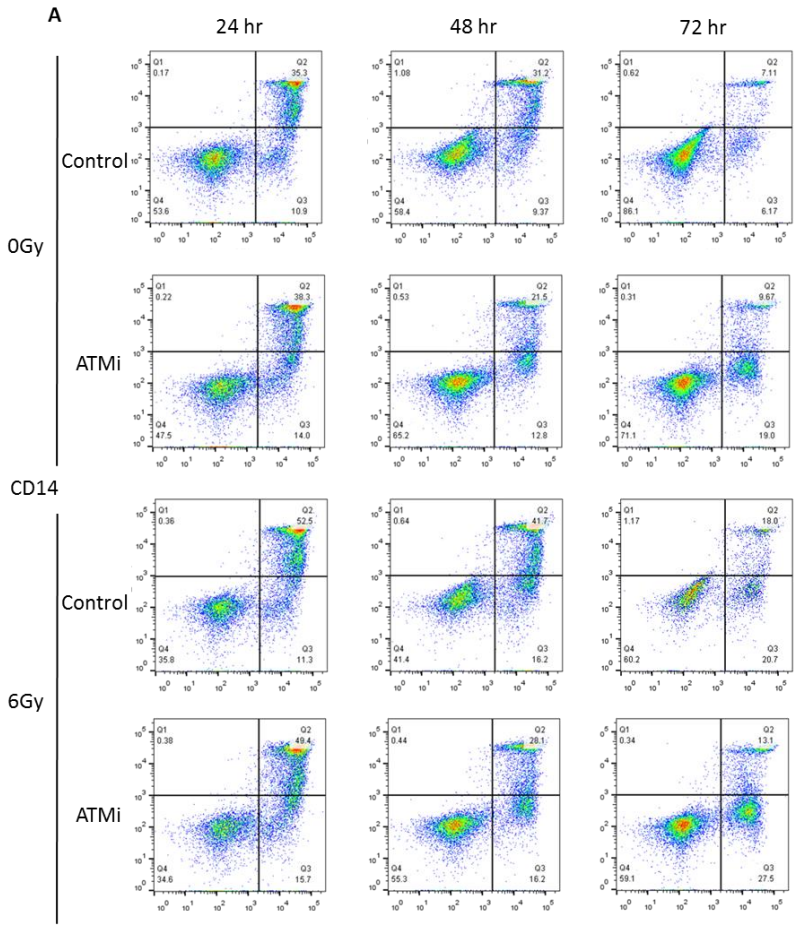


Figure 3.4A ATM favours cell survival in monocytes, but not in DC following exposure to IR

This figure demonstrates the survival benefit conferred by ATM blockade following exposure of monocytes to IR. Cell death was determined by annexin V/PI uptake assay. It show the late and early apoptosis of monocytes from single upon ATM inhibition and with or without radiation. Untreated monocytes were used as controls in both groups.

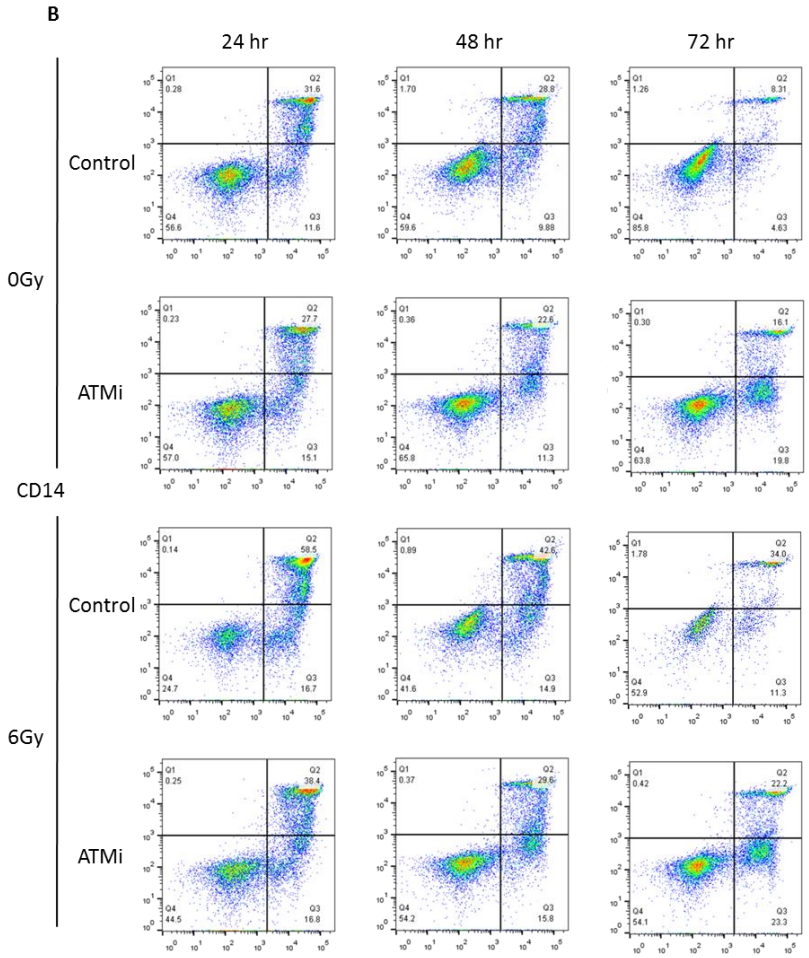


Figure 3.4B ATM favours cell survival in monocytes, but not in DC following exposure to IR

This figure demonstrates the survival benefit conferred by ATM blockade following exposure of monocytes to IR. Cell death was determined by annexin V/PI uptake assay. It show the late and early apoptosis of monocytes from single upon ATM inhibition and with or without radiation. Untreated monocytes were used as controls in both groups.

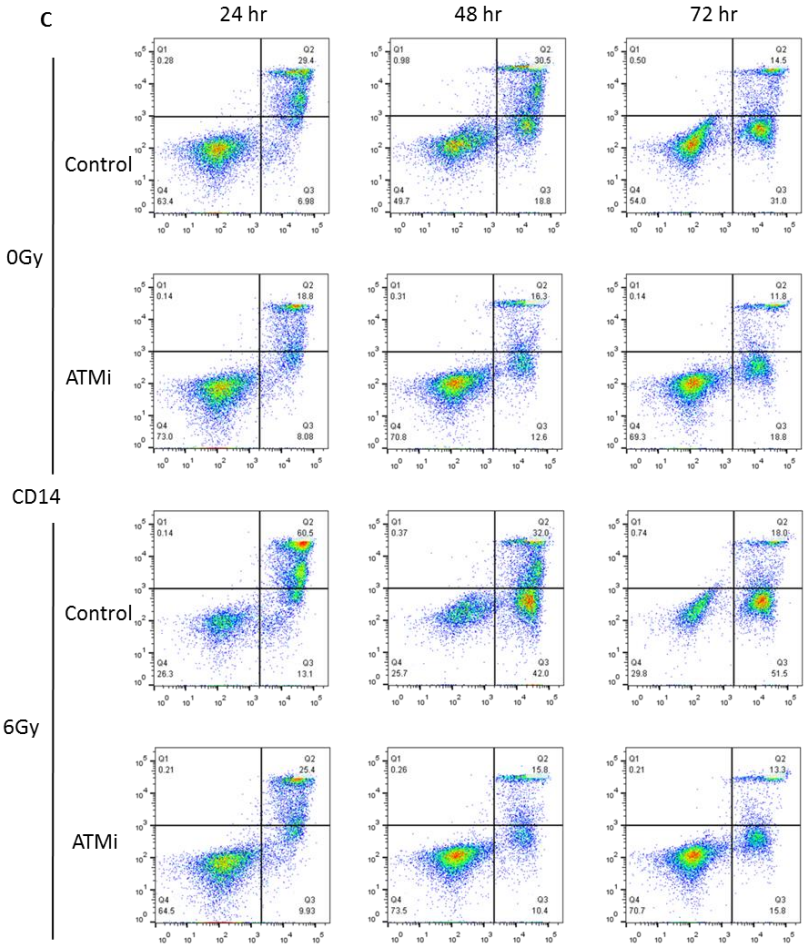


Figure 3.4C ATM favours cell survival in monocytes but not inDC following exposure to IR

This figure demonstrates the survival benefit conferred by ATM blockade following exposure of monocytes to IR. Cell death was determined by annexin V/PI uptake assay. It show the late and early apoptosis of monocytes from single upon ATM inhibition and with or without radiation. Untreated monocytes were used as controls in both groups.

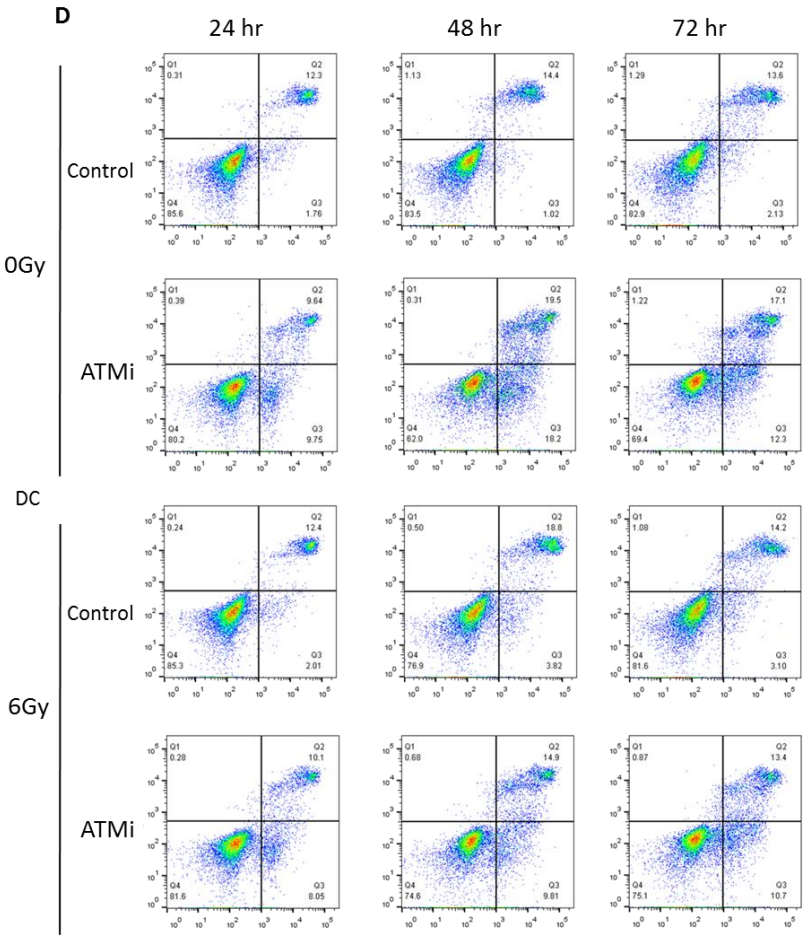


Figure 3.4D ATM favours cell survival in monocytes, but not in DC following exposure to IR

This figure demonstrates the survival benefit conferred by ATM blockade following exposure of monocytes to IR. Cell death was determined by annexin V/PI uptake assay. It show the late and early apoptosis of DC from single upon ATM inhibition and with or without radiation. Untreated DC were used as controls in both groups.

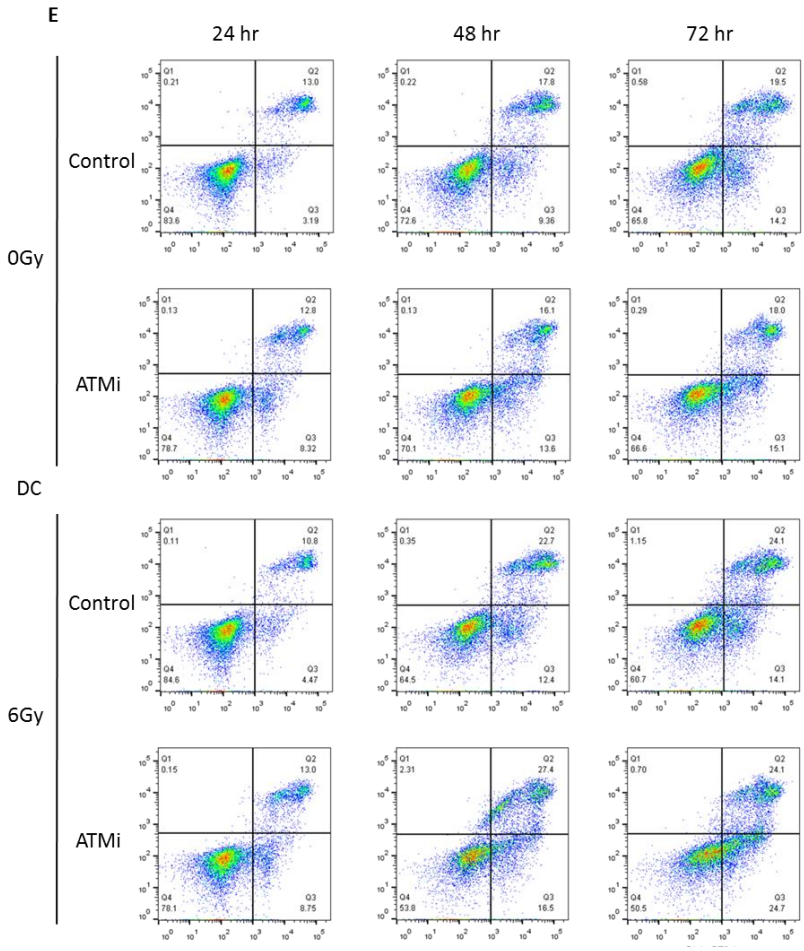


Figure 3.4E ATM favours cell survival in monocytes, but not in DC following exposure to IR

This figure demonstrates the survival benefit conferred by ATM blockade following exposure of monocytes to IR. Cell death was determined by annexin V/PI uptake assay. It show the late and early apoptosis of DC from single upon ATM inhibition and with or without radiation. Untreated DC were used as controls in both groups.

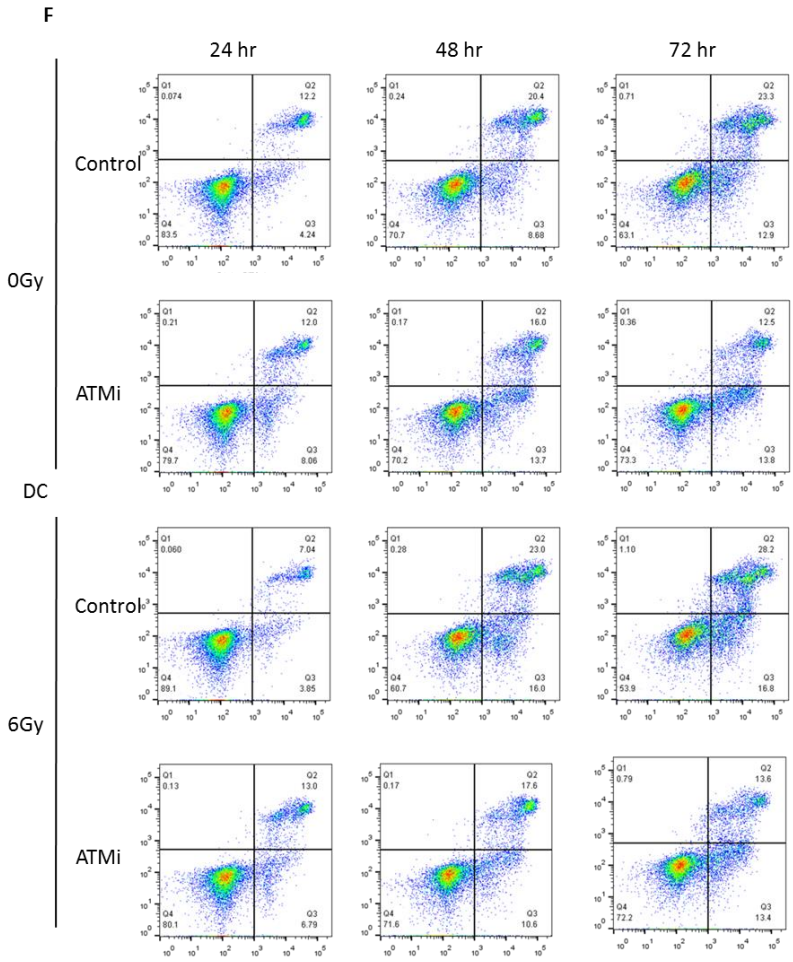


Figure 3.4F ATM favours cell survival in monocytes, but not in DC following exposure to IR

This figure demonstrates the survival benefit conferred by ATM blockade following exposure of monocytes to IR. Cell death was determined by annexin V/PI uptake assay. It show the late and early apoptosis of DC from single upon ATM inhibition and with or without radiation. Untreated DC were used as controls in both groups.

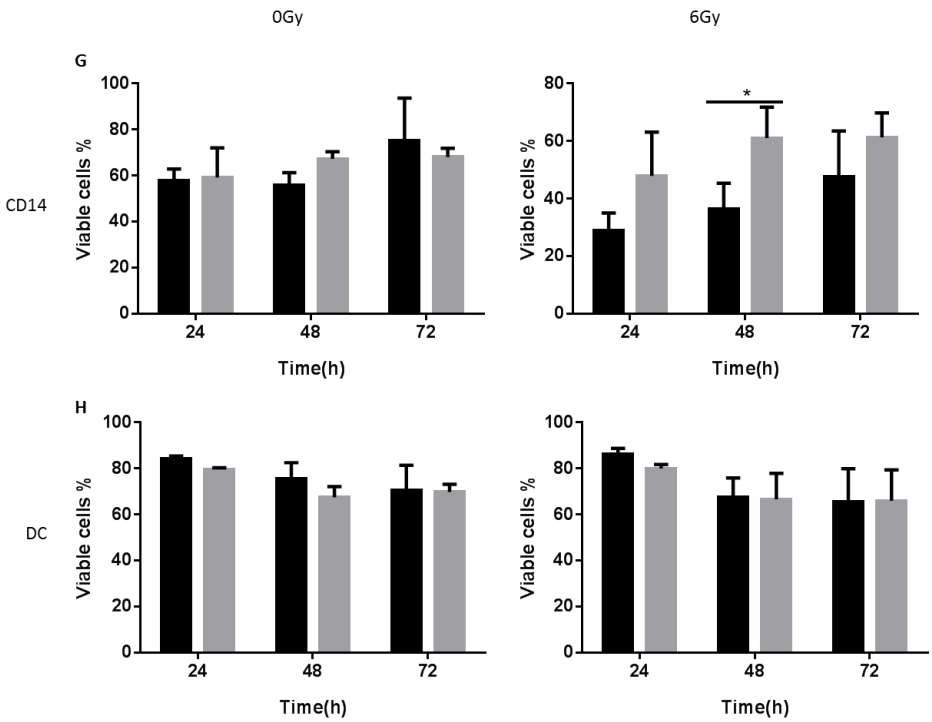


Figure 3.4G & H ATM favours cell survival in monocytes, but not in DC following exposure to IR

The overall summary of three donors and the comparison between control and treated groups have been represented as figure G and H (mean±SD) for monocytes and moDC respectively. Black bars represent either untreated CD14 or DC whilst grey bars show either CD14 or DC pretreated with ATMi. Two way Anova was performed to test the statistical significance between groups.

3.2.5 DNA-PK acts as the major double strand DNA break repair protein in resting monocytes and DCs

NHEJ is the principle DSB repair pathway in non-proliferating cells where the main player is DNA-PK [462]. So it was thought to be reasonable to determine the role of DNA-PK in cell survival in our cellular model systems following IR exposure. Cells were treated with 3µM DNA-PKi (NU7331) for 1 hr and then irradiated (6Gy). The DNA-PKi used in the study is an ATP-competitive

inhibitor [470]. Thus it inhibits the phosphorylation capacity of this kinase. This in turn inhibits its autophosphorylation which is the first step in the activation of DNA-PK [282]. Cell viability was severely impaired in monocytes upon DNA-PK blockade. As measured by the amount of DNA fragmentation, two donors showed less than 10% viable cells and the other one had approximately 20% viable cells after 72 hr (Figure 3.5 A, B, C). Overall, viability was lost significantly after 72 hr following DNA-PK inhibition even without radiation (Figure 3.6 G). DC were more radio-sensitive when treated with DNA-PKi, but the extent of cell death was nevertheless lower than in monocytes and not statistically significant (Figure 3.6 H). These observations were also supported by the alternate method of detection of apoptosis for monocytes (Figure 3.6 A, B, C) and DC (Figure D, E, F).

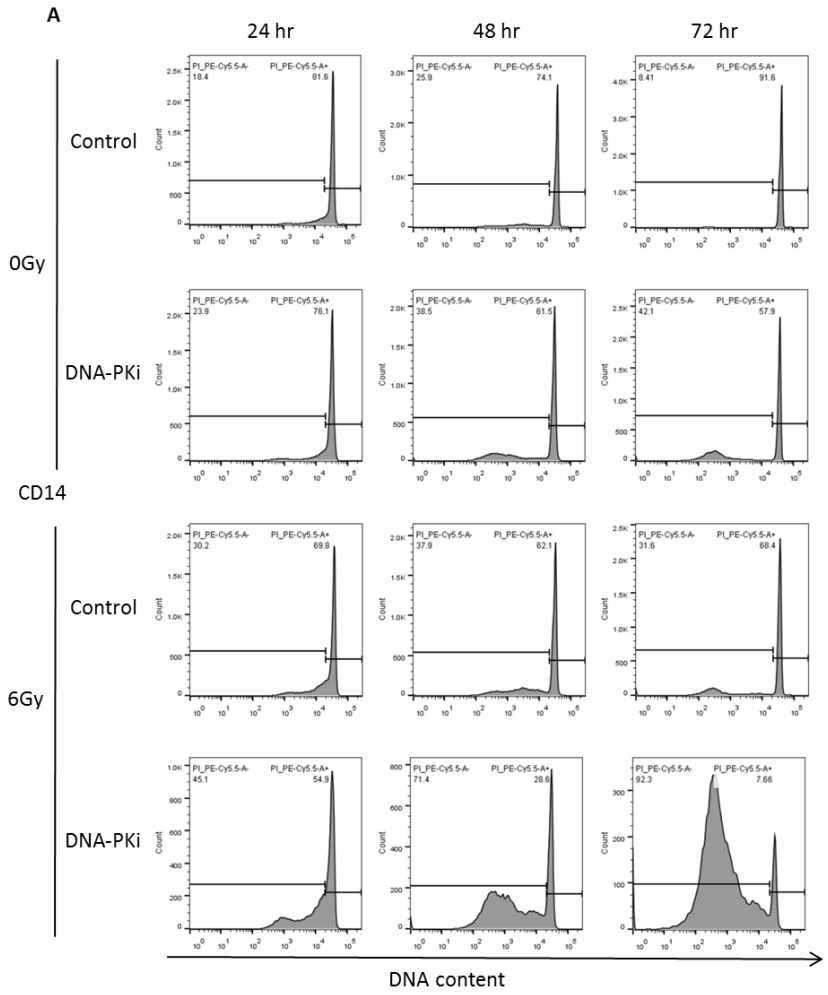


Figure 3.5A Inhibition of DNA-PK increases DNA damage and apoptosis in DC, but to a greater extent in monocytes

Freshly isolated monocytes were incubated with 3µM DNA-PKi inhibitor for an hour prior to exposure to IR. SubG1 fraction assay was carried out for 3 individual donors (A, B, C represent monocytes and D, E, F represent DC) at three time points. This figures represents one of the three donors. For each plot, Y axis denotes the number of events read by the flow cytometer and X axis indicates the intensity of the fluorescence in the red channel.

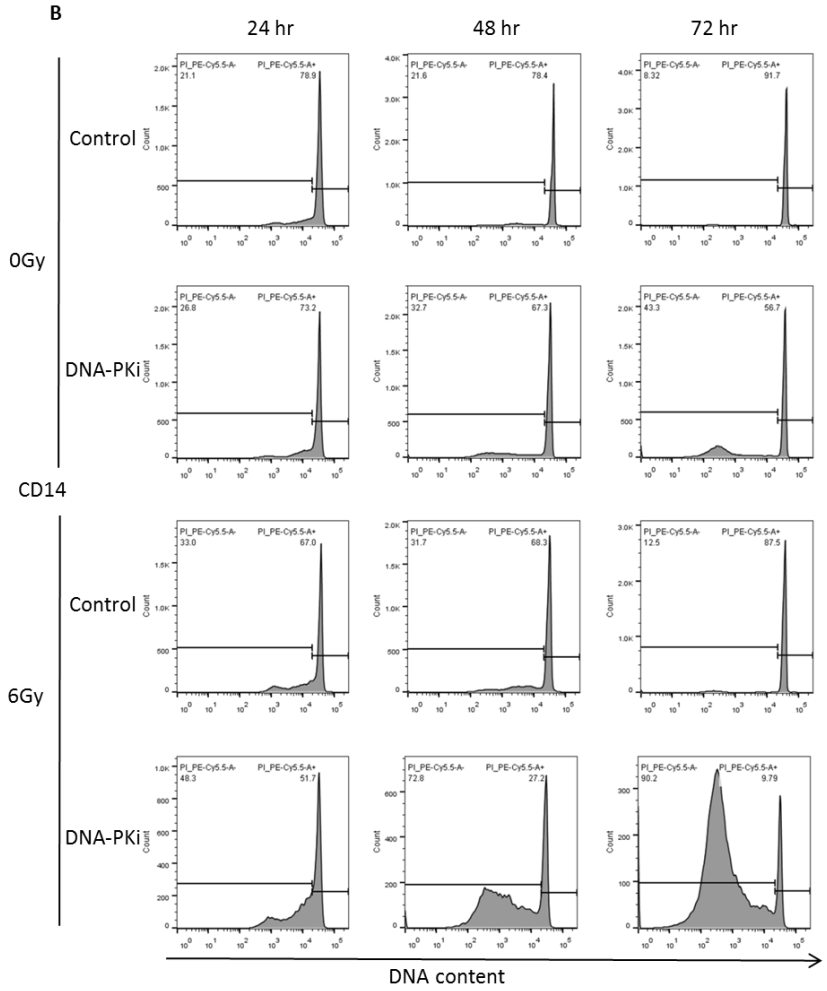


Figure 3.5B Inhibition of DNA-PK increases DNA damage and apoptosis in DC, but to a greater extent in monocytes

Freshly isolated monocytes were incubated with 3µM DNA-PKi inhibitor for an hour prior to exposure to IR. SubG1 fraction assay was carried out for 3 individual donors (A, B, C represent monocytes and D, E, F represent DC) at three time points. This figures represents one of the three donors. For each plot, Y axis denotes the number of events read by the flow cytometer and X axis indicates the intensity of the fluorescence in the red channel.

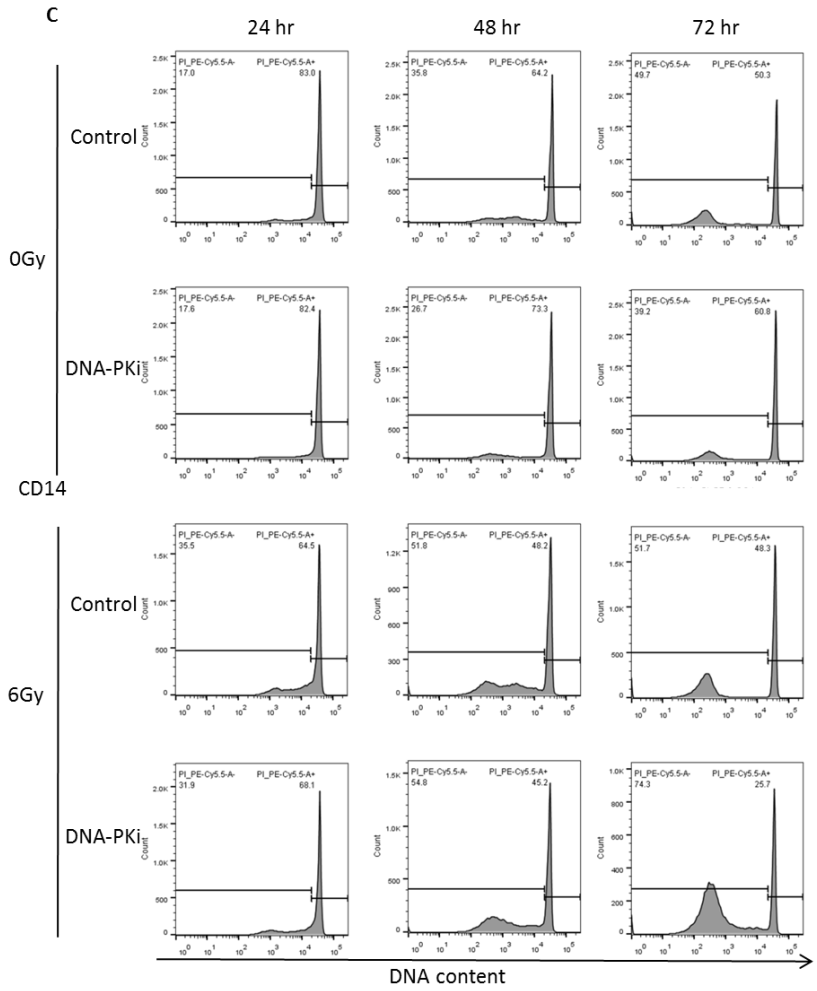


Figure 3.5C Inhibition of DNA-PK increases DNA damage and apoptosis in DC, but to a greater extent in monocytes

Freshly isolated monocytes were incubated with 3µM DNA-PKi inhibitor for an hour prior to exposure to IR. SubG1 fraction assay was carried out for 3 individual donors (A, B, C represent monocytes and D, E, F represent DC) at three time points. This figures represents one of the three donors. For each plot, Y axis denotes the number of events read by the flow cytometer and X axis indicates the intensity of the fluorescence in the red channel.

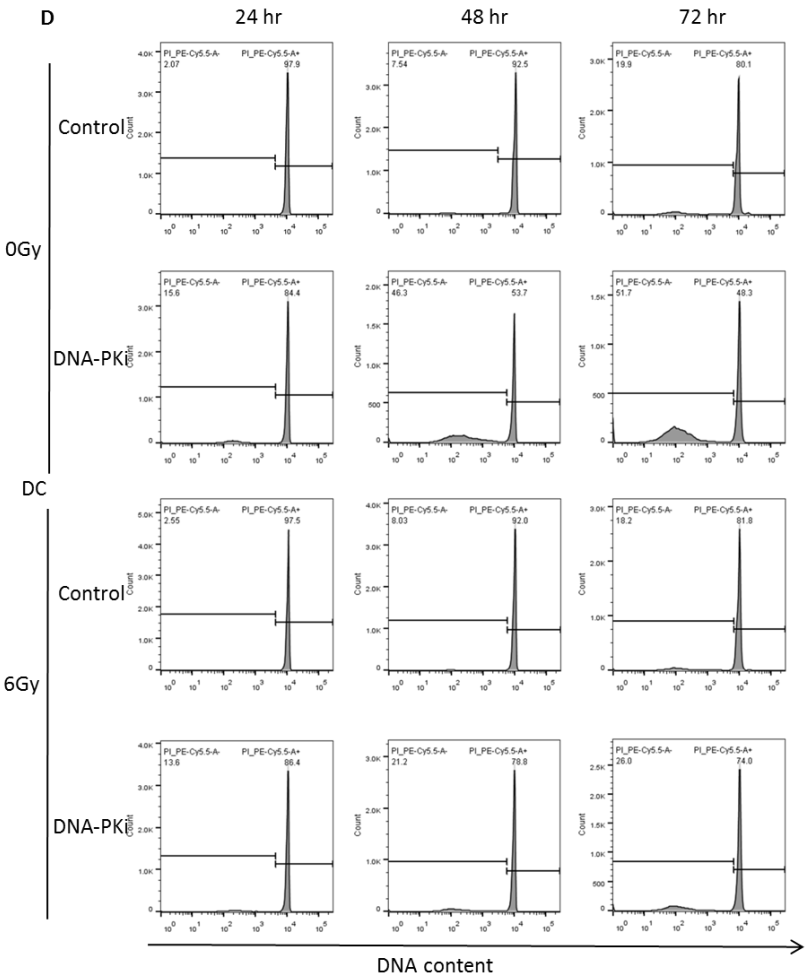


Figure 3.5D Inhibition of DNA-PK increases DNA damage and apoptosis in DC, but to a greater extent in monocytes

Monocyte derived DC were incubated with 3µM DNA-PKi inhibitor for an hour prior to exposure to IR. SubG1 fraction assay was carried out for 3 individual donors (A, B, C represent monocytes and D, E, F represent DC) at three time points. This figures represents one of the three donors. For each plot, Y axis denotes the number of events read by the flow cytometer and X axis indicates the intensity of the fluorescence in the red channel.

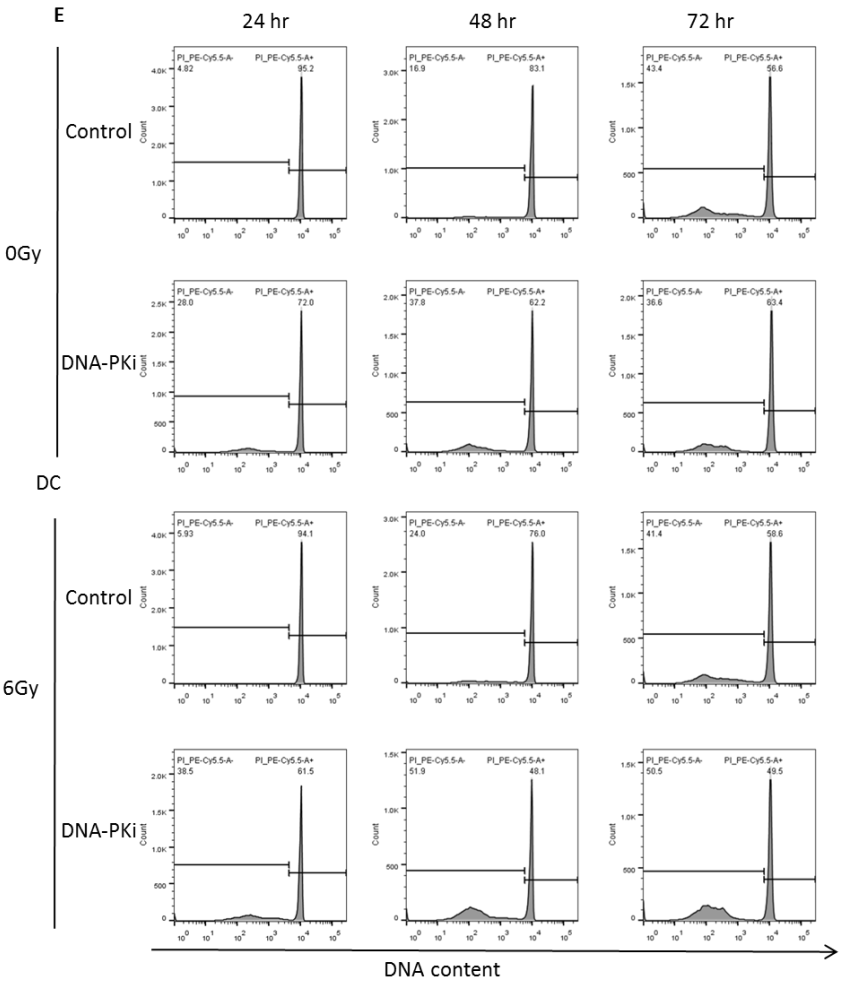


Figure 3.5E Inhibition of DNA-PK increases DNA damage and apoptosis in DC, but to a greater extent in monocytes

Monocyte derived DC were incubated with 3µM DNA-PKi inhibitor for an hour prior to exposure to IR. SubG1 fraction assay was carried out for 3 individual donors (A, B, C represent monocytes and D, E, F represent DC) at three time points. This figures represents one of the three donors. For each plot, Y axis denotes the number of events read by the flow cytometer and X axis indicates the intensity of the fluorescence in the red channel.

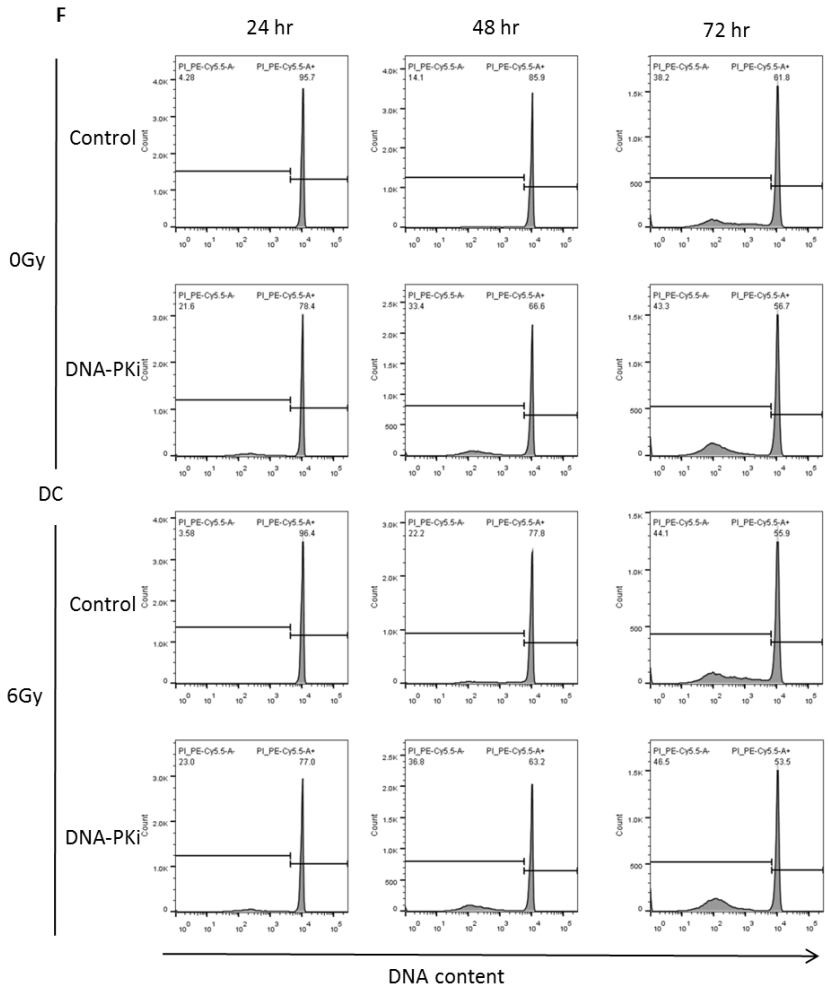


Figure 3.5F Inhibition of DNA-PK increases DNA damage and apoptosis in DC, but to a greater extent in monocytes

Monocyte derived DC were incubated with 3µM DNA-PKi inhibitor for an hour prior to exposure to IR. SubG1 fraction assay was carried out for 3 individual donors (A, B, C represent monocytes and D, E, F represent DC) at three time points. This figures represents one of the three donors. For each plot, Y axis denotes the number of events read by the flow cytometer and X axis indicates the intensity of the fluorescence in the red channel.

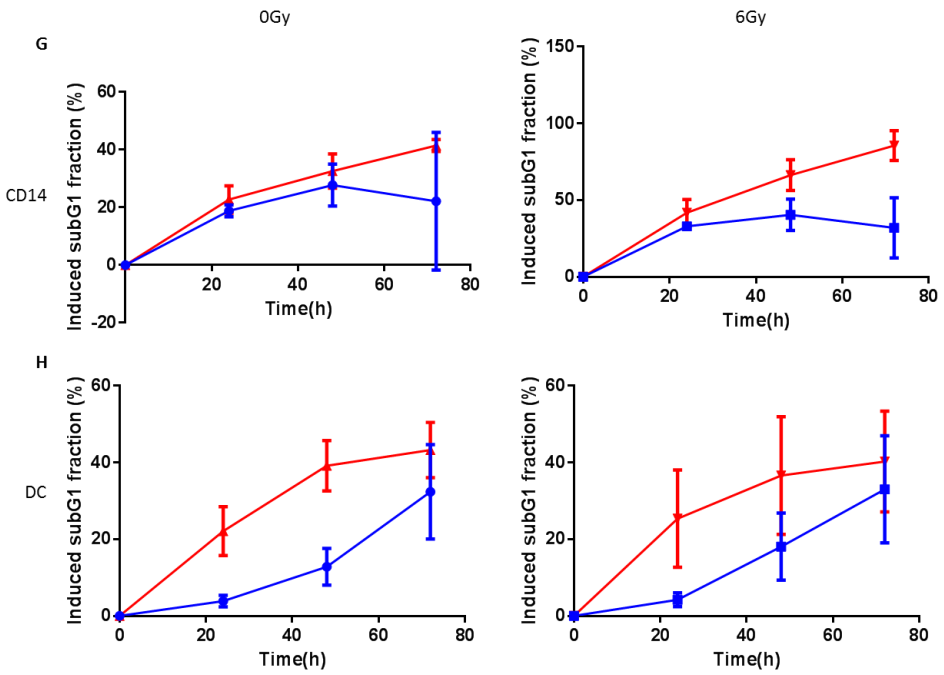


Figure 3.5G & H Inhibition of DNA-PK increases DNA damage and apoptosis in DC, but to a greater extent in monocytes

To summarize the whole experiment, mean±SD of the three donors was plotted for each group and compared with the untreated group (G, H). Blue line represent either untreated CD14 or DC and red line represents either CD14 or DC pretreated with DNA-PKi.

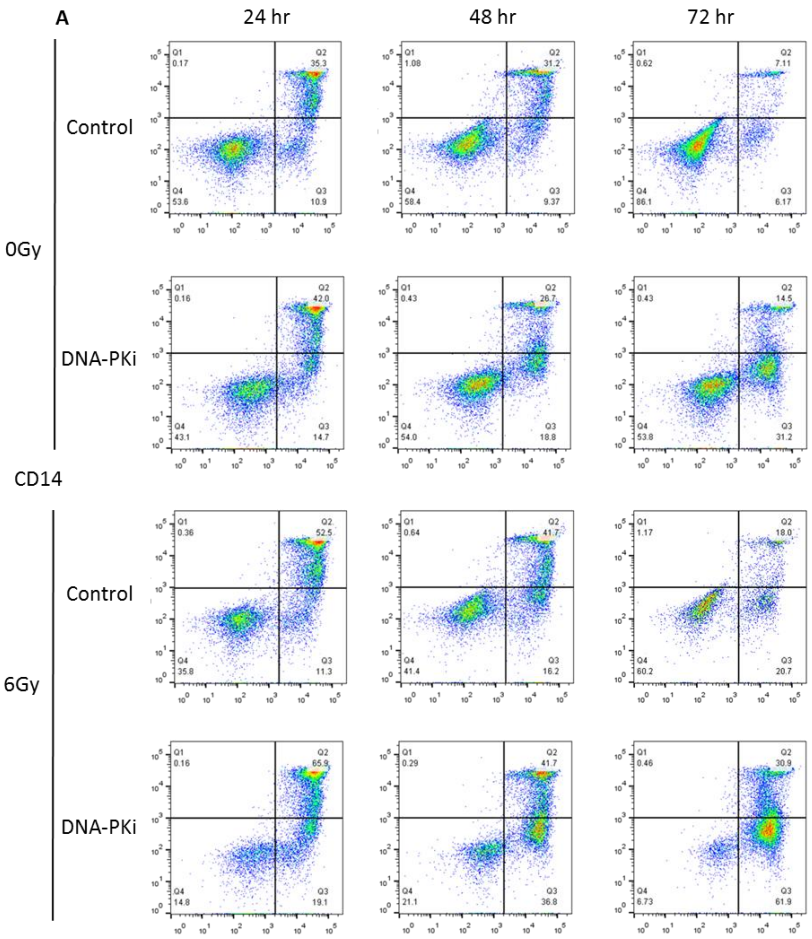


Figure 3.6A Determination of cell death upon DNA-PK inhibition by alternative method in monocytes and DC

The outcome of annexin V/PI double staining of DNA-PK inhibited monocytes following 6Gy irradiation for one donor is shown. On the quadrant plot, double negative cells are on the left bottom corner, annexin V single positive cells (early apoptotic) on the right bottom corner and double positive cells on the right upper corner.

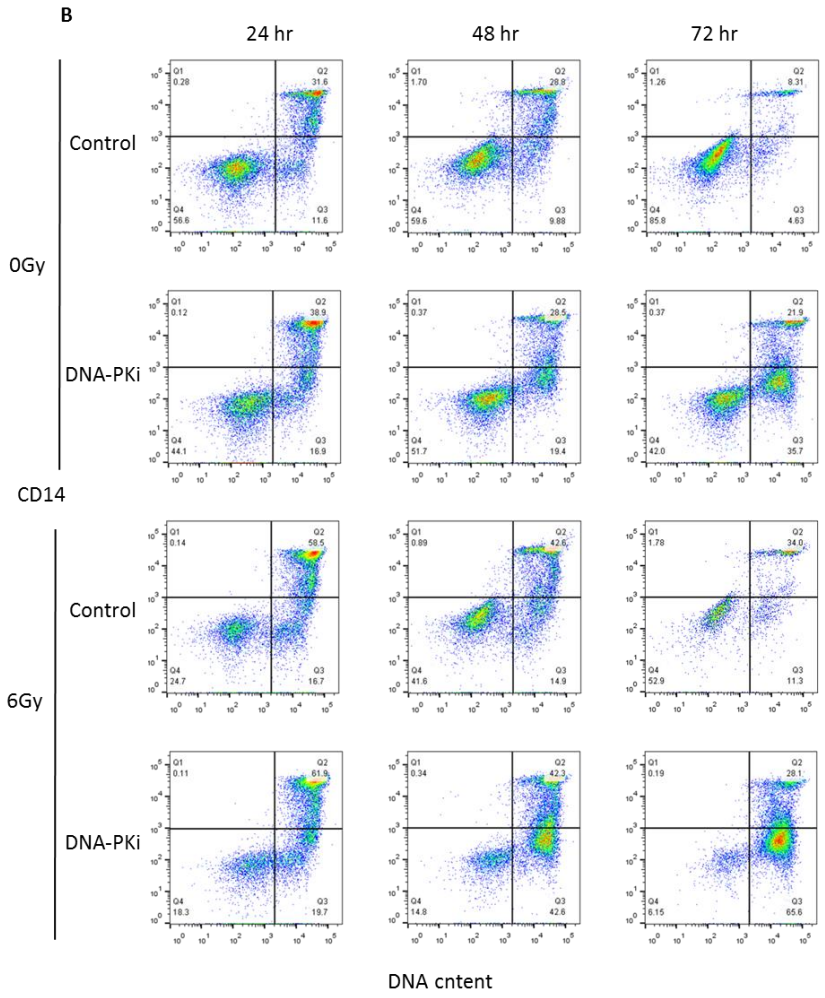


Figure 3.6B Determination of cell death upon DNA-PK inhibition by alternative method in monocytes and DC

The outcome of annexin V/PI double staining of DNA-PK inhibited monocytes following 6Gy irradiation for one donor is shown. On the quadrant plot, double negative cells are on the left bottom corner, annexin V single positive cells (early apoptotic) on the right bottom corner and double positive cells on the right upper corner.

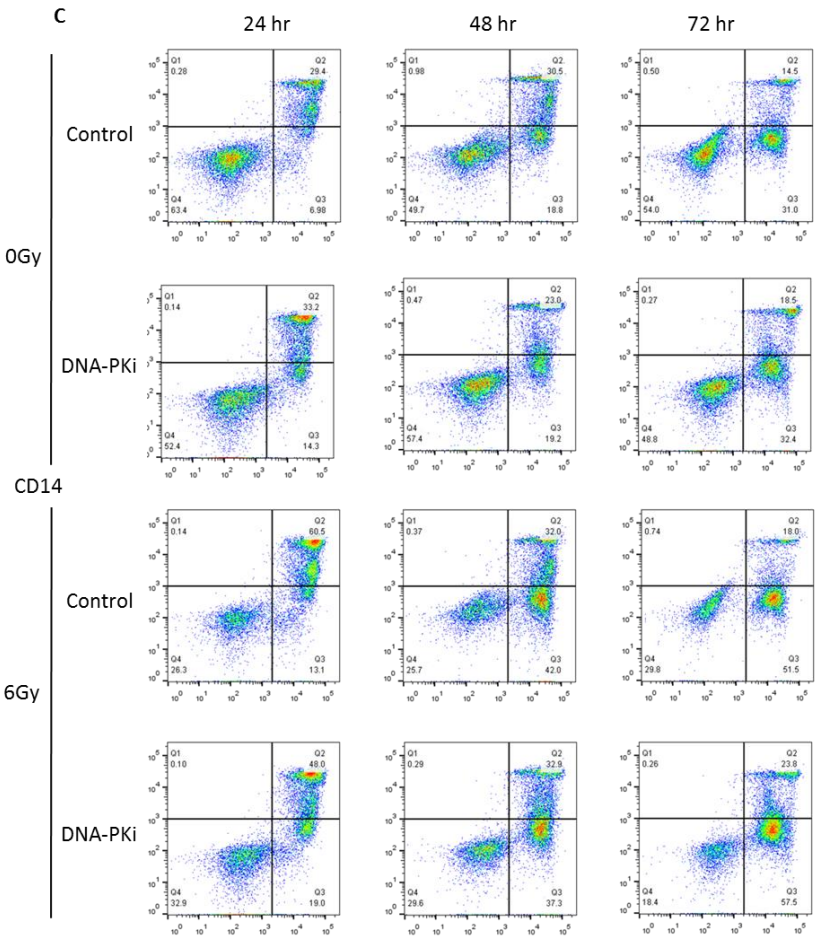


Figure 3.6C Determination of cell death upon DNA-PK inhibition by alternative method in monocytes and DC

The outcome of annexin V/PI double staining of DNA-PK inhibited monocytes following 6Gy irradiation for one donor is shown. On the quadrant plot, double negative cells are on the left bottom corner, annexin V single positive cells (early apoptotic) on the right bottom corner and double positive cells on the right upper corner.

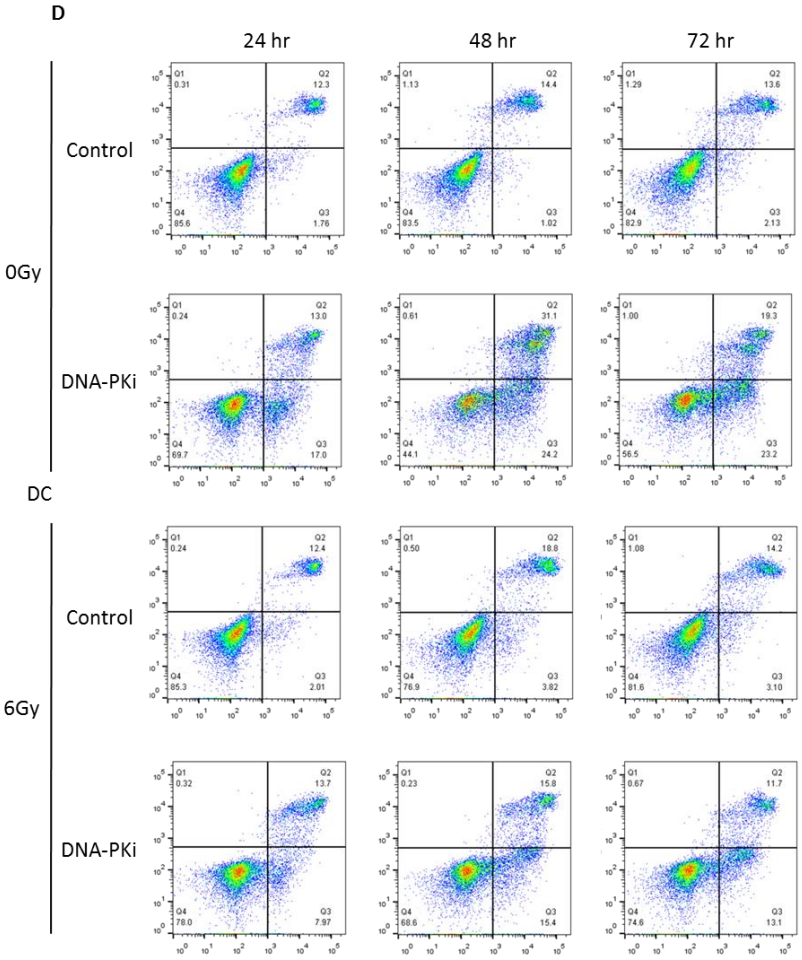


Figure 3.6D Determination of cell death upon DNA-PK inhibition by alternative method in monocytes and DC

The outcome of annexin V/PI double staining of DNA-PK inhibited DC following 6Gy irradiation for one donor is shown. On the quadrant plot, double negative cells are on the left bottom corner, annexin V single positive cells (early apoptotic) on the right bottom corner and double positive cells on the right upper corner.

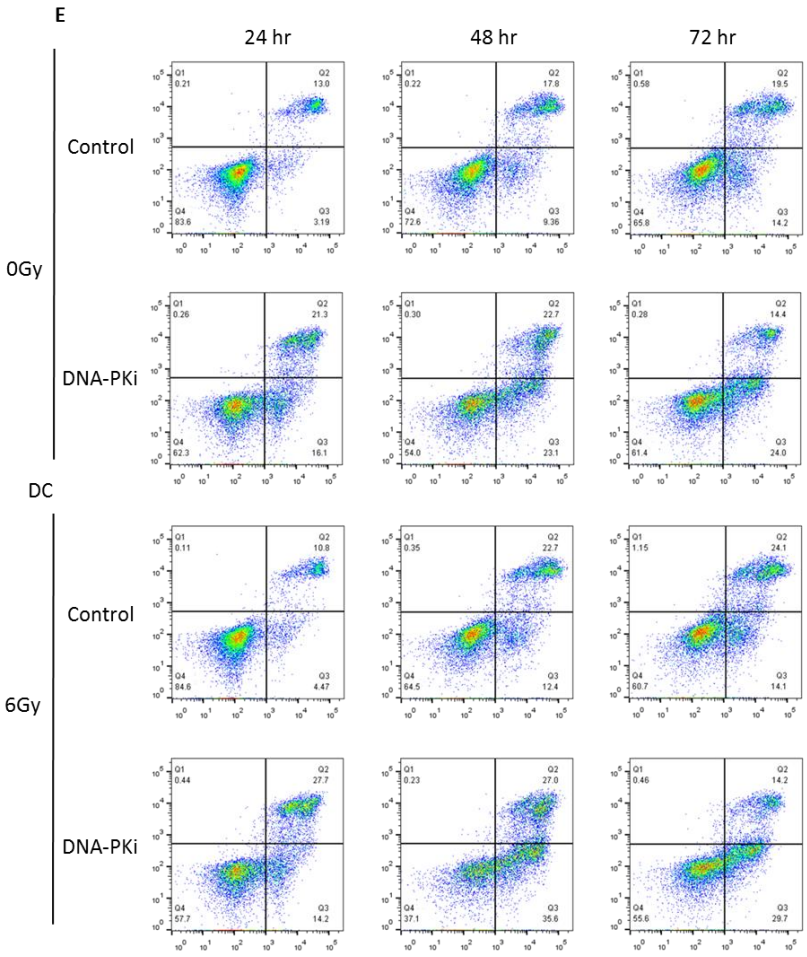


Figure 3.6E Determination of cell death upon DNA-PK inhibition by alternative method in monocytes and DC

The outcome of annexin V/PI double staining of DNA-PK inhibited DC following 6Gy irradiation for one donor is shown. On the quadrant plot, double negative cells are on the left bottom corner, annexin V single positive cells (early apoptotic) on the right bottom corner and double positive cells on the right upper corner.

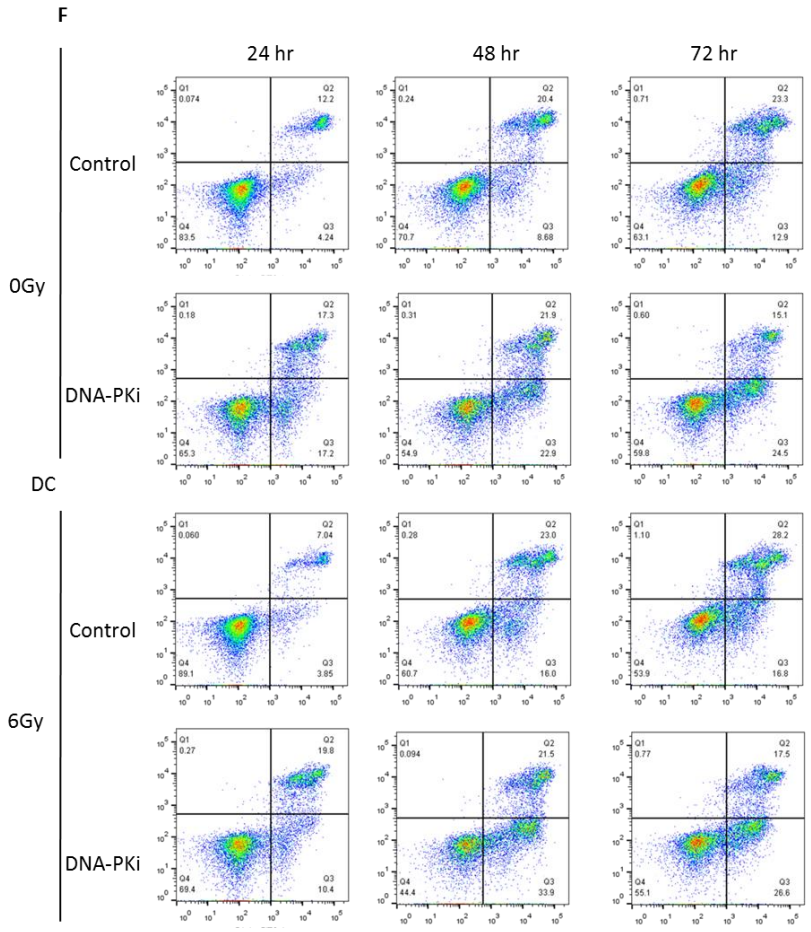


Figure 3.6F Determination of cell death upon DNA-PK inhibition by alternative method in monocytes and DC

The outcome of annexin V/PI double staining of DNA-PK inhibited DC following 6Gy irradiation for one donor is shown. On the quadrant plot, double negative cells are on the left bottom corner, annexin V single positive cells (early apoptotic) on the right bottom corner and double positive cells on the right upper corner.

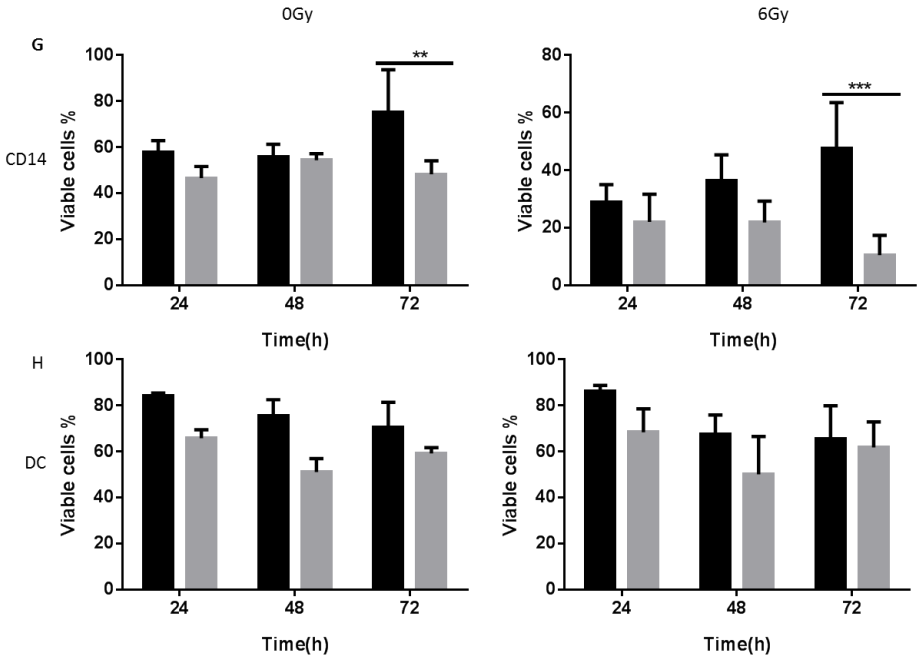


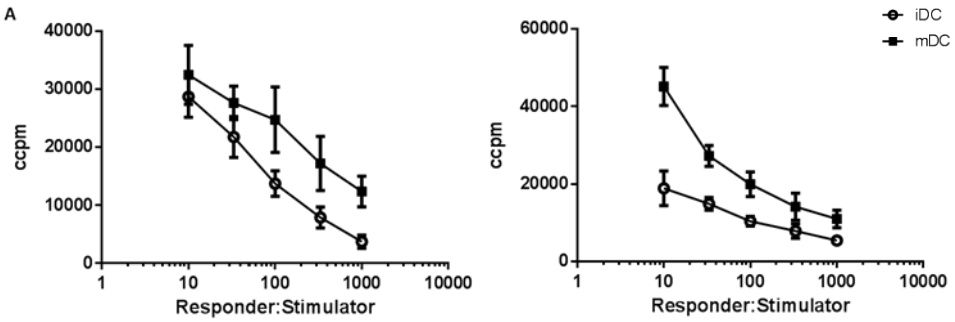
Figure 3.6 Determination of cell death upon DNA-PK inhibition by alternative method in monocytes and DC

For all three donors, the data have been presented in figure G for monocytes and figure H for DC. Black bars represent either untreated CD14 or DC and grey bar represents either CD14 or DC pretreated with DNA-PKi.

3.2.6 ATM or DNA-PK inhibited DC are unimpaired in their ability to stimulate T cell proliferation.

One of the main functions of DC is to initiate T cell responses to immunological danger. One of the key indicators of maturation status of DCs is their ability to stimulate T cell proliferation. Having observed the expression and important roles of ATM and DNA-PK in DC population, we set to determine the impact of inhibition of these molecules on classical DC function i.e. T cell proliferation. A mixed lymphocyte reaction (MLR) was carried out for immature DC, mature DC, ATM inhibited DC and DNA-PK inhibited DC. The cells were treated with the inhibitor, activated and then co-cultured with

PBMC as effector cells. T cell proliferation was then measured by radioactive thymidine incorporation assay. Initially, we undertook allogeneic MLR assays using immature DC (clear circle) and mature DC (black box) as stimulators in two donors (Figure 3.7 A) to demonstrate higher proliferation of T cells by mature DC. This was indeed the case especially from the donor on the right side. Then the effects ATMi and DNA-PKi were determined in allogenic setting for 3 donors (Figure 3.7 B). The inhibitors did not seem to affect the maturation status of the cells as there was no difference in the proliferative responses between LPS triggered DC and drug inhibited DC. Neither ATM inhibition nor DNA-PK inhibition had any negative effect on T cell stimulatory capacity of mature DC.



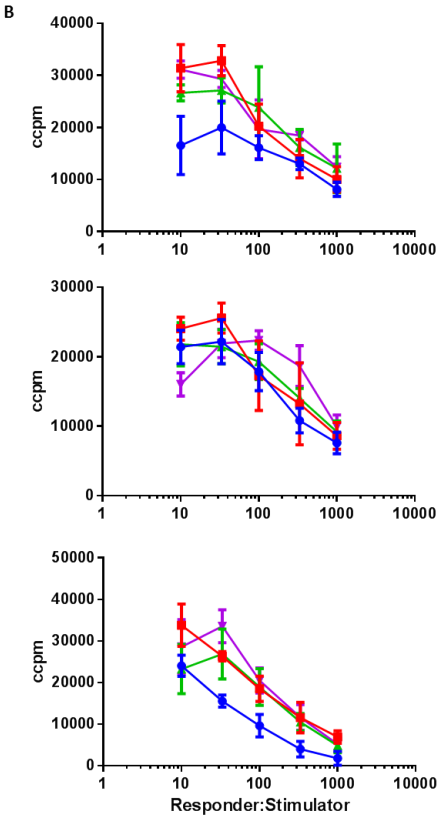


Figure 3.7 Inhibition of ATM or DNA-PK does not interfere with the ability of DCs to stimulate cell proliferation

DC were treated with either 10µM ATMi or 3µM DNA-PKi for 1 hr before activation with LPS. After 24 hr the cell were washed, re-counted and re-plated as a gradient of numbers with the highest number as 10^4 cells (stimulator) per well in six replicates. PBMC ($1x10^5$) (responder) either isolated freshly or revived from frozen stock were added to stimulators and cultured for 5 days. [^{3}H]thymidine incorporation was measured during the last 18 hr of culture to quantify T-cell proliferation. Figure A shows the allogeneic T cell proliferation from two donors stimulated by immature DC (clear circle) and matures DC (black square). Allogeneic MLR reactions between immature DC (blue circle), mature DC (red square), ATMi exposed DC (green triangle), DNA-PKi exposed DC (purple circle) and PBMC for 3 donors have been shown in B.

3.3 Discussion

Immune cells are always subject to intracellular ROS exposure following microbial infection and are thus prone to DNA damage. Most of the chemotherapeutic drugs used for cancer treatment target DNA and cause immunosuppression. Therefore, the DNA repair status of immune cells is of paramount importance to protect these cells during inflammation and drug treatment [471].

In this chapter, we compared the genotoxic response of human monocytes and DC following treatment with IR. We demonstrated that human monocytes isolated from the blood of healthy donors are highly radio-sensitive which might be explained by defects in DNA repair of these cells [469]. Monocytes have been reported to be extremely sensitive to the potent oxidative agent tert-butyl hydroperoxide (BOOH) and DNA methylating agent temozolomide (TMZ) due to lack of expression of several DNA repair molecules [472]. Lack of XRCC1, Lig IIIα and PARP-1 which are the key players in the last step of BER, impair the ability of these cells to repair SSB [469]. Conversely, all of these molecules are clearly expressed by DC. Consistent with this, we also found constitutively activated ATM and DNA-PK in DC, but not in monocytes. Cells used in our study are non-proliferating, so DSB cannot arise from DNA replication. The inability to repair SSBs in overlapping repair patches can lead to DSB formation even in non-proliferating cells. Indeed monocytes have been found to have higher amount of DSBs compared with DC following

chemical ROS or IR treatment [469]. So the hypersensitivity of monocytes to IR in our experiments can be well explained by the absense of these four repair proteins which ultimately leads to cell death.

We have demonstrated immune regulatory function of ATM and DNA-PK in dendritic cells. So it is of great importance that either inhibition of ATM or DNA-PK does not affect DC survival and function. At the same time we also compared the effect of inhibition of these molecules on viability of monocytes with DC. ATM inhibition did not modulate the sensitivity of DC towards radiation as demonstrated by both subG1 and annexin V/PI double staining assays. It was expected as ATM mainly functions in DSB repair through HR pathway that is only functional in proliferating cells [460]. DC are also competent in repairing SSBs through the BER pathway, thus minimizing the generation of DSBs that arise from two SSBs facing each other [469]. Strikingly we observed that inhibition of ATM conferred protected effect against IR induced apoptosis in monocytes. Our observation agrees with the finding that ATMi reduces the level of etoposide induce death of resting T cells whereas increases the propensity of apoptosis in cancer cells [473]. Etoposide treatment causes DNA damage in T cells and induces activation of the DDR pathway followed by p53- and caspase dependent apoptosis. ATM inhibition negatively regulates the apoptotic response by blocking the activation of DDR [473]. ATM inactivation also has positive effect on the survival of innate immune cells. Neutrophils from AT patients have prolonged life span which is also linked to the inadequate activation of p53 dependent

apoptotic pathway [474]. Bauer et al. have reported treatment of monocytes with ATMi reduces the percentage of induced subG1 fraction upon TMZ treatment. Following TMZ treatment, strong activation of ATM/ATR-Chk1/Chk2-p53 occurs in monocytes resulting in Fas (CD95, Apo-1) receptor upregulation and caspase-8 activation [475]. Activation of ATM and ATR is surprising in the non-proliferative cell system. However, this could be explained by partial redundant functions of DNA repair proteins meaning there is much overlap and crosstalk between different pathways and substrates. Thus a large amount of SSBs and DSBs produced by the faulty BER process in monocytes apparently activates several DDR pathways including p53 dependent Fas/CD95/Apo-1 controlled cell death mechanism [472]. Monocytes also lack the catalytic subunit of DNA-PK and thus are unable to repair DSB through NHEJ [469]. Similarly, we did not any phospho-DNA-PK expression either in resting monocytes or IR-exposed monocytes. However, upon inhibitionof DNA-PK, viability of monocytes was severely impaired, the underlying reason for which is unclear.

Immunosuppression during chemotherapy is partially attributable to the hypersensitivity of monocytes as this leads to a depletion of monocyte derived DC and macrophages, which are important players in anti-tumour immunity. The observation that ATM inhibition reduces the hypersensitivity of monocytes towards DNA damaging agents might of beneficial use to compensate some the immunosuppressive effects of chemotherapy.

Chapter 4 Immune regulatory role of ATM in DC

4.1 Introduction

ATM is a nuclear protein kinase which functions as a primary sensor of DSB [458]. The clinical phenotype associated with the loss of ATM activity is termed as ataxia telangiectasia (A-T), which is an eminent genomic instability syndrome characterized by progressive neuronal degeneration [458]. The A-T phenotype is very complex and includes ocular telangiectasias, variable immunodeficiency, growth retardation, premature aging, indications of mitochondrial dysfunction, insulin resistance, impaired response to oxidative stress and cancer susceptibility [476]. Cells from A-T patients show cytoskeletal aberrations, chromosomal instabilities, chromatin changes, abnormal check point controls and hypersensitivity to IR [476]. The gene encoding ATM is located on chromosome 11q22.3 and contains 66 exons transcribing into an approximately 12kb mRNA that encodes a protein of approximately 350 kDa [477]. More than 300 ATM mutations have been reported to be associated with AT, of which >80% are base substitution or insertions/deletions resulting in absence or severe reduction in protein expression. Approximately 10% of AT mutations in A-T patients have been characterized as missense mutations [478].

ATM exists as inert noncovalent homodimer or higher order multimer with its kinase domain attached to an internal domain containing Serine (Ser) 1981 of an adjacent ATM molecule [292]. This inter-domain interaction is essential for

correct folding and stability of ATM in intact cells. Following DNA damage, ATM autophosphorylation at Ser-1981 occurs in trans, dissociating highly active ATM monomers which are freed to phosphorylate other substrate in the cells [292]. Efficient activation of ATM also requires interaction with the MRN complex (MRE11-RAD50-NBS1). Three possible interaction surfaces between ATM and MRN have been reported:

1. through the C terminus of NBS1
2. through MRE11-RAD50
3. through contact with unwound DNA end.

Once bound to the damaged site, ATM initializes a signalling network to regulate cell cycle checkpoints, DNA repair and chromatin structure [479]. As a result of these, ATM activation also leads to apoptosis or senescence through phosphorylation of p53 [480]. The activated ATM is essential for the initiation of DNA end resection which is a preliminary event for DNA repair by HR pathway. Although in A-T cells, high levels of unresolved DNA breaks created by V(D)J and class switch recombination reflect an important role of ATM also in NHEJ, the underling mechanisms are not well understood [278]. ATM belongs to a conserved family of proteins termed phosphatidylinositol (PI) 3-kinase-like protein kinases (PIKKs), all of which contain a conserved kinase domain in their C-terminus and most of which possess a serine/threonine kinase activity [481, 482]. Although it principally functions in the DDR pathway, ATM has also been reported to be involved in a range of other cellular signalling pathways [277-279, 483]. Indeed the diversities of functions of ATM can be well explained by a large scale proteomic analysis of

proteins phosphorylated in response to DNA damage which identified over 700 target proteins for ATM [277]. Many of these proteins have no obvious link to DNA repair [277]. Recently ATM has been reported to function as a sensor of reactive oxygen species (ROS) and becomes activated by oxidative stress in the absence of DNA damage [278]. Oxidative activation of ATM is essential for reconstitutive capacity of hematopoietic stem cells that are hypersensitive to ROS [287]. Hypoxia is also known to activate ATM through alternate mechanism that does not involve DNA breaks [279, 283]. Cytoplasmic functions of ATM have been identified where ATM mediates NF-κB activation in response to genotoxic stress. Under genomic stress, ATM phosphorylates NF-κB essential modulator (NEMO), which is the regulatory subunit of IKK complex. This is required for mono-ubiquitination of NEMO which is essential for nuclear export of NEMO and ATM and for their subsequent association with the catalytic IKK subunit in the cytoplasm [483]. Potential cytosolic functions of ATM have been reported in the regulation of β-adaptin and phosphorylation of the eukaryotic initiation factor 4E-binding protein [484, 485].

In our group, it was previously reported that treatment of monocyte derived DC (moDC) with Wortmannin at 1μM markedly increases IL-23 production as compared to LPS alone [486]. But treatment with the more selective inhibitor LY294002 does not have any effect on IL-23 production. Inhibition of PI-3K occurs at all the concentrations of WM used in the experiment but only treatment with 1μM concentration resulted in elevated secretion of IL-23

[486]. The optimal target specificity of the PI-3K inhibitors Wortmannin and LY294002 are 0.1μM and 10μM, respectively [487]. Thus we hypothesized that increased IL-23 production is not a direct effect of PI-3K inhibition alone but due to other regulatory moieties. In many studies, Wortmannin is used at up to 20μM where it is understood to have many off-target effects [486-488]. These alternative targets include smooth muscle myosin light-chain (IC50 = 0.26μM) [487], Na/K-ATPase (inhibition at 0.5μM) [489], mTOR, DNA-PK (inhibition at 5-20 μM) [490], ATM, and PI4-kinase α and β [491]. Though none of these proteins have previously been described to play a role in the regulation of IL-23 response, recently there are some evidences suggesting the involvement of ATM in immune regulation. Defects in ATM are associated with autoimmune diseases. In patients with MS, there is a defect in the constitutive expression of ATM protein and as a consequence they are unable to stabilize p53 and are relatively resistance to cell death after genotoxic injury [492]. ATM deficiency is also reported in patients with RA. Both naïve and memory CD4 T cells from RA patients carry a large amount of damaged DNA due to insufficient expression of ATM [493]. ATM deficiency in mice results in increased sensitivity to dextran sulfate sodium-induced colitis and mounts a strong immune response characterized by inflammatory cytokines and circulating activated T cells [453]. Inhibition of ATM kinase causes increased expression of innate immune response genes and neurodegeneration in Drosophila [494]. It has been reported that damaged human fibroblasts developed persistent chromatin lesions, which initiated secretion of IL-6 and initiation and maintenance of this IL-6 production

required DDR proteins ATM, NBS1 and CHK2 [495]. In response to genotoxic stress ATM directly regulates the transcriptional activity of Ca^{2+}/cAMP response element (CRE) binding protein (CREB) which plays vital roles in neuronal development, function and survival [496, 497]. Phosphorylation of CREB on Ser-133 by PKA or other Ser-133 kinases in response to various stimuli is the canonical pathway of CREB activation [498]. Ser-133 phosphorylation promotes an interaction between CREB and its transcriptional coactivator, CREB binding protein (CBP), which stimulates the expression of CREB target genes [499-502]. IR and H_2O_2 induced ATM activation phosphorylates CREB on Thr-100, Ser-111 and Ser-121 which results in decreased CREB transactivational potential and inhibition of CBP binding probably by inducing a conformational change in CREB. Thus by altering the binding affinity with CBP, ATM modulates the expression of CREB dependent genes [496]. In addition to CREB, ATM interacts with other signalling molecules such as ATF1 [503], ATF2 [504], TRAF-6 [505] and PP2A [506].

The present study aimed to test the hypothesis that ATM kinase pathway plays a key role in immune regulation in DC. We have demonstrated that the use of a highly selective ATM antagonist, KU55399 (KU) resulted in increased transcription of the IL-23p19 gene and increased IL-23 secretion. However, inhibition of closely related kinase ATR did not enhance IL-23. Activating ATM signalling with ionizing radiation suppressed IL-23 responses and this was prevented by inhibiting ATM-activation. Our data showed that ATM regulated pathway only cooperates with TLR4 signalling for enhanced IL-23 production

but not with TLR1/2, TLR3, TLR5, TLR7/8, Dectin-1 and Dectin-1/TLR2. Moreover, we demonstrated that transcriptional factor CREB is phosphorylated at S121 when DC were exposed to IR. The expression of phospho-CREB-S121 was completed blocked by ATM inhibitor (ATMi) which was associated with recovery of IL-23 production. While ATM inhibition did not affect LPS induced phospho-CREB-S133, forskolin induced CREB activity was enhanced by ATM blockade. CREB transactivation activity was markedly reduced by a specific CREB-CBP interaction inhibitor (C-Ci) in luciferase reporter assay in HEK293T cells. Most importantly, the increased level of TLR4 dependent IL-23 caused by ATM inhibition in DC was completely blocked by the C-Ci. This study describes a novel function of ATM kinase in the regulation of IL-23 in DC.

4.2 Results

4.2.1 Inhibition of ATM but not ATR regulates IL-23 production in moDC

With the selective inhibition of ATM, moDCs responded to LPS/IFNγ stimulation with significantly increased IL-23 secretion. This effect was consistent between donors and increased with the concentration of inhibitor up to 10μM which is widely used as an accepted fully inhibitory concentration of ATMi (KU55933). Although, moDCs treated with 1μM ATMi secreted IL-23 at a similar level to LPS/IFN-γ treatment only, conditioning with 3μM significantly enhanced IL-23 secretion ($p < 0.01$). Compared to LPS/IFN-γ treatment alone, preconditioning with 10μM consistently increased IL-23 secretion up to 8-fold (Figure 4.1 A). Cell viability was not affected by treatment with up to 10μM of ATMi. Thus, in all subsequent studies, ATMi was used at 10μM.

The effect of KU55933 in enhancing IL-23 production was dependent on TLR-signalling as IL-23 secretion was not observed when DCs were treated with ATMi alone or in combination with IFN-γ (Figure 4.1 B). So, the presence of IFN-γ did not influence IL-23 production. Although, there was a difference in the basal level of IL-23 from TLR4 triggered moDCs between donors, significant increase in IL-23 levels were detected from all 20 donors studied. For DC treated with LPS alone, the level of IL-23 secretion was 3.67±1.7ng/ml which was increased significantly to 17.36±8.88ng/ml after incubating with 10μM of ATMi (Figure 4.1 C).

As ATR is functionally closely related to ATM and both ATM and ATR predominantly function in DNA repair in replicating cells [507], we tested the hypothesis that inhibition of ATR does not impact IL-23 expression in our non-dividing DC. Treatment of DC with a series of concentrations of ATRi (NU6027) in the presence of maturation signal did not provoke IL-23 production (Figure 4.2 A). Inhibition of ATR had no significant impact on IL-23 secretion (Figure 4.2 B & C). As expected, inhibition of ATM with the highly-selective inhibitor KU55933 potently induced IL-23 secretion in LPS-treated DC.

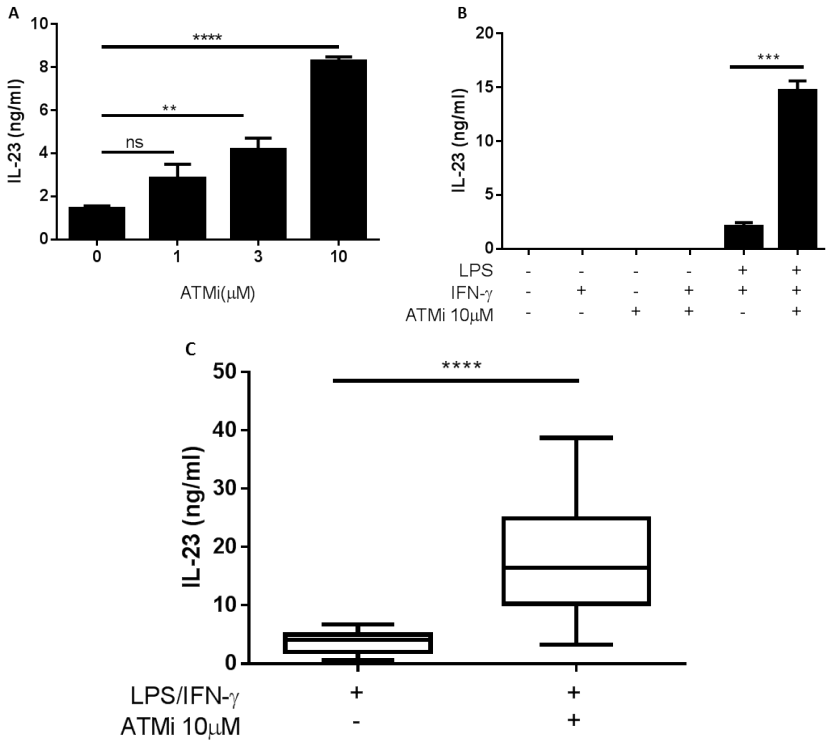


Figure 4.1: Stimulation of IL-23 secretion by the ATM inhibitor KU55933-conditioned moDC.

Dose-dependent increase in IL-23 secretion by moDC treated with ATMi andactivated by LPS/IFN-γ (**A**). moDCs were treated with KU, prior to stimulation with LPS/IFN-γ, and IL-23 levels were measured at 24-hour by ELISA. **(B)** The effect of ATM inhibition on IL-23 secretion by moDC in the

presence or absence of various activating ligands. Missing bars indicate where cytokine secretion was below the limits of sensitivity for the ELISA. **(C)** Enhanced level of IL-23 production by moDC isolated from 20 independent donors when the moDC were conditioned with LPS/IFN-γ alone compared to 10μM ATMi conditioned moDC. Error bars indicate the standard deviation (SD) of triplicate experiments. In all figures, statistically significant differences were determined by the unpaired t-test with Welch's correction. (**:0.01>p value≥0.001, ***: p value<0.001).

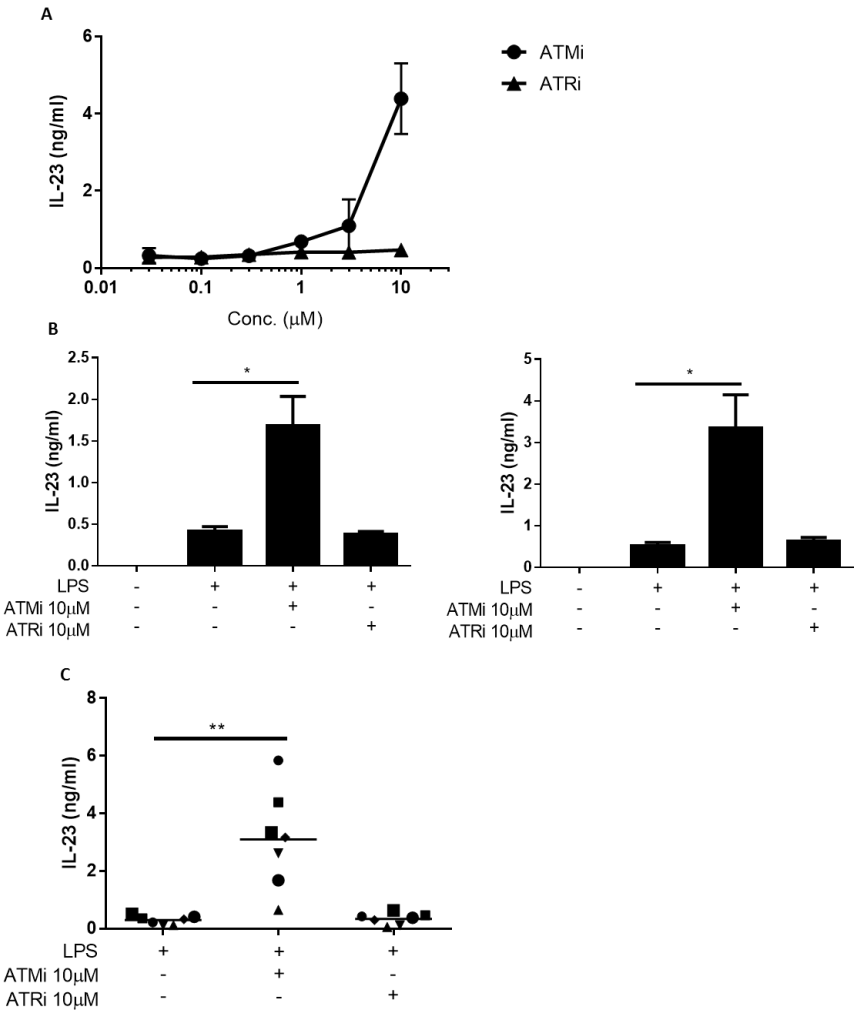


Figure 4.2 ATR inhibition does not affect IL-23 secretion by TLR4 triggered DC moDC were treated with LPS in the presence or absence of the indicated concentrations of ATM or ATR inhibitor and cytokine secretion was determined after 24 hours by ELISA. (A) IL-23 secretion increased with increasing concentrations of ATM inhibitor, but not ATR inhibitor. Data are the

mean of triplicates from one representative donor ± SD. Data from two representative donors (from 7) are presented in figure 4.2 B showing significant increase in IL-23 production by ATM inhibition compared to ATR inhibition at 10μM. The summary figure (4.2C) compares the effect of ATM inhibition or ATRi inhibition with LPS stimulation alone in all the donors studied.

4.2.2 ATM inhibition induces IL-23 production in macrophages and murine BMDC

We also examined the effect of ATM inhibition on monocyte derived macrophages. In all the four donors, treatment of M1 macrophages with ATMi significantly increased IL-23 release (Figure 4.3 A). For M2 macrophages, ATMi increased IL-23 production from undetectable level to detectable level (Figure 4.3 B). Our observation was also agreeable in BMDC from mouse. Upon activation with LPS and pre-treatment with ATMi, DC from all three mice showed several fold increase in IL-23 secretion (Figure 4.3 C).

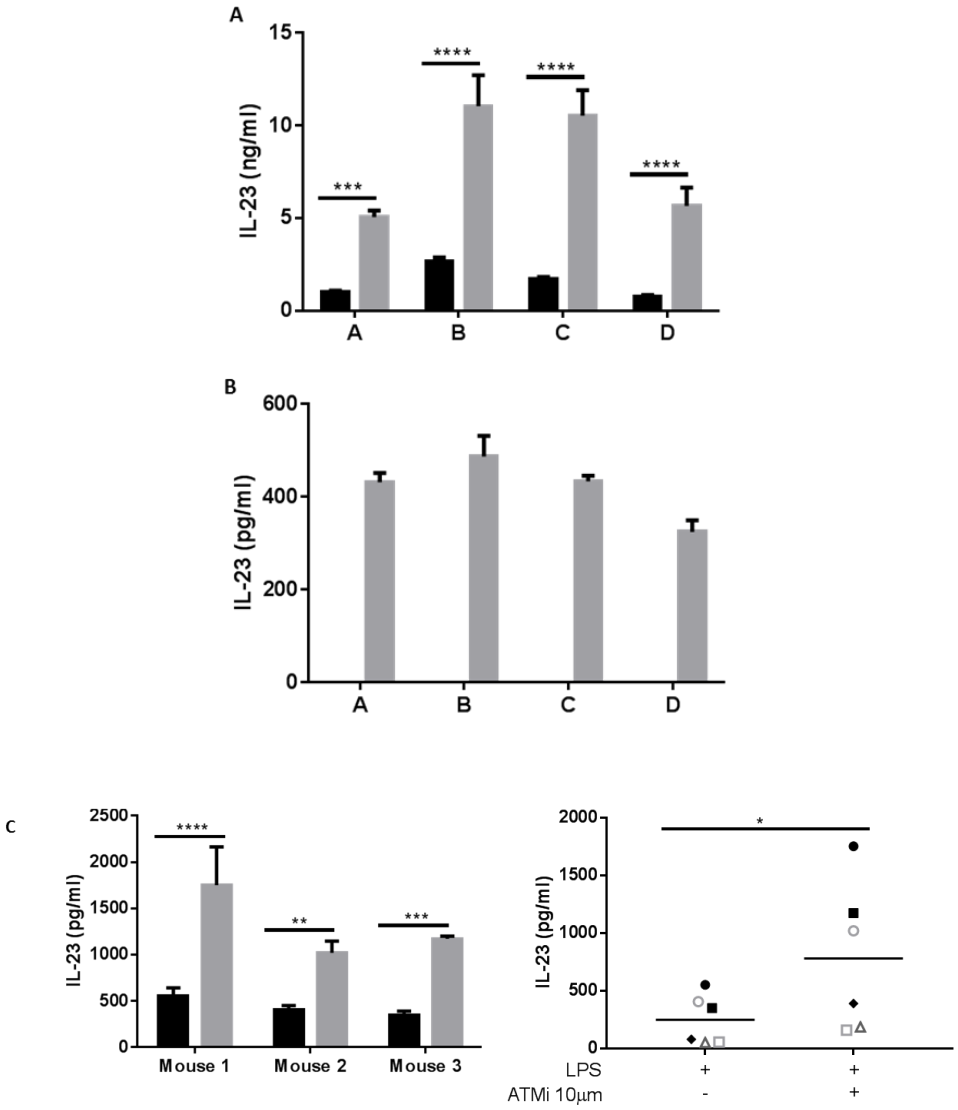


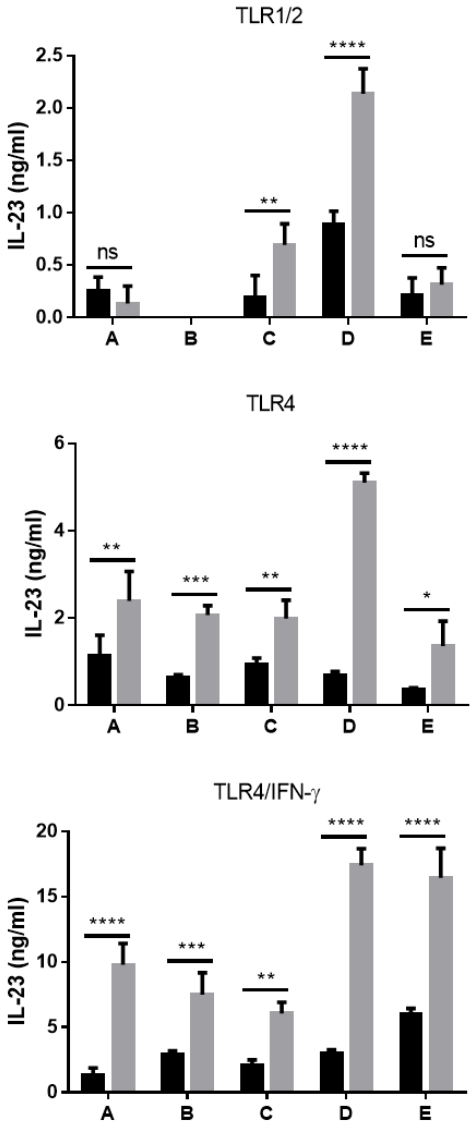
Figure 4.3 ATM regulates IL-23 production in monocyte derives macrophages and in murine BMDC

M1 and M2 macrophages were derived by culturing monocytes with GMCSF and MCSF respectively for 7 days. M1 macrophages were activated with LPS/IFN-γ alone or after preincubation with10μM ATMi and M2 macrophages were stimulated with LPS only or in combination with ATMi. Macrophages from 4 donors (Figure 4.3 A for M1 and Figure 4.3B for M2) showed enhanced IL-23 secretion with ATM inhibition compared to TLR4 triggering only. BMDC were isolated from mouse bone marrow, rested and treated with ATMi (10μM) for 1 hr before stimulated with LPS for 24 hr, supernatant was then

collected to measure IL-23 level by ELISA. Three representative experiments have been shown (Figure 4.3 C left) and all the 6 mice done were presented in a summary graph in Figure 4.3 C right. In all the graphs, black bar represents LPS/IFN-γ or LPS treatment and grey bar represents cells preconditioned with ATMi.

4.2.3 ATM regulation on IL-23 production has been observed only by TLR4 triggered moDC

Next we tested the association between different TLR signaling pathways and ATM-dependent IL-23 induction in DC. IL-23 was secreted when DC were exposed to agonists of the TLR1/2, 4, 7/8, Dectin 1 or Dectin 1/TLR2 receptors. However, ATM inhibition did not significantly increase IL-23 in response to TLR1/2, 7/8, Dectin 1 or Dectin 1/TLR2 compared to the respective stimulation only (Figure 4.4). In contrast to other TLRS, TLR3 and 5 agonists did not elicit detectable levels of IL-23. Inhibition of ATM kinase resulted in a significant increase in cytokine release only when DC were triggered with TLR4 agonist. We also tested the effect of IFN-γ on TLR4 driven IL23 induction. The role of IFN-γ is dispensable as similar median fold (3.4) increase was observed from LPS triggered cells without IFN-γ (Figure 4.4B).


TLR1/2
IL-23 (ng/ml)
2.5
2.0
1.5
1.0
0.5
0.0
ns
**
****
ns
A
B
C
D
E
TLR4
IL-23 (ng/ml)
6
4
2
0
**
***
**
****
*
A
B
C
D
E
TLR4/IFN-γ
IL-23 (ng/ml)
20
15
10
5
0
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A
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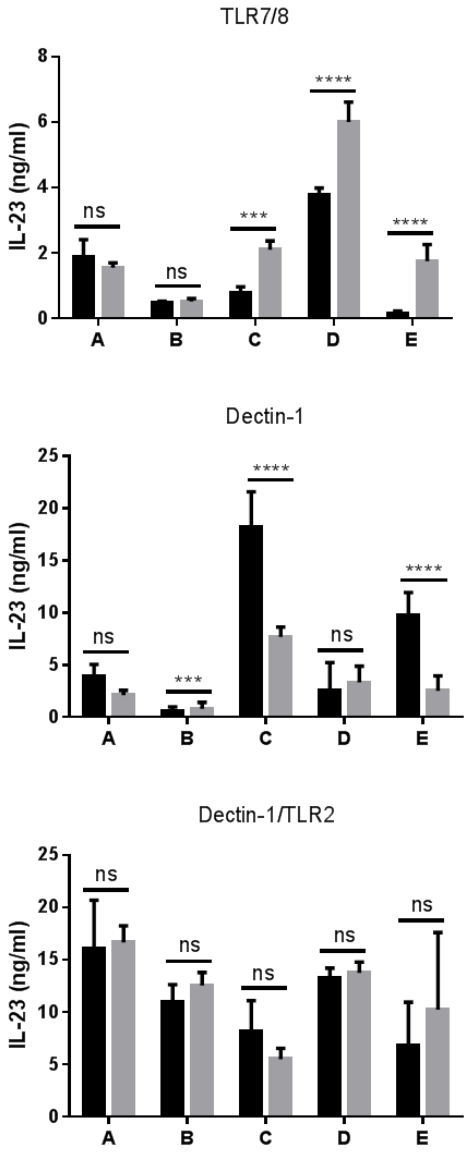


Figure 4.4A Inhibition of ATM enhances IL-23 secretion induced by TLR4 agonist only

The response of ATMi conditioned moDC (KU55933 10µM) from 5 donors to TLR1/2, TLR3, TLR4, TLR5, TLR7/8, dectin-1 and dectin-1/TLR2 was studied. Pam3CSK4, poly I:C, LPS, flagellin, R848, curdlan and zymosan were used as TLR1/2, TLR3, TLR4, TLR5, TLR7/8, dectin-1 and dectin-1/TLR2 agonists respectively. moDC were stimulated for 24 hours with the indicated TLR agonist in the presence or absence of 10µM ATMi and IL-23 secretion was determined by ELISA. IFN-γ was only used in conjunction with TLR4 to compare the effect with LPS only stimulation. Black bar indicates TLR stimulation only and grey bar represents ATMi pre-incubation followed by TLR

activation. A, B, C, D, E represent five individual donors. Error bars indicate the SD of biological triplicates. Missing bars mean that the cytokine secretion was below the limit of the sensitivity of the ELISA. The results for TLR3 and TLR5 were not presented as there was no IL-23 section for all donors studied.

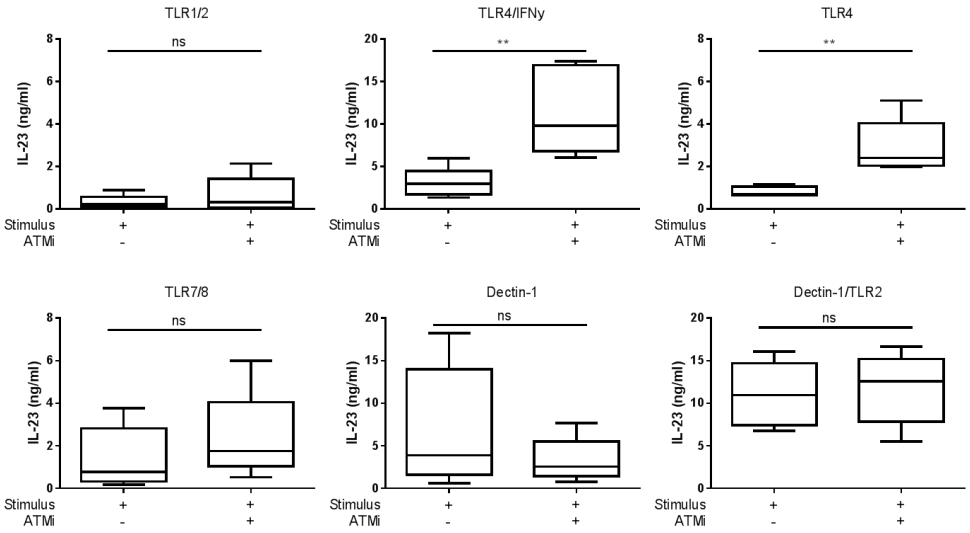
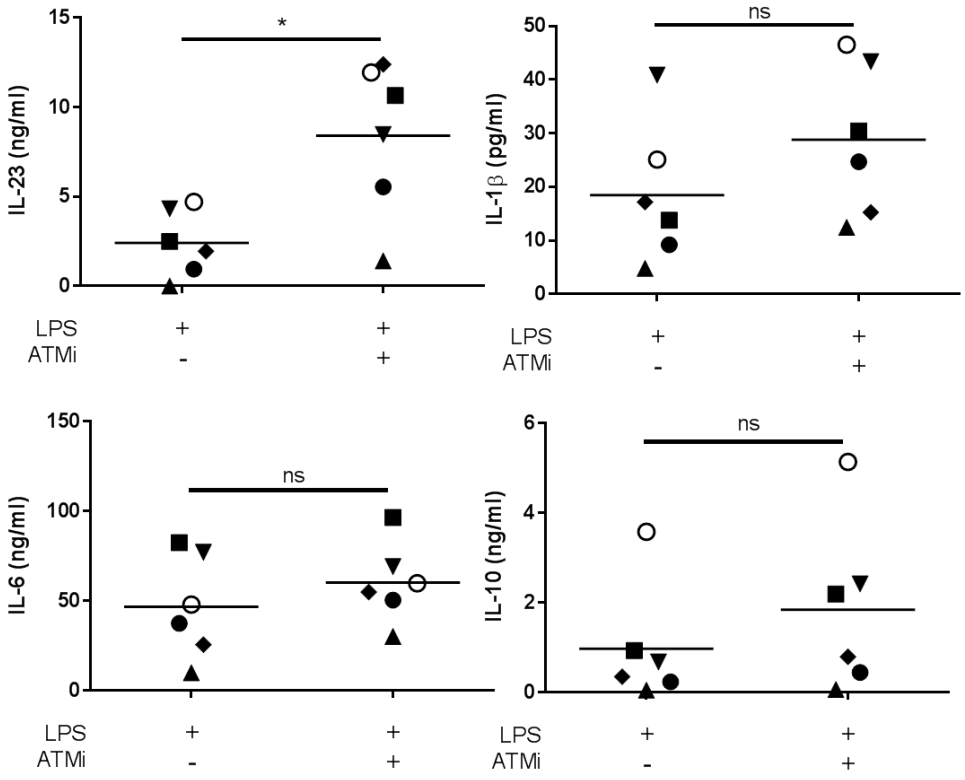


Figure 4.4B IL-23 regulation by the ATM signalling pathway is restricted to TLR4 only

The box and whisker plots comparing the effect of ATM inhibition on IL-23 with the indicated TLR agonist in all the 5 donors analyzed. The line through each box represents the median value while the error bars indicate the lower and upper limits. Statistical significance was determined by non-parametric unpaired t-test known as Mann-Whitney test.

4.2.4 Regulation of other cytokines by ATM

While inhibition of ATM with the highly selective ATMi (KU55933) potentially enhanced IL-23 secretion in moDC in response to LPS, it did not significantly affect secretion of other cytokines such as IL-12, IL-10, IL-6, Il-1β and IL-27. However, only in the case of TGFβ, significant changes were observed upon ATM inhibition (p=0.026). ATM inhibition resulted in average three fold change in TGFβ secretion compared to LPS stimulation alone.



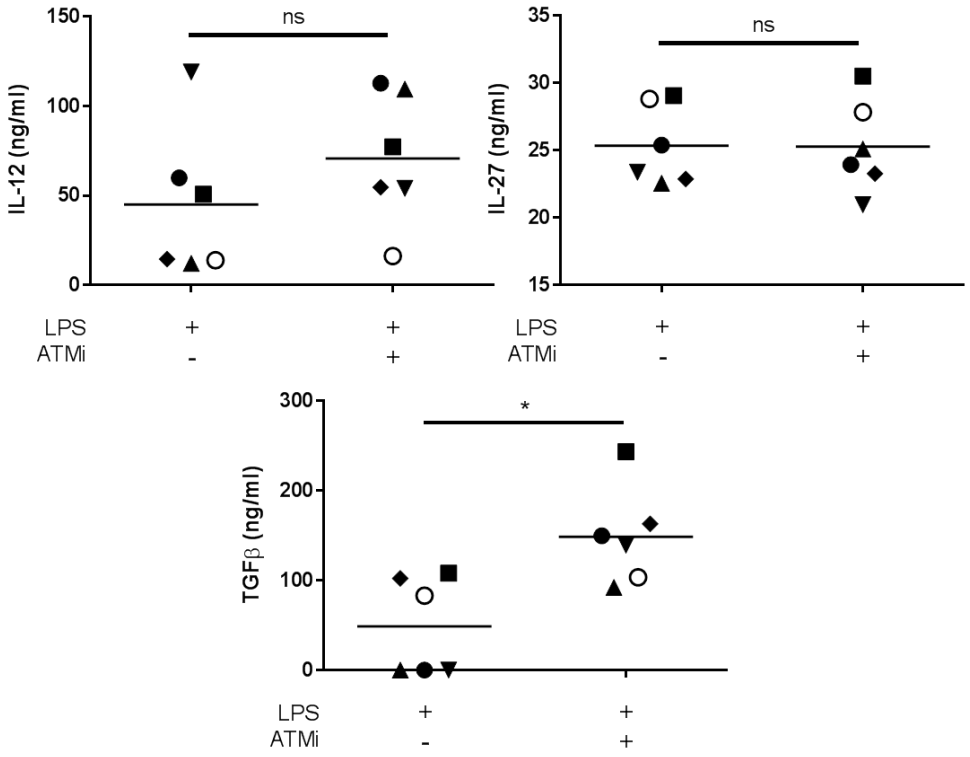


Figure 4.5 Impact of ATM inhibition on other cytokine production

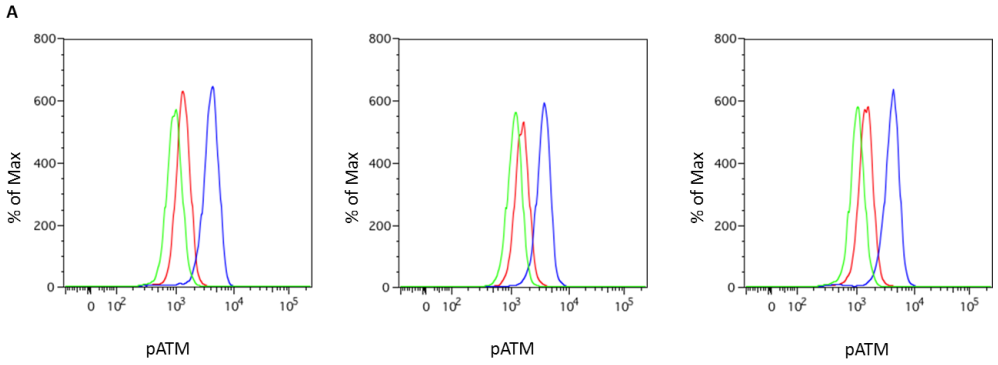
The role of ATM signalling for IL-1β, IL-6, IL-12, IL-27 and IL-10 along with IL-23 was determined by ELISA. TGFβ level was assessed by TMLC co-culture assay. Data shown are from 6 donors for moDC treated with LPS alone or in combination with 10μM ATMi. Each symbol represents an individual donor. Non-parametric unpaired t-test was carried out to test the significance between two groups.

4.2.5 Ionizing radiation activates ATM and has a suppressive effect on IL-23 production by moDC, but has no effect on BMDC

To determine whether further activation of ATM exerted a suppressive effect on IL-23, we exposed DC to 6Gy IR as a means of activating ATM. Following treatment with IR, the level of phospho-ATM (Ser 1981) was markedly increased in moDC as demonstrated by intracellular staining with flow cytometer. Treatment with ATMi (KU55933 10μM) prior to radiation blocked

IR induced ATM activation and the level of phospho-ATM was similar to the level observed in non-irradiated cells (Figure 4.6 A).

Irradiation of moDC with 6Gy resulted in significant suppression of IL-23 secretion in response to TLR4 stimulation in 3 of the 4 donors studied. Blocking activation of ATM by treatment with the inhibitor prior to IR abolished the inhibitory effect of radiation on IL-23 (Figure 4.6 B). Over all 11 donors studied, there was 50% mean reduction in IL-23 level following conditioning by ionising radiation, whereas when pre-treated with ATMi, the cells retained 100% mean production of IL-23 (Figure 4.6 C). It should be noted that although the basal levels of IL-23 secreted by ATMi treated DC were considerably higher than in control cells, the absence of ATM function prevented the suppressive effect of ionizing radiation. Importantly, exposure of DC to X-rays had no effect on their viability either immediately following radiation as shown in previous chapter. Surprisingly, IR did not alter IL-23 response in murine BMDC. As shown in Figure 4.7, although ATM inhibition clearly increased IL-23 production over LPS activation, IR had no effect on IL-23 levels in these cells.



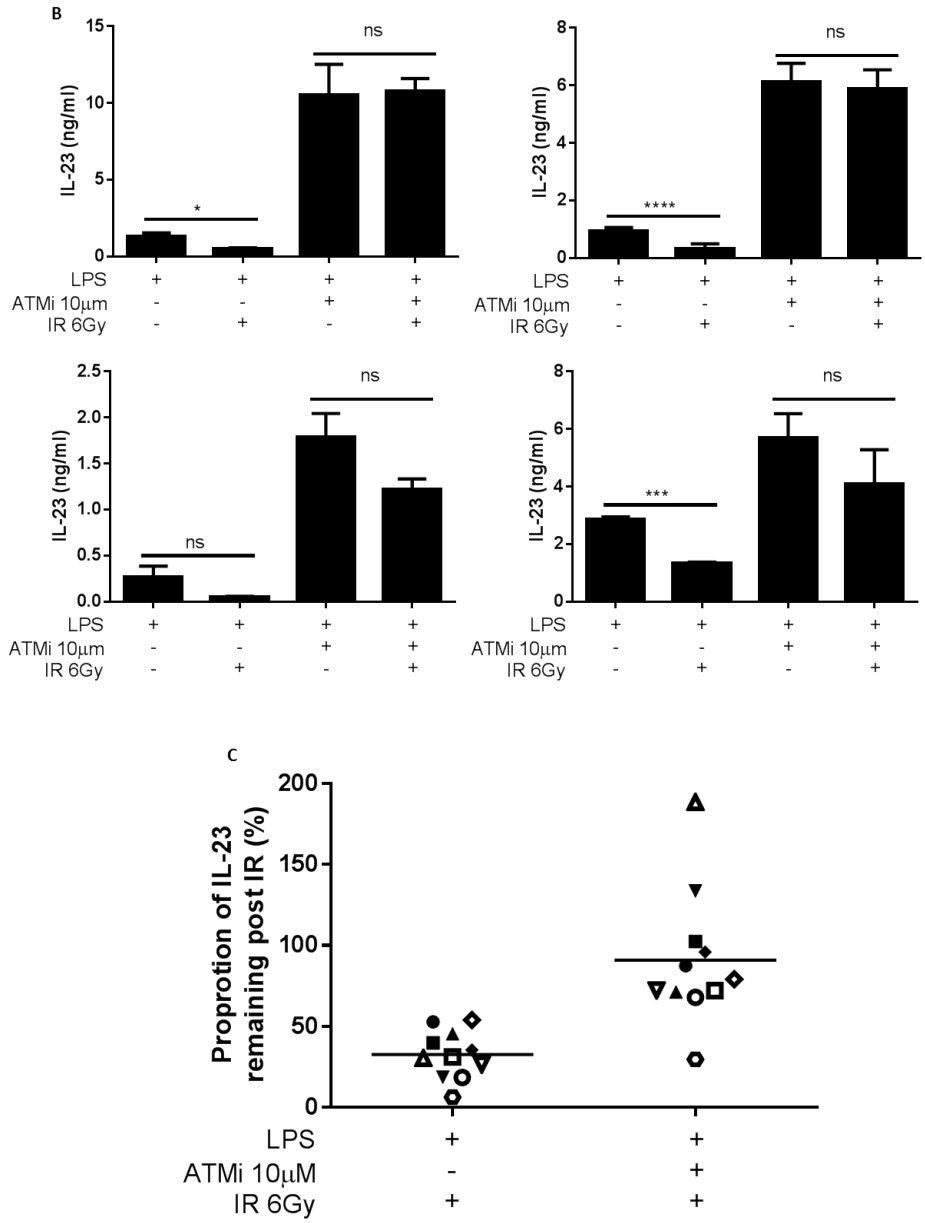


Figure 4.6 IR-induced activation of ATM suppresses IL-23 production which can be reversed by pre-treatment with ATMi

The phosphorylation status of ATM was confirmed by intracellular staining (4.6 A). DC were exposed to 6Gy of ionizing radiation with or without pre-treatment with KU55933. The phosphorylation of ATM was examined 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.

moDC were exposed to ATMi (10µM) for 1 hr or left untreated prior to 6Gy radiation and then immediately activated with LPS. Data from 4 donors out of 11 are presented in figure 4.6 B. The effect of IR was shown for LPS stimulation alone and also when ATM activation was prevented by pre-treating with the ATMi. Figure 4.6 C 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.

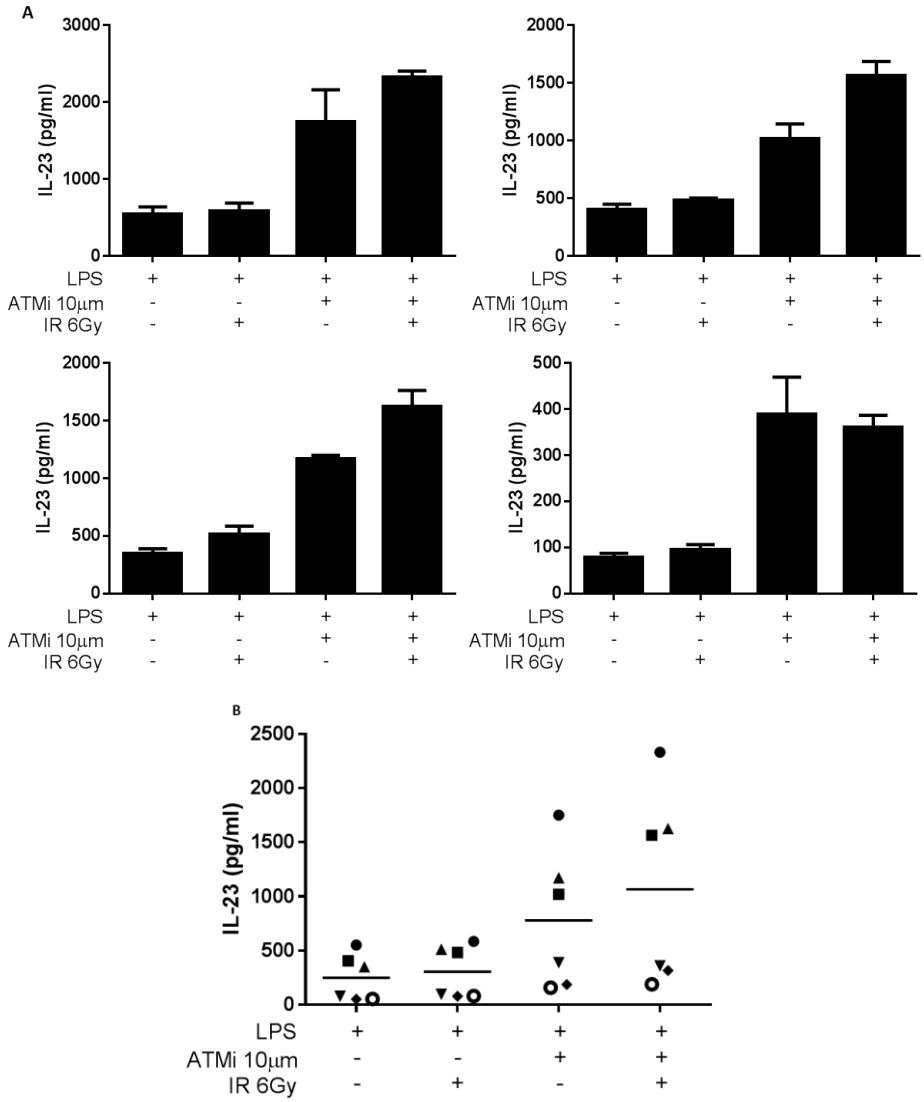


Figure 4.7 IR does not affect IL-23 secretion by ATM inhibited murine BMDC

BMDC were treated as described before and exposed to x-ray prior to activation with LPS. Data form 3 individual mice have been shown in 4.7 A to

demonstrate the effect of radiation with or without ATM blockade. The overall summary for all 6 mice are presented in B.

4.2.6 Regulation of phosphorylation and transactivation activity of CREB by ATM

ATM has been reported to regulate the activity of the transcription factor CREB in response to IR [496]. Therefore to test the hypothesis that ATM mediates its effect on IL-23 expression through the CREB transcription factor, we first examined the phosphorylation of CREB under different experimental conditions. Although TLR4 stimulation did not affect phosphorylation of ATM kinase, phosphorylation of CREB at Ser133 was observed, the modification widely recognized to be associated with increased transcriptional activity of CREB (Figure 4.8 A). However, ATM became activated immediately (30 min) following exposure to IR as apparent by the phosphorylation of Ser1981. Phosphorylation of ATM coincided with phosphorylation of CREB at Ser121, one of the residues associated with decreased CREB function. Importantly, in the absence of LPS, no phosphorylation at Ser133 of CREB was observed. CREB was phosphorylated at both Ser121 and Ser133 following addition of LPS to irradiated cells. As expected, treatment with ATMi abrogated activation of ATM as well as CREB phosphorylation without affecting Ser133 activation. Changes in IL-23 secretion following IR mirrored these observations, so we next determined the outcome of ATM inhibition on CREB function in a CREB reporter assay. Forskolin (Fsk) was used as a positive control to activate CREB function through the PKA-cAMP pathway [508]. In HEK293T reporter cells,

1μM of Fsk induced CREB reporter activity and that was further augmented in the presence of ATMi (Figure 4.8 B). Addition of a small molecule inhibitor (C-Ci) that prevents interaction between CREB and CBP, ablated CREB reporter activity in ATMi and ATMi/Fsk treated cells. This observation further demonstrated the role of ATM as regulator of CREB function.

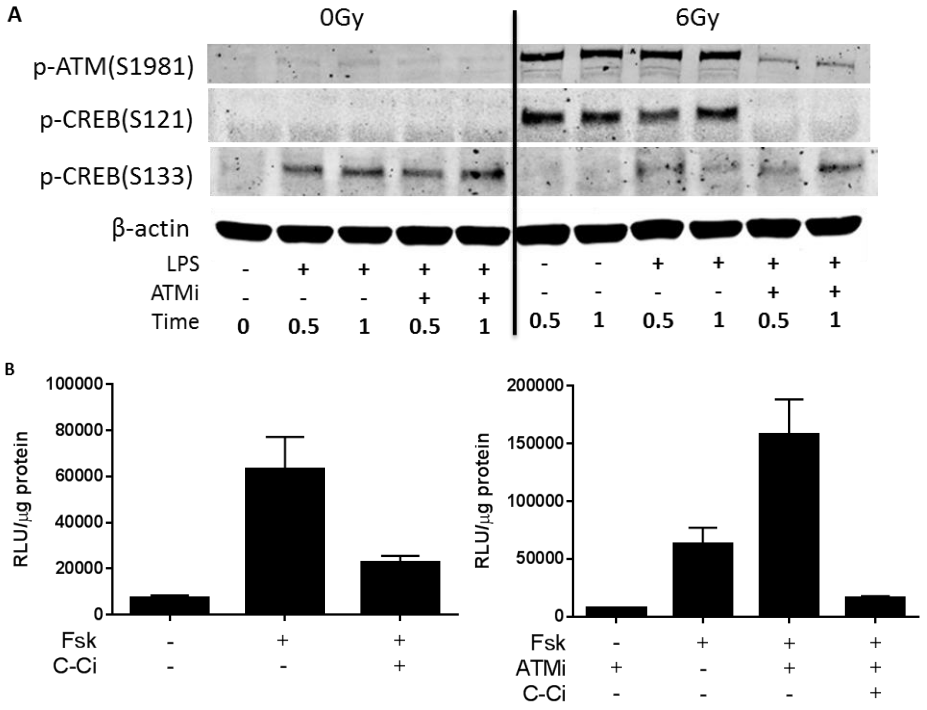


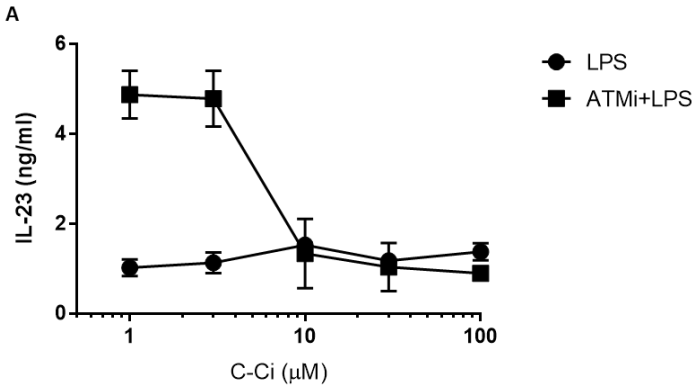
Figure 4.8 ATM mediated phosphorylation regulated CREB function

moDC were media exposed, LPS exposed or pretreated with ATMi and then exposed to LPS. For IR treatment, cell, were exposed to 6Gy immediately before activation with LPS. Cell extracts were collected at 30 min and 1 hr post IR and analyzed by western blotting for protein expression of p-ATM(Ser 1981), p-CREB(Ser 121) and p-CREB(Ser 133). β-actin was used as loading control. Data shows one representative donor (Figure 4.8 A). Figure 4.8 B shows the measurement of CREB transcriptional activity as investigated by luciferase reporter assay. CRE-RLuc-transfected HEK293T cells were left untreated or treated with Fsk (1μM) alone or treated with ATMi (10μM) for 30

min before addition of Fsk or pretreated with ATMi (10μM) and C-Ci (10μM) prior to addition of Fsk. The Renilla luciferase activity was measured 5 hr after Fsk stimulation and was expressed as relative luciferase unit/μg protein (RLU/μg protein).

4.2.7 The effect of ATM on IL-23 is mediated CREB/CBP interaction

CREB is an essential transcription factor for prostaglandin E2 mediated IL-23 expression [509]. Therefore we hypothesized that CREB might be the terminal mediator of ATM repressed IL-23 production. Inhibition of CREB-CBP interaction by treatment with C-Ci did not affect LPS-dependent IL-23 in any of the concentrations used (Figure 4.9 A). However the ATMi-enhanced IL-23 production 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 4.9 B).



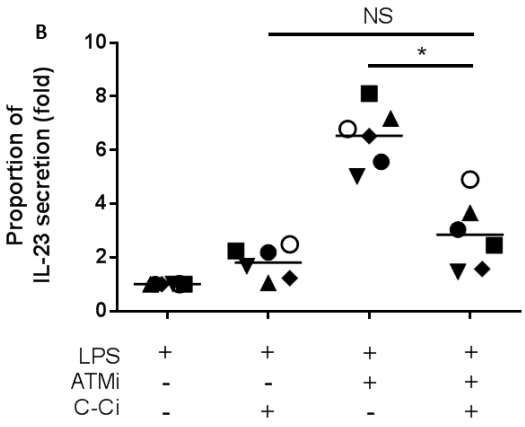


Figure 4.9 ATM dependent IL-23 regulatory pathway signals through CREB

The result of inhibition of CREB-CBP interaction on IL-23 secretion when DC received different concentrations of C-Ci inhibitor (1µM, 3µM, 10µM, 30µM, 100µM) in the presence of LPS only or LPS plus ATMi. IL-23 level was determined by ELISA after 24 hr and one representative donor has been shown in figure 4.9 A. Figure 4.9 B represents the summary graph for six donors for a single dose of C-Ci (10µM).

4.3 Discussion

Identification of novel strategies to modulate IL-23 secretion would have important implications in controlling immune responses as well as in therapeutic approaches. However, knowledge of IL-23 regulation and the upstream signalling network controlling its expression is limited. In this study, a new mechanism has been hypothesized for increased IL-23 secretion by human DCs. We described that DNA repair protein functions as a novel regulator of the pro-inflammatory IL-23 response in DC. ATM inhibition augmented IL-23 secretion and its activation had a negative effect on LPS induced IL-23 response. Importantly we have demonstrated that IR induced suppression on IL-23 is mediated by ATM through negative regulation of CREB transcriptional activity.

The serine/threonine kinase protein ATM is a member of the PI-3K related kinase family and it co-ordinates cellular responses to repair the damages following ionizing radiation induced DNA double strand breaks [480]. Despite of its roles in the control of cell cycle progression, an important relationship has been established linking ATM with autoimmune conditions [492, 493]. The ATM inhibitor KU55933 used in this study has been widely implicated at 10μM as target specific concentration [510, 511]. KU55933 is a potent, highly selective and competitive inhibitor with an IC_{50} for ATM of 13nM. In contrast, 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. The 200-fold difference in IC_{50} between ATM and the next target assures that the findings obtained with KU55933 are restricted to ATM inhibition. It is important to consider off-target effects when using kinase inhibitors to dissect function. Thus the increase in IL-23 caused by KU55933 may be due to ATM inhibition only and not for other off-target effects. Previously we have clearly demonstrated that KU55933 abolishes activation of ATM following exposure to IR [512], while a DNA-PK inhibitor does not (data not shown). Thus it is reasonable to speculate that the observed increase in IL-23 caused by KU55933 is due to ATM inhibition only and not for other off-target effects. Alternatively a second ATM inhibitor could be used to confirm the phenotype observed with KU55933. There are several ATM inhibitors that would be suitable for this, such as, KU60019 (analogue of KU55399) [513], CP466722 [514] and KU59403 [515]. Our data have shown that ATM inhibition results in enhanced IL-23 production not only in moDC, but also in circulating myDC and monocyte derived macrophages. We were also able to demonstrate our observation in murine DC. Moreover, blockade of ATM activation (induced by IR) restored IL-23 levels to those of non-irradiated cells. This further shows that ATM negatively regulates IL-23 pathway in DC.

ATR is another PI3KK family member that functions at the core of DNA damage signalling apparatus and initiates a rapid activation of signalling cascades in response to DNA damage. In contrast to ATM, ATR senses SSBs formed by stalled replication fork, as well as those generated by processing of

DSBs [277]. Despite of being activated by very different types of stimuli, several groups have demonstrated that ATR also responds vigorously to DSBs in an ATM dependent manner. The study by Stiff et al. [516] shows that ATR dependent activation of ATM by UV and hydroxyurea which are potent activators of ATR signalling. DNA damage responses have been reported to regulate cytokine production in neutrophils. Simultaneous inhibition of ATM and ATR synergistically increases IL-8 production in neutrophils more than inhibition of either protein alone [474]. However, our data indicates that LPS dependent IL-23 enhancement is dependent on ATM, not ATR. It is suggestive that ATM may act in a very different way to its established role in DNA repair in terms of signalling IL-23 response. This could be confirmed in future by looking at activation of DDR such as phosphorylation of histone H2AX.

In addition to the canonical roles of ATM and DNA-PK in safeguarding genome integrity, these proteins are now considered to take part in other cellular stress pathways. Oxidative stress activates ATM whereby it functions as a cellular redox sensor. In contrast to ATM activation by ionizing radiation, the oxidized form of ATM is a disulphide linked dimer and in this form ATM acts as a sensor of ROS [278, 289]. ATM is also activated under conditions of low oxygen tension, high salt and chlorine concentrations [279, 283]. ATM deficiency induces ER stress in astrocytes [517] which can be explained by the blockade of tunicamycin induced ER stress by ATM [518]. Furthermore, ATM also interacts with NF-κB essential modulator (NEMO) and by increasing nuclear translocation, promotes NF-κB-dependent signalling [519]. An immune regulatory role of ATM has been observed in the persistent activation

of immune responses in mice deficient of ATM [453]. Thus ATM acts as a more generalized stress sensor and may regulate immune responses by controlling inflammatory signalling mechanisms.

Our study delineates a novel role of ATM in immune regulation and also a potentially new mechanism of controlling IL-23 responses in DC. We have shown that CREB phosphorylation is the ultimate regulator of ATM dependent IL-23 signalling. CREB is a basic leucine zipper family transcription factor that is activated in response to various cellular stimuli such as, cAMP, $Ca^{2+,}$ hypoxia, UV light and growth factors [520, 521]. The transactivational potential of CREB is upregulated through phosphorylation of Ser-133 which lies within the kinase inducible domain (KID) [521]. Phosphorylation of Ser-133 promotes an interaction between KID and KIX domain of CBP resulting in CREB dependent gene expression [499, 522]. The important role of CREB in transcriptional control has been reflected in a study which identified approximately 4,000 genes in human genome that are regulated by CREB [523]. Following exposure to IR, ATM phosphorylated CREB at Ser-121 in DC, which is consistent with the finding of Thr-100, Ser-111 and Ser-121 as putative ATM phosphorylation sites in vivo in response to genotoxic stress [496]. Treatment with ATMi prevented IR induced ATM activation and also 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, whilst ATMi reverses that suppression as observed. CREB phosphorylation on Ser-111 and Ser-121 by ATM is mediated through casein kinases 1/2 (CK1/2) in response to

DNA damage. In the presence of DNA damage, ATM phosphorylates CREB at Ser-111 which triggers subsequent phosphorylation of Ser-108 by CK2 and Ser-114/117 by CK1. The Ser-108/111/114/117 phosphorylated form then renders CREB permissive for ATM dependent phosphorylation on Ser-121 [524]. Thus observing the expression of IR induced phospho-CREB-108/111/114 with or without ATMi would provide insights into the mechanism of the regulatory role of ATM on CREB function controlling IL-23 secretion.

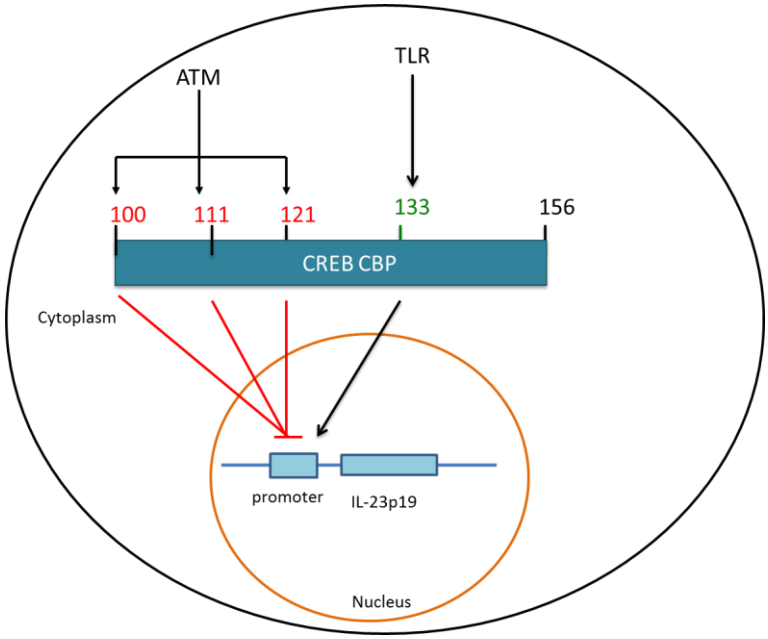


Figure 4.10 Proposed mechanism of regulation of IL-23 by cytoplasmic ATM through CREB.

TLR4 stimulation favours IL23 production through CREB phosphorylation on Ser133. Activated ATM negatively regulates the transcriptional activity of CREB by phosphorylating at Ser111, Ser121 and Thr100, thereby suppressing the activation of IL23p19.

Negative regulation of CREB activity by ATM has been confirmed in a Renilla luciferase reporter assay in HEK293T cells transfected with CRE-RLuc. ATM inhibition further increased CREB mediated gene transcription induced by Fsk. A small molecule inhibitor that inhibits the KID-KIX interaction abolished CREB reporter function [525] induced by Fsk alone or in combination with ATM. This inhibitor also significantly ablated ATMi enhanced IL-23 level confirming that ATM mediated IL-23 signalling is dependent on CREB. Although LPS treatment phosphorylated CREB at Ser-133, inhibiting KID-KIX interaction did not affect LPS induced IL-23 level. This could be explained by the fact that despite being a key determinant of CREB transcriptional activity, Ser-133 phosphorylation does not always correspond CBP binding activity or transactivational potential [526]. Also there is evidence of CREB activation through CBP-independent mechanisms [527-530]. Our future investigation will involve looking at the effect of ATM inhibition on IL-23 gene expression in CREB Ser111 or Se-121 knocking mice as well as monocytic tumour cell line. Confirmation of this new regulatory pathway will create an opportunity to modulate IL-23 dependent immune responses for therapeutic approaches in autoimmune diseases and cancer.

Chapter 5 The role of DNA-PK in regulating IL-23 signalling in DC

5.1 Introduction

Interleukin-23 (IL-23) is a member of IL-12 superfamily cytokines that plays an important role in the regulation of T helper type 17 (Th17) responses [531]. It is a heterodimeric protein comprising p19 and the p40 subunit shared with IL-12p70 [532]. This cytokine is secreted predominantly by antigen presenting cells (APC), including moDC, myDC [533], macrophages [534], and microglia [535] in response to immune danger. Initially IL-23 was thought to play a role in promoting proliferation of the memory T cell population. But subsequent work in experimental model demonstrates involvement of IL-23 in inflammatory responses as well [223]. DC are the principal APCs that play a central role in priming naïve T cells and link innate and adaptive responses through communication with natural killer (NK) cells and neutrophils [536]. The differentiation of CD4+ T cells into different Th cell subsets is markedly influenced by the cytokines derived from DC, so called "signal 3". For example, interferon-γ (IFN-γ) producing Th1 cells are generated in response to IL-12 and activate cytotoxic T lymphocytes (CTL), NK cells and MΦs [537]. On the other hand, IL-4 drives the development of Th2 responses that provide help to B lymphocytes to become activated and secrete antibody while IL-1 and IL-6 are known to induce inflammatory Th17 responses [538, 539]. Although IL-23 was initially thought to play important role as an inducer, it is generally accepted that this cytokine is more important for the survival and expansion of Th17 cells. It has been found that animals with deficiency of IL-23p19 do not

develop experimental autoimmune encephalomyelitis (EAE), collagen-induced arthritis (CIA) and inflammatory bowel disease (IBD). These findings point to key involvement of IL-23 in induction of pathogenic Th17 cells. Moreover, IL-23 also rapidly induces IL-17 secretion from γδT-cells suggesting these may be amongst the first cells to respond to IL-23 during the course of inflammation [540].

The critical roles of Th17 cells in inflammation and autoimmune diseases are well established which are mediated through the cytokines IL-17A, IL-17F, IL-21, IL-22, IL-6 and tumour necrosis factor (TNF) [237, 541]. Elevated secretion of IL-17 is found in a number of organ-specific and autoimmune diseases such as, multiple sclerosis (MS), rheumatoid arthritis (RA), psoriasis and inflammatory bowel disease [531]. Th17 cells also play an important role in protective immunity at mucosal surfaces and against infectious agents such as certain bacteria, fungi, virus and protozoa [542, 543]. In contrast to autoimmunity the roles of Th17 cells in cancer are somewhat conflicting. Some reports show that tumour growth is enhanced by IL-17, possibly through stimulation of tumour vascularization and tumour growth [544, 545]. On the other hand, several lines of evidences suggest that these cells play positive roles in protective anti-tumour immunity [260, 546-549]. Within the same tumour microenvironment, the number of Th17 cells is inversely correlated with the number of regulatory T cells (Treg) and positively correlated with other immune effector cells, including IFN-γ secreting effector T cells, CTL and NK cells [237, 547, 548].

In addition to its established role in promoting Th17 response, IL-23 itself plays an important role in immunity against infections agents including fungus by facilitating differentiation of MΦ to the M1 type which is characterized by production of high levels of nitric oxide (NO) and tumour-necrosis-factor-α (TNF-α) [148, 532, 550]. Transgenic mice which express the IL-23p19 subunit in multiple tissues suffer from severe multi-organ inflammation, runting, infertility and premature death with increased secretion of TNF-α and IL-1 [551]. In contrast to IL-12 which induces naïve T cells to produce large amounts of IFN-γ, IL-23 mainly acts on memory T cells to secrete high levels of IFN-γ and restrict the generation of Treg and IL-10 expression. Thus, IL-23 may result in autoimmune disorders by restricting the functions of tissue protective lymphocytes [6, 20].

Although IL-12 has structural and functional resemblance to IL-23, its functions are nevertheless distinct. Furthermore the signalling events involved in IL-23 regulation are, in contrast to IL-12, not clearly understood [92, 153]. The two well-known [92] stimulation signals for IL-23 secretion are Toll-like receptor 4 (TLR4) and TLR2 agonists [143, 552, 553]. Mitogen-activated protein kinase (MAPK) and nuclear-factor-K-B (NFKB) are induced in response to TLR4 signalling which leads to differential activation of p44/p42 extracellular signal-related kinase (Erk) 1/2, p38 MAPK and Jun N-terminal kinase (JNK) and IL-12 gene expression [552, 554, 555]. For regulation of the IL-12 family cytokine genes, p38 MAPK is assumed to play a positive role [556]. In cord blood derived DCs, enhanced phosphorylation of Erk1/2 and

p38 MAPK result in synergistic increased in LPS induced IL-23, although Erk1/2 is known to negatively regulate IL-12 expression [555, 556]. Consistent with this, inhibition of p38 MAPK has been shown to block LPS/IFN-γ induced IL-23 secretion [486]. In addition, phosphatidyl inositol 3-kinase has been implicated in the regulation of the IL-12 family gene expression. It has been demonstrated by Butchar *et al.* [148] that human monocytes are induced to produce IL-23 by *Francisella tularensis* which is down-regulated by pharmacological inhibition of PI-3K. In the previous chapter, we have described ATM as an important regulator of IL-23 signalling in DC. Here we reported that ATM is not the sole member of the DDR that regulates IL-23 production and provide evidence to support a role for a second DNA damage repair protein, DNA-PK, in signal 3 cytokine regulation in DC.

DNA-PK is a serine threonine kinase which is composed of the 465-kDa catalytic subunit, DNA-PKcs and the regulatory subunits Ku70/80 subunits. It plays a central role in repairing DSBs by NHEJ which is assumed to be the fundamental pathway for DSB repair in human somatic cell. This is an important cellular mechanism in repairing DSB induced by ionizing radiation as well as reactive oxygen species (ROS). Primarily, DSBs are recognized by the Ku subunits following by the recruitment of DNA-PKcs and formation of the DNA-PK complexes on both ends of the DSB. A conformational change occurs in DNA-PKcs as a result of assembly of the DNA-PK complex in such way that two globular domains interact creating channels that enclose the two DNA ends to protect from degradation. Activation of DNA-PKcs requires association with Ku70/80 and DNA-termini. In order to release DNA termini and to

regulate DNA end access, DNA-PKcs undergoes autophosphorylation on several serine and threonine residues. These residues are phosphorylated by DNA-PKcs itself and occasionally by ATM or ATR, which releases DNA-PKcs from the DNA and allows NHEJ to proceed. As being a critical enzyme in the NHEJ pathway, DNA-PK also takes part in HR. However, it is largely unknown that how a cell chooses to repair a DSB between by NHEJ or HR. It has been anticipated that NHEJ is the primary default pathway for DSB repair and HR may take over when NHEJ fails [282, 462]. DNA-PKcs is also important in additional DSB repair processes such as V(D)J recombination and class switch recombination thus imparting in immune system development [557]. Mice that have mutation (*scid* mutation) in the *Prkdc* gene that encodes for DNA-PKcs are incompetent in generating function immunoglobulin and T-cell receptors and have defective numbers of mature B and T lymphocytes [558-561]. This missense mutation gives rise to a premature stop codon that results in reduced expression of the DNA-PKcs protein. This particularly affects the differentiation of stem cells into mature lymphocytes, whilst leaving myeloid differentiation unaffected [560, 562, 563]. In addition to DNA repair, DNA-PK also regulates genes involved in metabolism, cell proliferation and radiation sensitivity [462, 564]. Understanding the activation of ATM and DNA-PK for DNA repair may provide useful clues to their proposed alternate roles as cytoplasmic signalling mediators that govern inflammatory gene expression in DC.

Although its canonical role is to repair DSB through NHEJ, DNA-PK has been described to control innate immune response and pro-inflammatory pathways. Phosphorylation of p50 NF-κB by DNA-PK is essential for the formation of p50/p50 and p50/p65 NF-κB dimers and induction of NF-κB dependent genes such as VCAM-1 [565]. DNA-PK also modulates NK cell activity by interacting with CD158d, a killer-cell immunoglobulin-like receptor (KILR), found on all NK cells, and by promoting recruitment and activation of Akt and NF-κ induce cytokine production (e.g. IL-1β, IL-6, IFN-β and IL-27) [566, 567]. DNA-PK is involved in human immunodeficiency virus-1 (HIV-1) mediated killing of CD4+ T cells [568]. Double-stranded DNA damage response is induced following integration of viral genome into the host chromosome. This activates DNA-PK and phosphorylates p53 and histone H2AX leading to death of CD4+ cells. Importantly, pharmacological inhibition of DNA-PK could be a therapeutic approach to prevent HIV-1 mediated CD4+ T cell death. *Listeria monocytogenes* causes host DNA strand breaks and activates DNA damage/replication checkpoint responses through the phosphorylation and activation of DNA-PK, H2AX and CDC25A. This increases the host cell cycle duration to allow DNA repair and/or fork protection and thereby favours propagation of this intracellular pathogen [569].

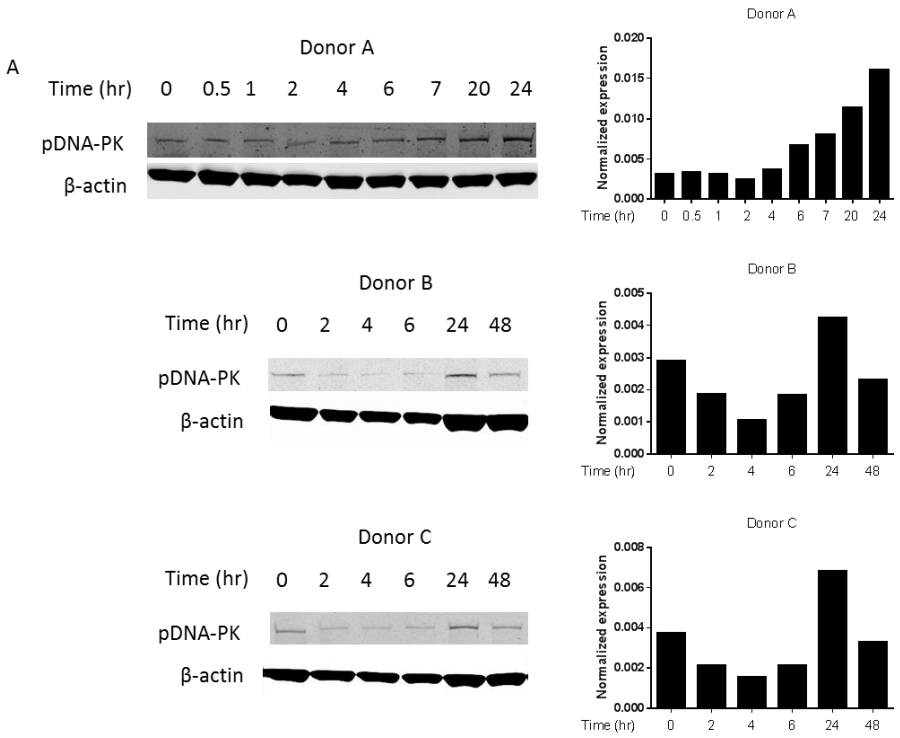
Here we investigated the expression of DNA-PK in resting DC as well as TLR4 triggered DC. We examined the contribution of DNA-PK to the production of IL-23 by DC following different TLR stimulations using a highly selective small molecule inhibitor, NU7441 (DNA-PKi). Similar to ATM inhibition, DNA-PK

inhibition significantly increased IL-23 expression in DC which was restricted to TLR4 only. In contrast to ATM, DNA-PK was found to regulate other cytokine production. Furthermore, we investigated whether this increased IL-23 had any association with IL-1β signalling or NFκB activation. Although the molecular mechanism linking DNA-PK with IL-23 regulation has yet to be determined, our findings point out DNA-PK as a novel therapeutic target for IL-23 associated pathologies.

5.2 Results

5.2.1 Phosphorylation kinetics of DNA-PK upon LPS stimulation

Activation of DNA-PK in response to LPS treatment was measured by determining the phosphorylation of DNA-PKcs at Ser 2056. Resting moDC were found to express phospho-DNA-PKcs and the level was increased following addition of LPS over a period of 24 hr. Phosphorylation of DNA-PKcs level appeared to decrease after 24 hr in two of the donors (Figure 5.1 A) examined for 48 hr. Treatment with DNA-PKi, but not with ATMi substantially decreased LPS induced DNA-PKcs phosphorylation at 4 hr in all of the three donors studied (Figure 5.1B).



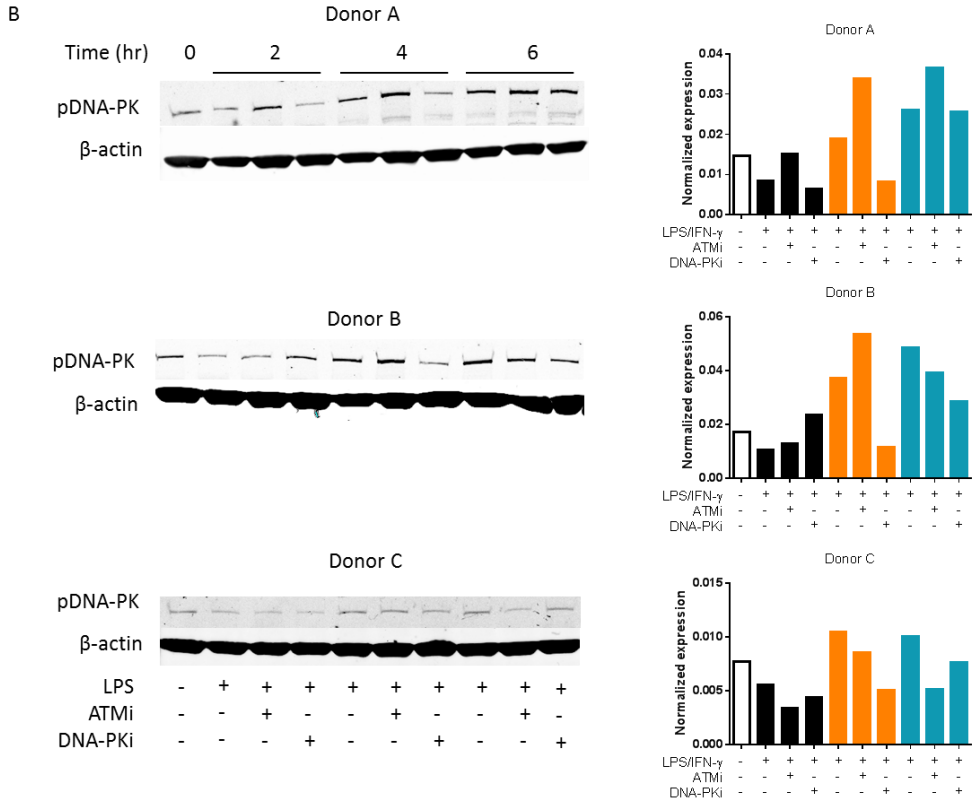


Figure 5.1 Activation of DNA-PK in response to LPS treatment

Western blot of phospho-DNA-PKcs (Ser 2056) in media exposed DC, in DC during first 24 hr of maturation for one donor and up to 48 hr of maturation for two donors have been presented in figure 5.1 A left panel. moDC were treated with either ATMi or DNA-PKi prior to LPS stimulation and DNA-PKcs phosphorylation at Ser-2056 was examined at 2 hr, 4 hr and 6 hr of maturation. Figure 5.1 B left panel represents the western blots of all the three donors studied. For both Figure 5.1 A and B, right panels show the normalized phospho-DNA-PKcs levels of the corresponding blots by using the ratio of reading of phospho-DNA-Pkcs bands to the corresponding β-actin bands. In figure 5.1 B graphs, white bar for untreated sample, black bars for samples at 2 hr, orange bars for samples at 4 hr and blue bars for samples at 6hr.

5.2.2 DNA-PK regulates IL-23 secretion in DC

To determine a role for DNA-PK in IL-23 secretion, DC were treated with a selective DNA-PKi, NU7441 prior to activation with LPS. Inhibition of DNA-PK resulted in increased IL-23 production consistently in a dose dependent manner (Figure 5.2A). IL-23 secretion was significantly elevated at 3μM ($p<0.001$) concentration of DNA-PKi. Cell viability was unaffected up to 3μM of DNA-PKi as shown in Table 5.1. Therefore, 3μM NU was used in all subsequent experiments.

As with ATM-inhibition, IL-23 enhancement was observed to be dependent on the activation of DC through TLR4 as incubating DC with the inhibitor alone or with IFN-γ did not increase IL-23 levels (Figure 5.2B). This again indicted that IFN-γ is not involved in enhancing IL-23 secretion. When the level of IL-23 production by 3μM DNA-PKi conditioned DC was compared with the level of IL-23 produced by LPS treated DC alone for twenty five donors, there was (Figure 5.2C) marked increase with p value less than 0.0001. The level was increased from 3.55±1.69ng/ml for LPS treated DC alone to 23.85±12.62ng/ml for DC conditioned with DNA-PKi which is higher than the amount observed for ATM-inhibition.

Next we determined whether ATM and DNA-PK-inhibition synergized to augment IL-23. Inhibiting ATM and DNA-PK induced an additive effect on IL-23 expression that was higher than treatment with either inhibitor or LPS/IFN-γ alone (Figure 5.3A). To determine the optimum level of IL-23 produced by ATM or DNA-PK conditioned moDC, we measured IL-23 level at different time

points. The highest level of IL-23 was produced at 24 hours which decreased with time (Figure 5.3B). This may due to the degradation of the protein or may be internalized by DC itself as this cell is known to express IL-23 receptor [99].

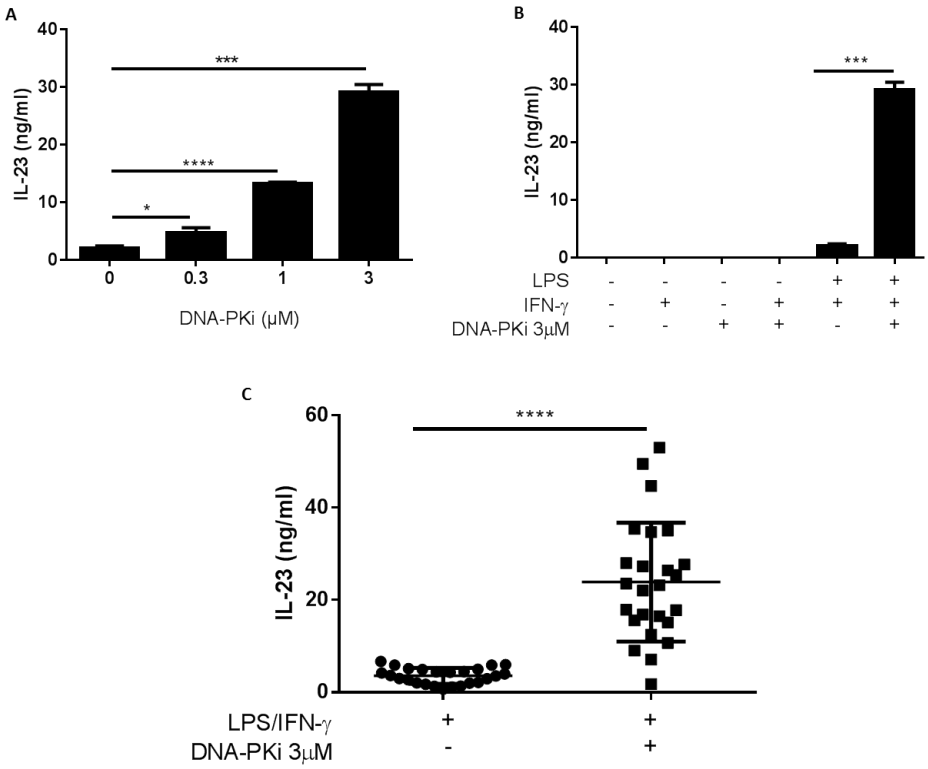


Figure 5.2 Stimulation of IL-23 secretion by DNA-PKi-conditioned moDC.

Dose-dependent increase in IL-23 secretion by moDC treated with DNA-PKi (NU7441) and activated by LPS/IFN-γ (Figure 5.2 A). moDCs were treated with a series of concentrations of DNA-PKi, prior to stimulation with LPS/IFN-γ, and IL-23 levels were measured at 24-hour by ELISA. B shows the effect of LPS and IFN-γ on IL-23 secretion enhanced by DNA-PK inhibition in moDC. Missing bars indicate where cytokine secretion was below the limits of sensitivity for the ELISA. Error bars indicate the standard deviation (SD) of triplicate experiments from the same donor. (C) Significant increase of IL-23 production by moDC isolated from multiple donors (25) when pre-conditioned 3μM DNA-PKi compared to LPS/IFNγ triggering only. Each donor is represented by an individual spot. In all figures, statistically significant differences were

determined by the non-parametric unpaired-t-test. (**:0.01>p value≥0.001, ***: p value<0.001).

Table 5.1 Effect of ATM inhibitor KU, DNA-PK inhibitor NU and PERK inhibitor (PERKi) on viability of cells

Condition	**Cell viability (%)**
Immature dendritic cells	100
Mature dendritic cells	95
KU 3µM	100
KU 10µM	97
KU 30µM	0
NU 3µM	90
NU 10µM	25

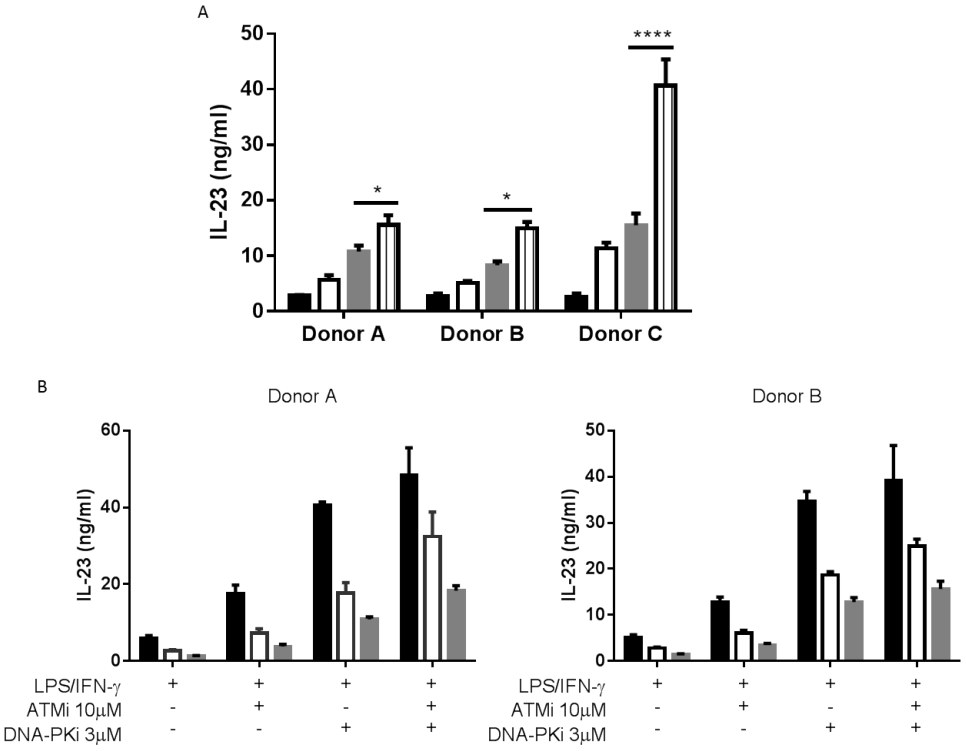


Figure 5.3 Effect of inhibition of both ATM and DNA-PK on IL-23

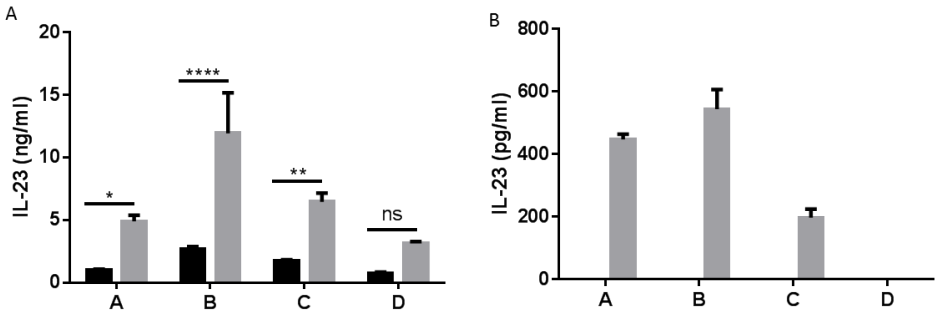
moDCs from three donors were preincubated with ATMi, DNA-PKi, with both of the inhibitors or left untreated before activation with TLR4 agonist and IL-23 levels were measured at 24 hours. For each donor, black bar: LPS/IFN-γ treatment alone, white bar: 10μM ATMi pretreatment, grey bar: 3μM DNA-PKi pretreatment and stripy bar: 10μM ATMi and 3μM DNA-PKi pretreatment (Figure 5.3 A). The IL-23 production levels by moDC were assessed at three time points for two donors at the indicated treatment conditions (Figure 5.3 B); 24 hr (black bars), 48 hr (white bars), and 72 hr (grey bars). Error bars indicate the standard deviation (SD) of triplicate experiments from the same donor.

5.2.3 DNA-PK blockade also results in significant increase in IL-23 production macrophages and BMDC

We next determined whether the effect of DNA-PK inhibition extended to monocyte derived macrophages. These cells also produced elevated levels of

IL-23 when received DNA-PKi. However, the magnitude of the effect was lower than in moDC. In all the four donors, treatment of M1 macrophages with DNA-PKi and LPS/IFN-γ significantly increased IL-23 release (Figure 5.4 A). For M2 macrophages, DNA-PKi increased IL-23 production from undetectable to detectable levels in the presence of LPS (Figure 5.4 B).

In the previous chapter we showed that ATM inhibition elicits significant enhancement in IL-23 in murine DC. Therefore, we performed the same experiment using DNA-PKi. Media exposed BMDC did not secret any detectable IL-23. Corresponding to same three mice in figure 4.3 D, DNA-PKi (3µM) also induced a significant, 2-3 fold increase in mIL-23 from DC of those mice (Figure 5.4 C).



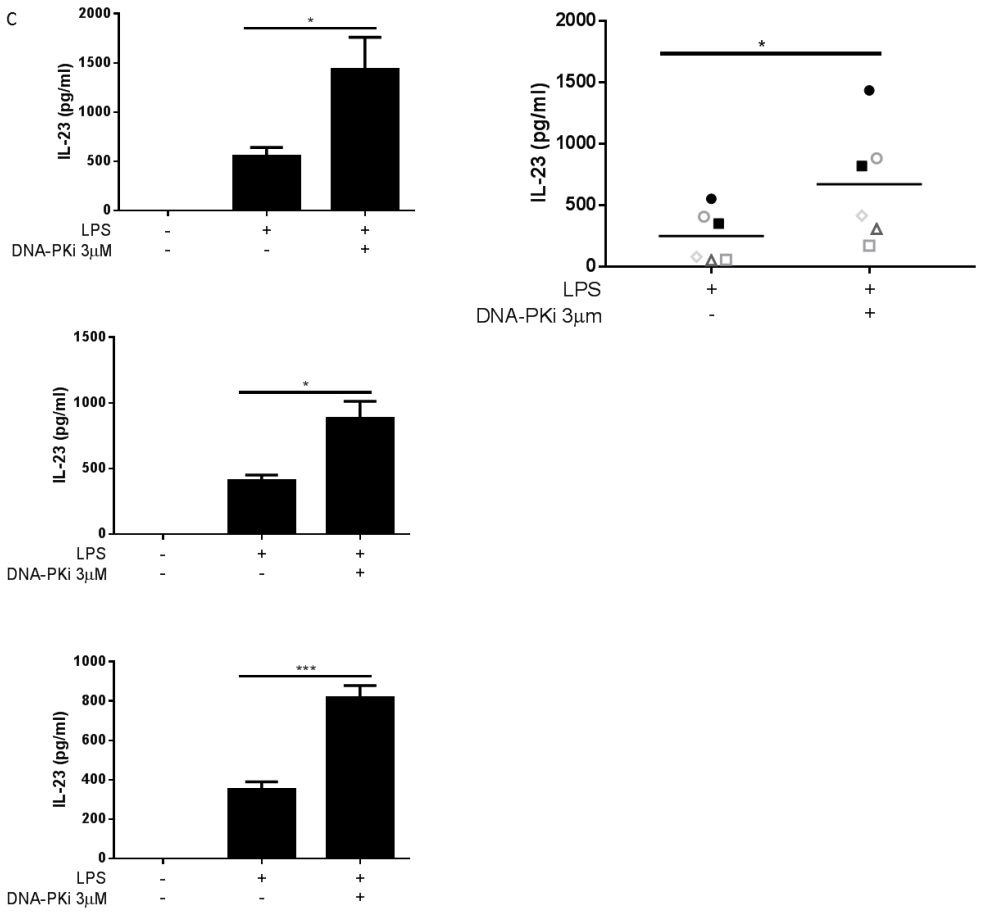
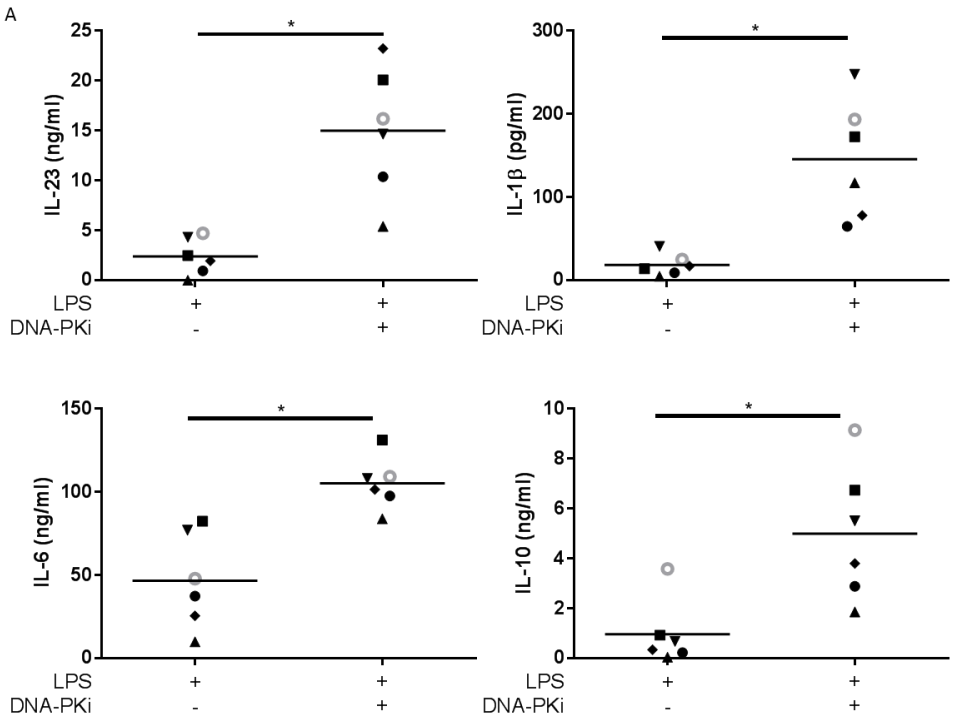


Figure 5.4 DNA-PK dependent IL-23 response in other cell types

M1 and M2 macrophages were generated from monocytes as described previously. M1 macrophages were activated with LPS/IFN-γ alone or after preincubation with 3μM DNA-PKi and M2 macrophages were stimulated with LPS only or in combination with DNA-PKi. Macrophages from 4 donors (Figure 5.4A for M1 and Figure 5.4B for M2) showed enhanced IL-23 secretion with DNA-PK inhibition compared to TLR4 triggering only. BMDC were isolated from mouse bone marrow, rested and treated with DNA-PKi (3μM) for 1 hr before stimulated with LPS for 24 hr, supernatant was then collected to measure IL-23 level by ELISA. Three representative experiments have been shown (Figure 5.4C left) and all the 6 mice done were presented in Figure 5.4C right. In the graphs 5.4 A and , black bar represents LPS/IFN-γ or LPS treatment and grey bar represents cells preconditioned with DNA-PKi.

5.2.4 In addition to IL-23, inhibition of DNA-PK also regulates other cytokine secretion

In contrast to ATM inhibition, inhibition of DNA-PK with the highly selective DNA-PKi (NU7441) potentially enhanced IL-1β, IL-6 and IL-12 secretion by moDC in response to LPS, along with IL-23. In the 6 donors tested, there was mean 6.2-fold increase in IL-23 level compared to LPS exposed DC. The secretion of IL-1β, IL-6 and IL-10 was enhanced by mean 7.9, 2.3, 5.1 fold respectively over the 6 donors. In contrast DNA-PKi did not significantly affect the secretion of IL-12, IL-27 or TGFβ (Figure 5.5).



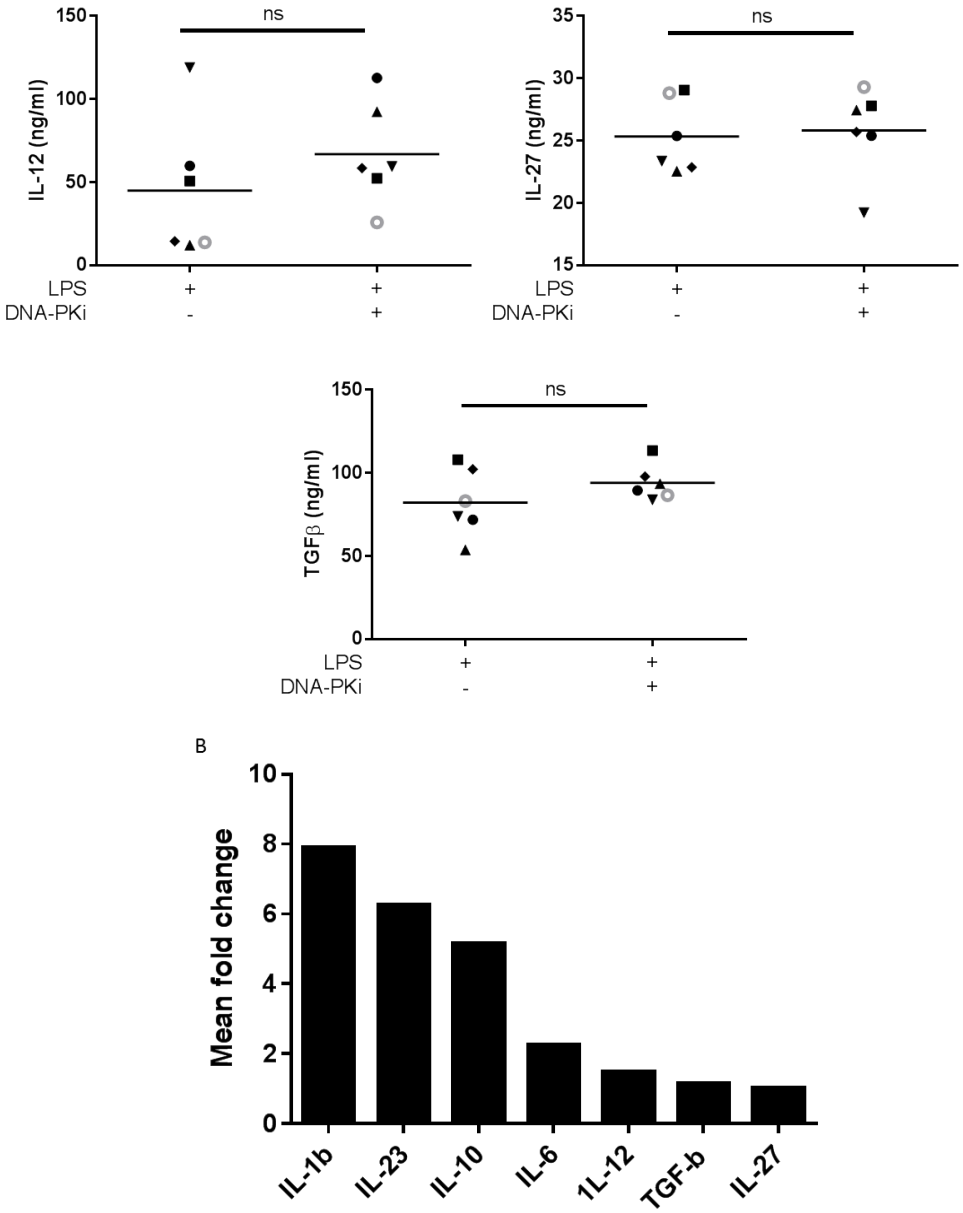


Figure 5.5 DNA-PK inhibition modulates IL-1β, IL-6 and IL-10 secretion along with IL-23

In addition to IL-23, the levels of IL-1β, IL-6, IL-10, IL-12, IL-27 and TGFβ were examined in DNA-PKi inhibited DC supernatant and compared with the levels produced by mature DC (LPS stimulated) from 6 donors. . Each symbol represents an individual donor. Non-parametric unpaired t-test was carried out to test the significance between two groups (Figure 5.5 A). The mean fold change in each cytokine has been presented in figure 5.5 B.

5.2.5 Involvement of different TLR signalling pathways in DNA-PK regulated IL-23 response

Examining the cooperation between different TLR pathways and DNA-PK dependent IL-23 induction in DC, we observed that DNA-PK regulated cytokine release only when DC were triggered with TLR4 agonist. Although exposure to ultrapure agonists of the TLR1/2, 4, 7/8, Dectin 1 or Dectin 1/TLR2 receptors resulted in IL-23 secretion (Figure 5.6A), DNA-PK inhibition did not significantly affect the overall level in all the 5 experiments performed (Figure 5.6B) . Treatment with DNA-PKi reduced TLR7/8 induced IL-23 secretion in 4 out of 5 donors, among which three were significant (Figure 5.6A). But the overall effect was non-significant over the donors done (Figure 5.6B). The presence of IFN-γ only increased the magnitude of IL-23 produced in response to TLR4. The overall significance (p value of 0.0079) was same irrespective of addition of IFN-γ(Figure 5.6B) .

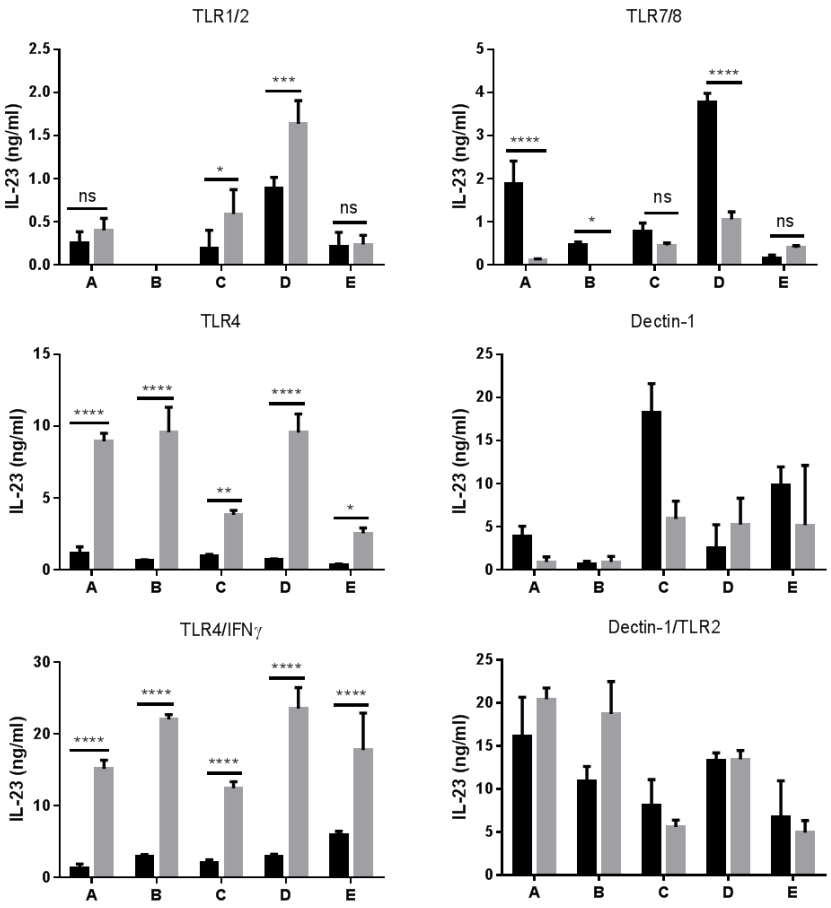


Figure 5.6A Enhanced IL-23 signalling by DNA-PK is only cooperated by TLR4

The response of DNA-PKi conditioned moDC (NU7441 3µM) from 5 donors toTLR1/2, TLR3, TLR4, TLR5, TLR7/8, dectin-1 and dectin-1/TLR2 was studied. Pam3CSK4, poly I:C, LPS, flagellin, R848, curdlan and zymosan were used as TLR1/2, TLR3, TLR4, TLR5, TLR7/8, dectin-1 and dectin-1/TLR2 agonists respectively. moDC were stimulated for 24 hours with the indicated TLR agonist in the presence or absence of 3µM DNA-PKi and IL-23 secretion was determined by ELISA. IFN-γ was only used in conjunction with TLR4 to compare the effect with LPS only stimulation. Black bar indicates TLR stimulation only and Grey bar represents DNA-PKi pre-incubation followed by TLR activation. Error bars indicate the SD of biological triplicates. Missing bars mean that the cytokine secretion was below the limit of the sensitivity of the ELISA. The results for TLR3 and TLR5 were not presented as there was no IL-23 section for all donors studied.

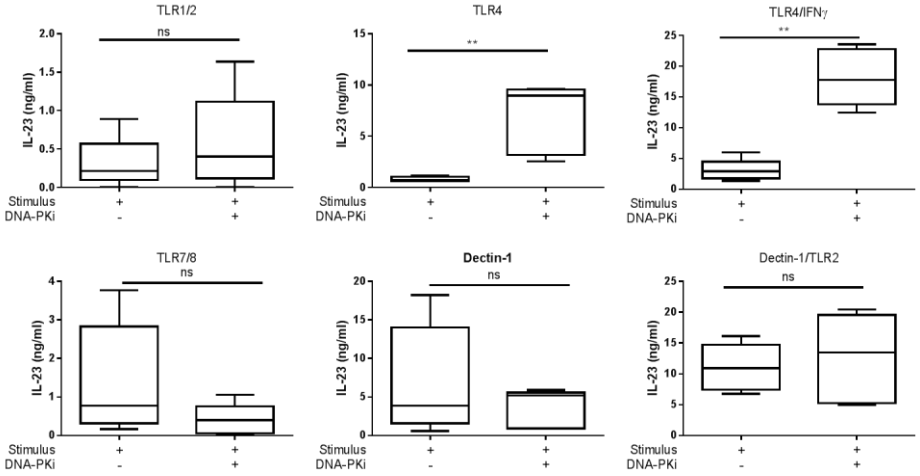


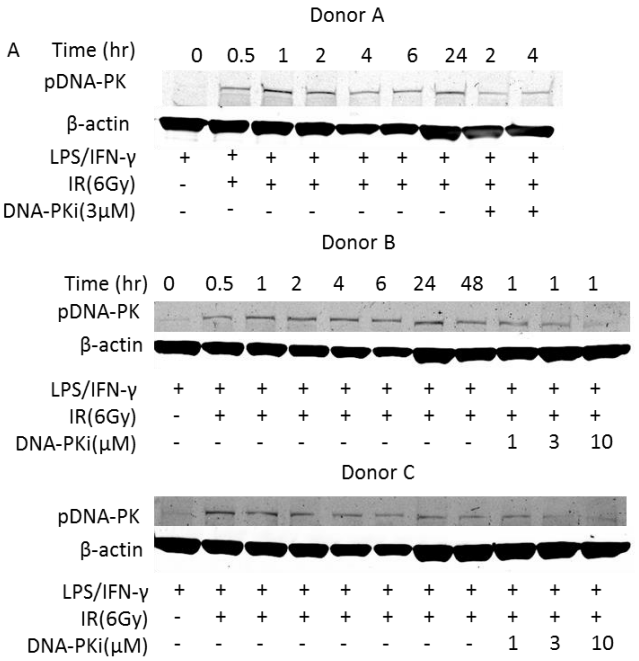
Figure 5.6B TLR4 restricted IL-23 secretion by DNA-PK inhibited DC

The effect of individual TLR signaling on DNA-PK regulated IL-23 release have been presented as box and whisker plots for all the 5 donors tested. The line through each box represents the median value for corresponding treatment group while the error bars indicate the lower and upper responders. Statistical significance was determined by non-parametric unpaired t-test known as Mann-Whitney test.

5.2.6 DNA-PK inhibited DC are unable to overcome the suppressive effect of radiation on IL-23 secretion

DC have been described to express basal levels of DNA-PK activation which increases in response to exposure to ionising radiation. We also observed phosphorylation of DNA-PKcs (Ser 2056) in resting DC in all the three donors (Figure 5.8 A). A rapid increase in DNA-PKcs phosphorylation was seen after 30 min following exposure to 6Gy X-rays. DNA-PKcs remained phosphorylated for some time after this (depending on donors) and then decreased over a period of 48hr. Pretreatment with DNA-PKi prior to exposure to X-rays abolished the activation of DNA-PK.

Prevention of ATM activation by treatment with ATMi rescued IL-23 suppression in radiation exposed DC as shown in the previous chapter. However, in the same four donors, addition of DNA-PKi prior to radiation did not recover IL-23 level to that secreted by non-irradiated dug pretreated cells. Nevertheless, in all of the donors, DNA-PKi restored IR suppressed IL-23 levels to that of the mature cells (LPS treated only).



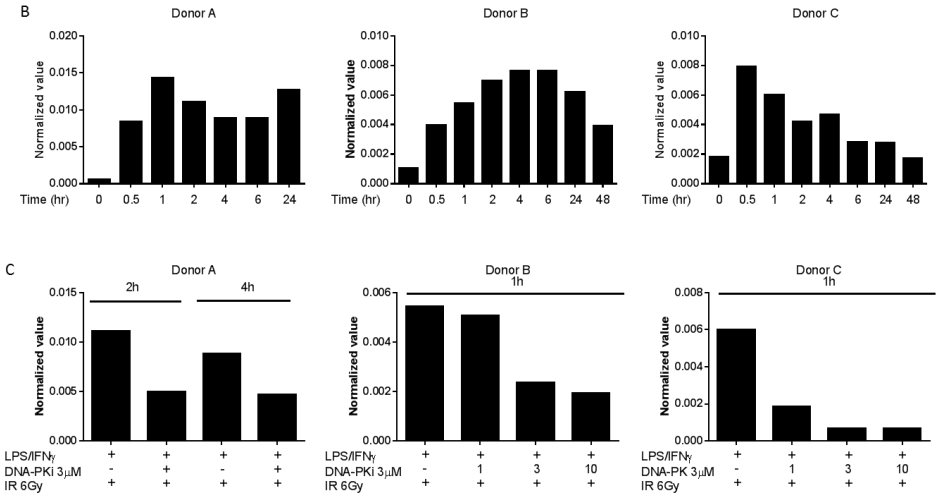


Figure 5.7 DNA-PKi blocks radiation induced activation of DNA-PK

Radiation induced activation of DNA-PK (expression of phospho-DNA-PKcs) was investigated in mature DC following exposure to 6Gy x-ray. Western blots of phospho-DNA-PKcs (Ser 2056) for three independent experiments have been shown for indicated conditions at indicated time points (Figure 5.7 A). In figure 5.7 B and C, normalised expression of the phospho-protein was calculated for the corresponding donors in figure 5.7 A as the ratio of reading of phospho-DNA-PKcs bands to the corresponding β-actin bands. IR induced expression of phospho-DNA-PKcs in mature DC have been shown in figure 5.7 B, whereas the effect DNA-PKi on IR induced phosphorylation of DNA-PKcs has been presented separately in figure 5.7 C for the same three donors.

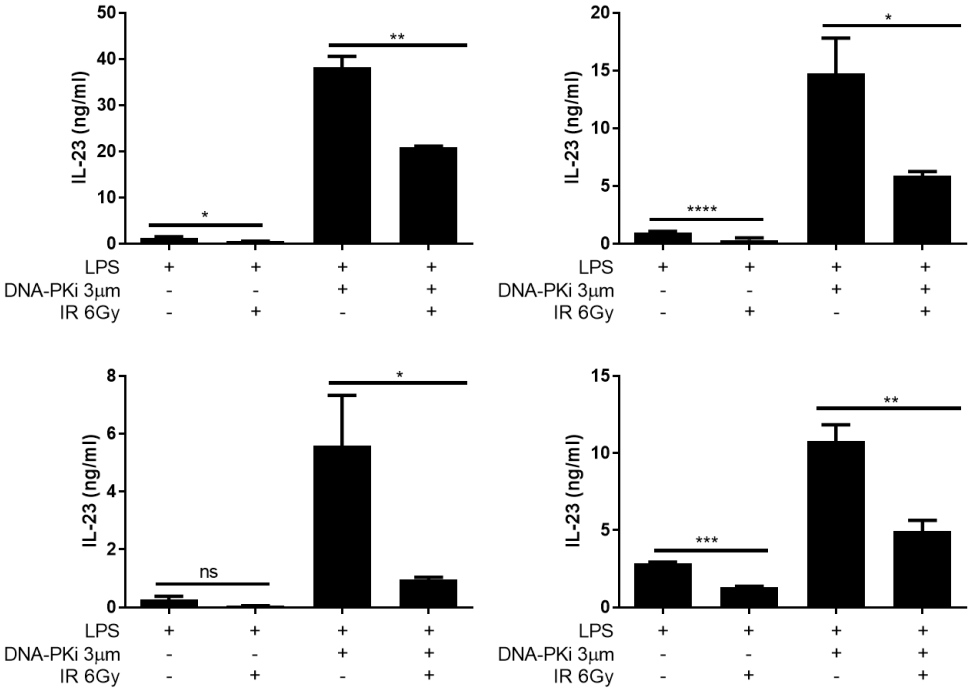


Figure 5.8 Treatment with DNA-PKi fails to abrogate the suppressive effect of radiation on IL-23

moDC were exposed to DNA-PKi (3µM) for 1 hr or left untreated prior to 6Gy radiation and then immediately activated with LPS. Data from 4 representative donors have been presented in figure 5.8. The effect of IR was

shown for LPS stimulation alone and also when radiation induced DNA-PK activation was prevented by pre-treatment with the inhibitor.

5.2.7 Consequence of blocking IL-1β receptor signalling on ATM and DNA-PK mediated IL-23 signalling

Inhibition of ATM and DNA-PK led to increased IL-1β production, notably approximately 8 fold increase in IL-1β upon DNA-PK inhibition compared to LPS treatment alone. Thus to rule out the hypothesis that enhanced IL-23 response in our experimental model is IL-1β dependent, we used IL-1 receptor antagonist (IL-1ra) to abolish IL-1β receptor signalling. First we showed that recombinant IL-1β elicited IL-23 production from TLR4 triggered moDC in a concentration dependent manner (Figure 5.9 A) and this response was abrogated in the presence of IL-1ra (Figure 5.9 B). We then test the effect of IL-1ra on ATMi or DNA-PKi regulated IL-23 response. Clearly (Figure 5.10 A and B) it was observed that blocking IL-1β receptor had no effect on ATM or DNA-PK-dependent IL-23 production. We repeated the same experiment (Figure 5.10 C), where we again showed that in the same donor IL-1β induced IL-23 secretion which was blocked by IL-1ra, but ATMi or DNA-PKi induced IL-23 secretion was unaffected by IL-1ra.

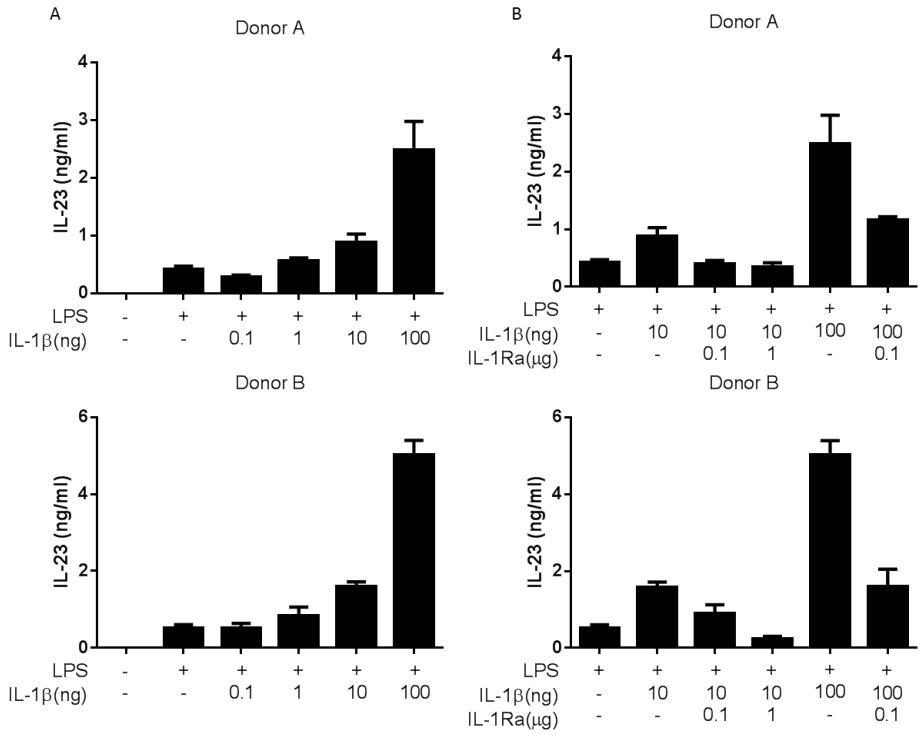
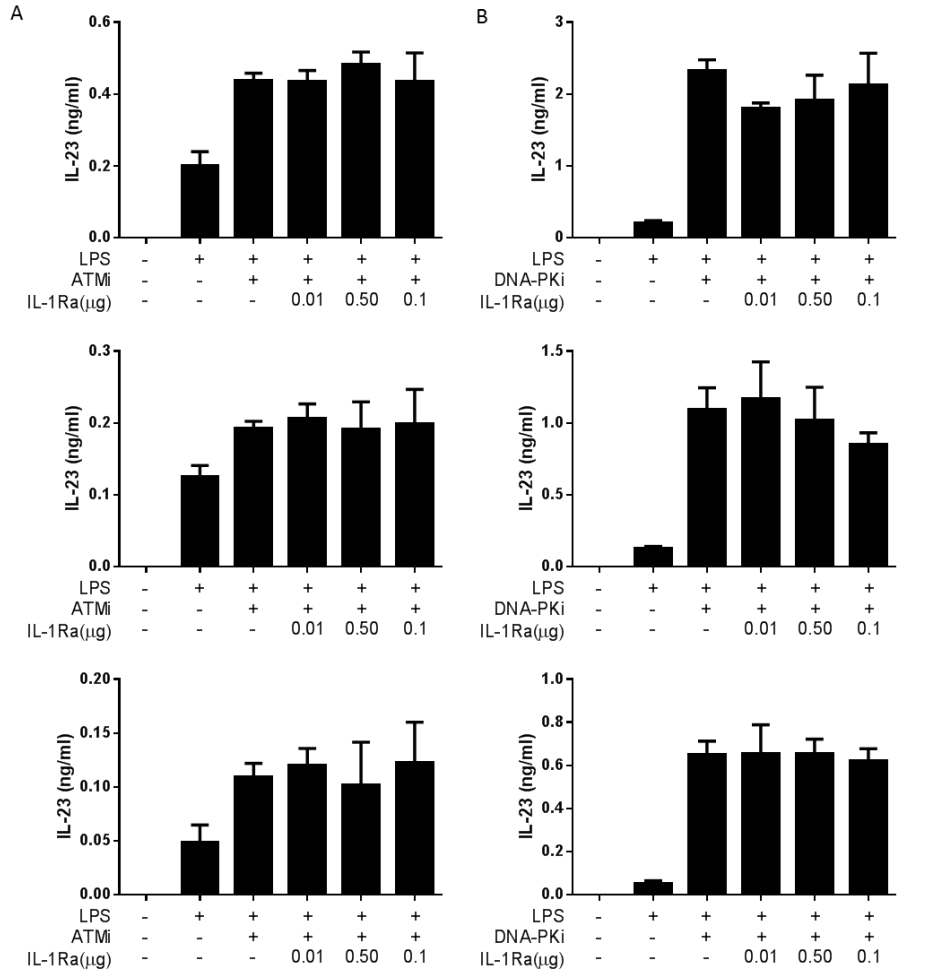


Figure 5.9 Effect of recombinant IL-1β on IL-23 production

moDC were stimulated with LPS in the presence of recombinant IL-1β for 24 hr and the level of IL-23 was determined by ELISA. IL-1β driven IL-23 secretion from two donors have been demonstrated in figure 5.9 A when DC received 0.1, 1, 10 0r 100 ng/ml IL-1β. To test that IL-1ra could block IL-1β dependent IL-23 secretion, moDC were treated with IL-1ra for 1 hr prior to stimulation with IL-1β and LPS. Indicated concentrations of IL-1ra were used for only 10 ng/ml and 100ng/ml IL-1β to determine IL-23 production (Figure 5.9 B) for the same two donors as in figure 5.9 A.


A
B
IL-23 (ng/ml)
LPS
ATMi
DNA-PKi
IL-1Ra(μg)
0.01
0.50
0.1

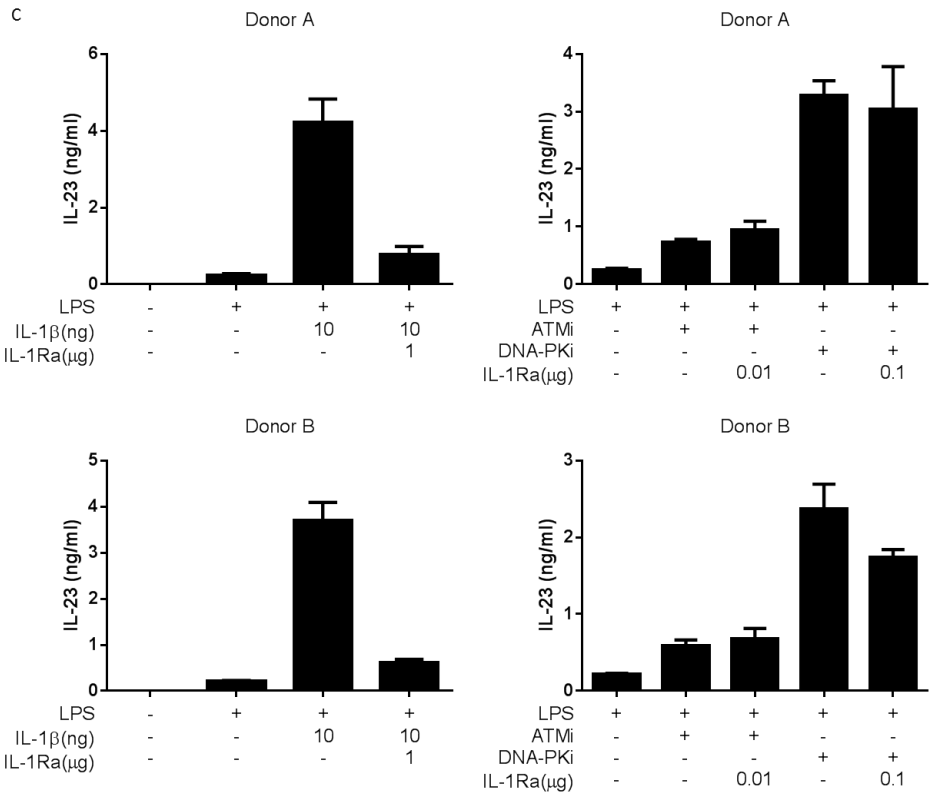

C
Donor A
Donor B
IL-23 (ng/ml)
LPS
IL-1β(ng)
IL-1Ra(μg)
ATMi
DNA-PKi
10
1
0.01
0.1

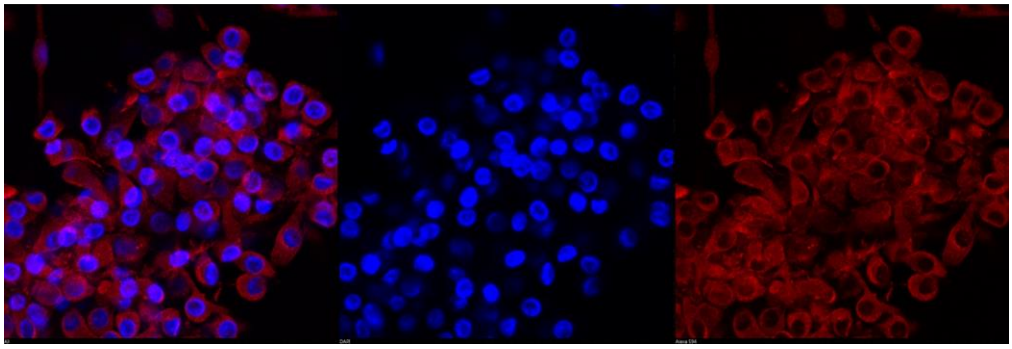
Figure 5.10 ATM or DNA-PK dependent IL-23 response is not mediated by IL-1β

IL-1ra and ATMi or DNA-PKi were added to moDC fo1 1 hr and then triggered with TLR4 agonist. The effect of varying concentrations of IL-1ra on IL-23 secretion have been documented from the same three independent experiments in figure 5.10 A for ATM inhibition and figure 5.10 B for DNA-PK inhibition. In figure 5.10 C, the effect of 0.1 µg IL-1ra on IL-1β induced IL-23 production as well as on ATM or DNA-PK regulated IL-23 has been represented for same two donors.

5.2.8 Analysis of NF-κB activation upon inhibition of ATM and DNA-PK

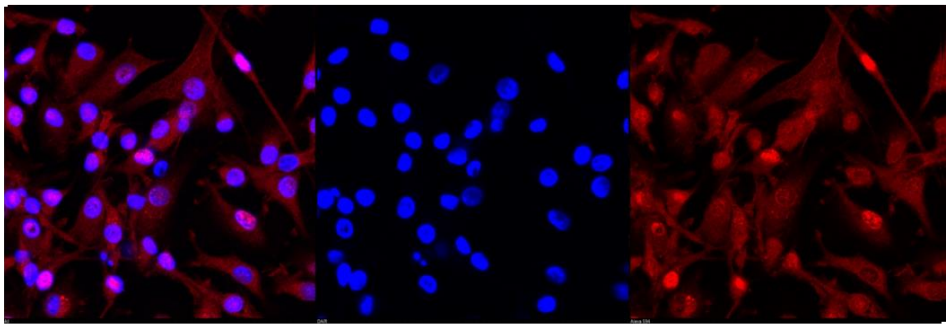
The involvement of NF-κB in IL-23 signalling has been reported by several groups. To find out whether inhibition of ATM or DNA-PK enhanced IL-23 production through increasing NF-κB activation over TLR4 signalling alone, we examined the nuclear translocation of NF-κBp65 by confocal microscopy. In response to LPS stimulation, the translocation of p65 was higher at 2 hr and then decreased over time. At 6 hr, all p65 was completely out of the nucleus. Following inhibition of ATM or DNA-PK, maximal translocation was also observed at 2 hr. However, in case of DNA-PK inhibition, cells seemed to retain p65 even at 6 hr.

iDC

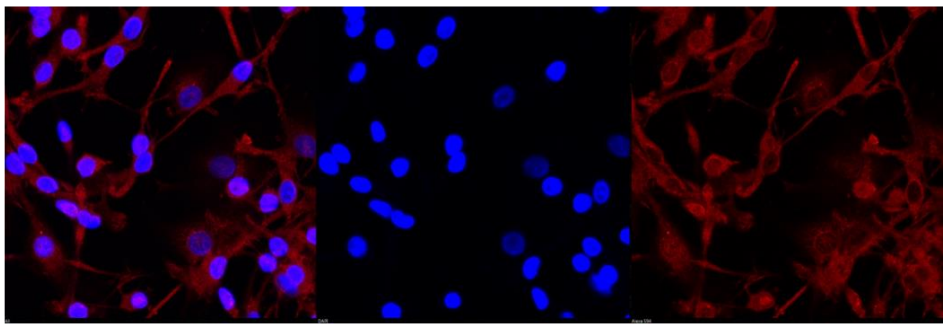


LPS

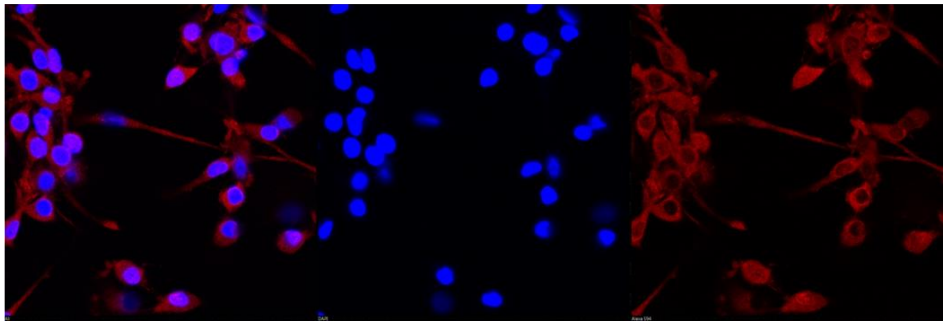
2h



4h

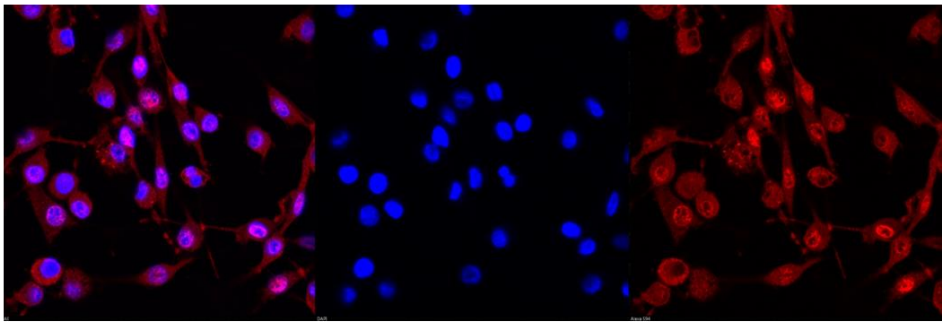


6h

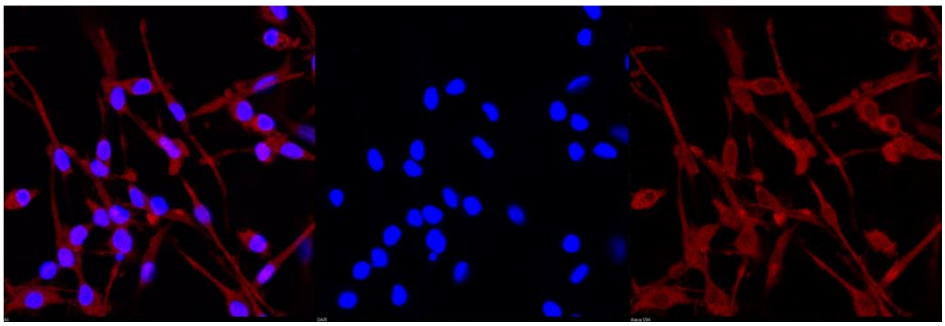


ATMi+LPS

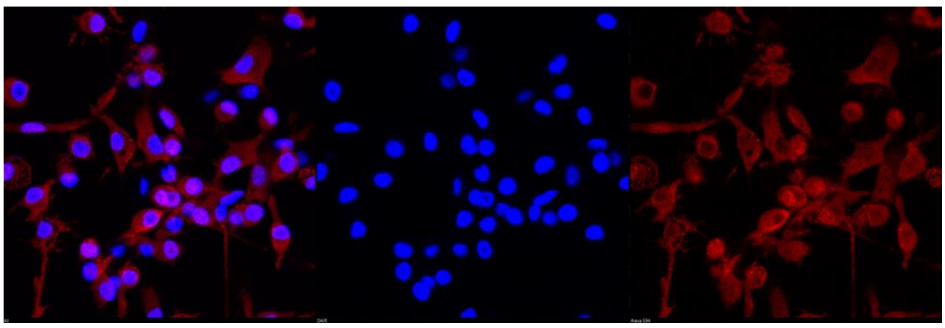
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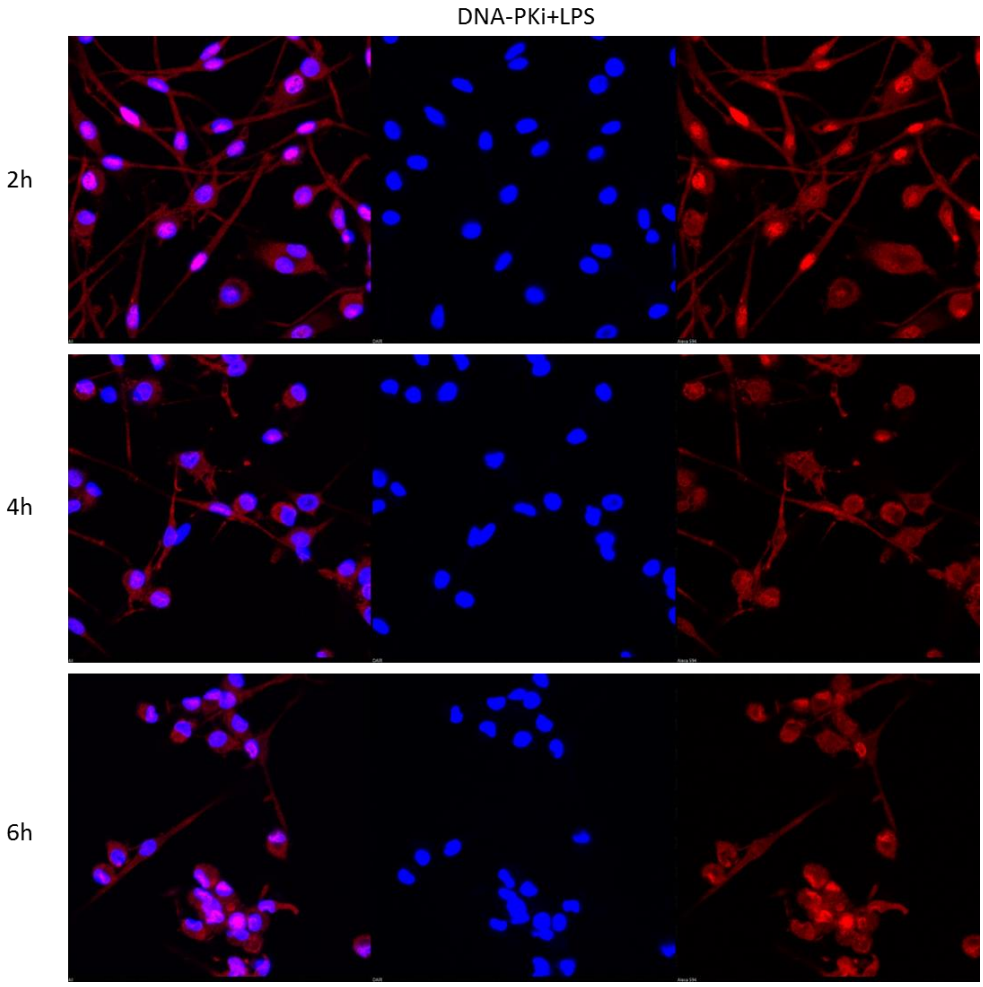


Figure 5.11 Inhibition DNA-PK increases nuclear retention of NF-κB

moDC were treated with either ATMi or DNA-PKi prior to LPS stimulation and NF-κBp65 translocation to the nucleus was examined at 2 hr, 4 hr and 6 hr of maturation. Cell were viewed as 40X magnification and DPI was used as nuclear stain. Observations from one representative donor have been shown.

5.3 Discussion

DNA-PK is a protein kinase of paramount importance for repairing DNA double strand breaks. In contrast to this major role, we observed that DNA-PK also plays a role as an important regulatory factor for IL-23 production by DC which might well be explained by alternate functions of DNA-PK in DC other than DNA repair. Understanding the alternate roles of DNA-PK in DC is important because of their central role in cross-priming, and naïve T cell differentiation through different cytokine production. Identifying regulatory molecules and checkpoints in the IL-23 axis could therefore have profound implications for autoimmunity, immunisation and cancer immunotherapy.

DNA-PKcs is a member of the PI-3K family and functions in double strand break repair through NHEJ. There is homology between the catalytic domains of DNA-PKcs and PI-3K [470]. It has been demonstrated that ATM is required for the activation of DNA-PKcs [570]. This leads to a suggestion that both of these proteins might function in the same signalling pathway with ATM upstream of DNA-PKcs. NU7441 is a highly potent and selective ATP-competitive inhibitor of DNA-PKcs with an IC50 of 12nM. Furthermore the potency of NU7441 is 20-100 times more than other DNA-PK inhibitors and at least 100-fold more selective for DNA-PK than other PI-3K family members [470, 571]. NU7441 inhibits autophosphorylation of DNA-PK at Ser2056, which is known as the most robust and specific target for DNA-PK activity. NU7441 was reported to potentiate cell death through cellular radiosensitization and chemosensitization by inhibiting DNA-PK mediated DSB repair [571]. All these

features make NU7441 the drug of choice for dissection of DNA-PK activity. Therefore, we can conclude the elevated levels of IL-23 produced by 3μM DNA-PKi conditioned moDC were likely as a result of DNA-PKcs inhibition only. Alternatively other small molecule inhibitors [572, 573]such as NU7026, KU0060648 and IC86621, could be used to re-examine our observation of the effect of DNA-PK inhibition on IL-23. NU7026 is 50 fold more selective for DNA-PK than ATM and ATR. It has an IC50 of 0.23 μM for DNA-PK, 13 μM for PI-3K and >100 μM for ATM or ATR [574]. IC86621 is also a strong inhibitor of DNA-PK and does not have activity against closely related PI-3Ks even at concentrations of 100μM [572]. Results should be interpreted more carefully when using KU0060648 as it targets both DNA-PK and PI-3K [575].

We have shown for the first time that pre-treatment of DC with DNA-PKi followed by TLR4 stimulation augments IL-23 production from human or murine DC. Interestingly, LPS triggering increased phospho-DNA-PKcs expression over time from that expressed by resting DC. Rapid activation of DNA-PKcs was observed immediately after exposure to 6Gy IR. This is in agreement with Bauer *et al.* who described basal expression of DNA-PKcs in DC and macrophages both at the mRNA and protein level. DNA-PKcs activity was further significantly enhanced when DC or macrophages were exposed to radiation [469]. Although addition of DNA-PKi prevented LPS or IR induced phosphorylation of DNA-PKcs, it did not recover IL-23 level suppressed by IR to that of the drug treated non-irradiated cells. However, it did recover IL-23 secretion to that of the LPS treated non-radiated cell proving our hypothesis

that activated DNA-PK is a negative regulator of IL-23. In the previous chapter we have demonstrated activation of ATM upon IR and its role in CREB phosphorylation regarding IL-23 production. Following exposure to IR, whilst DNA-PKi could block activation of DNA-PK, it did not affect phosphorylation of ATM (data not shown) enabling ATM to negatively regulate IL-23 through CREB. This may explain the failure of DNA-PK inhibited DC to reverse the suppressive effect of IR on IL-23 and also provide the evidence that ATM and DNA-PK function through discrete signalling pathway to control IL-23 response. In all our experiments, we only looked at phosphorylated form of ATM or DNA-PK in response to different activation signals. It would be nice to confirm that the change in phosphorylated ATM or DNA-PK is not due to change in the total protein by comparing total ATM or DNA-PK to the phosphorylated form.

Although the association between DNA-PK and immune responses has not yet been well documented, recent evidence suggest a role for this protein in inflammation [462, 576]. Cytoplasmic role of DNA-PKcs has been reported where it regulates IL-10 and IL-12p70 production in macrophages. Whilst DNA-PKcs is required for oligodeoxynucleitdes containing unmethylated CpG motifs (CpG-ODNs) induced production of IL-10 in peritoneal macrophages, it negatively regulates IL-12 production [576]. By directly stimulating macrophages, DC and B cells, CpG-ODNs induce the production of cytokines including IL-6, IL-10, IL-12, IFN-α/β and TNF-α, and facilitate the induction of Th1 immune response [577, 578]. Evidences show that immune activation

induced by CpG-ODN is mediated through TLR9 dependent as well as TLR9 independent signalling pathways [579, 580]. DNA-PKcs induces IL-10 production from CpG-ODN stimulated macrophages through ERK activation which has been shown to be impaired in macrophages from SCID mice (with mutation in DNA-PKcs) and also in siRNA mediated DNA-PKcs KO mouse monocyte/macrophage cell line RAW264.7. This study suggests that DNA-PKcs in the cytoplasm contributes to CpG-ODN induced cytokine production and also by manipulating intracellular trafficking of CpG-ODN, it is possible to regulate production of anti-inflammatory cytokine IL-10 and inflammatory cytokine IL-12 in macrophages through the DNA-PKcs-ERK pathway [576]. We observed that DNA-PK inhibition reduced IL-23 and IL-10 (data not shown) production upon TLR7/8 stimulation in four of the five donors tested. As TLR7/8 and TLR9 signal through the common signalling pathway [298], future investigation into DNA-PKcs-ERK pathway could provide insights into the mechanism of the inhibitory role of DNA-PKi on TL7/8 induced cytokine production.

Recently DNA-PKcs has been reported to be involved in CpG-ODN induces IL-6 and IL-12 responses from murine BMDC. This study showed that by binding with DNA-PKcs, CPG-ODN induced its association with TRAF6 [455]. TRAF6 is a key relay molecule that transduces signals from TLR9 and MyD88 to its downstream effectors NF-κB and JNK leading to expression of proinflammatory cytokines [581]. Loss of DNA-PKcs resulted in impaired

phosphorylation of IKK, IκBα, NF-κB and JNK and also DNA-PKcs deficient DCs displayed defective IL-6 and IL-12 response in response to CpG-ODN [455]. The Interferon regulatory factor-3 (IRF3) plays a central role in host defence against viral infection by inducing IFN-α and IFN-β transcription [582]. It is also responsible for enhanced IL-23 production in systemic lupus erythematosus (SLE) [583]. Interestingly, DNA-PK is also activated by viral infections and phosphorylates IRF-3, protecting it from degradation and promotes IRF-3 dependent proinflammatory responses. DNA-PK functions as a pattern recognition receptor by binding cytoplasmic DNA and modulates host defence against viral infection. This triggers transcription of type I interferons, cytokines and chemokine genes via a pathway involving IRF-3, TANK-binding kinase and stimulator of interferon genes [456]. Vaccinia virus has been reported to evolve a strategy to escape this mechanism of immune induction [584]. A highly conserved virulence protein of this virus called CD16, binds directly to the Ku heterodimer and blocks DNA-PK binding to DNA. This prevents DNA-PK dependent DNA sensing and decreases production of cytokines and chemokines. DNA-PK also induces pro-inflammatory responses by NK cells by phosphorylating Akt on Ser473 through an endosomal signalling pathway [569].

Limited information is available regarding the role of DNA-PK in adaptive immunity mediated by DC. A recent study has described that DNA-PK is involved in the induction of Th2-mediated airway inflammation to house dust mite (HDM) in DC [585]. In response to HDM, DNA-PK was phosphorylated

and activated in DC by intracellular ROS. ROS generation was dependent on DNA-PK as BMDC from SCID mice were defective in production of ROS which plays important role in MHCII-restricted antigen presentation and activation of CD4+ Th2 cells [586-590]. Treatment with DNA-PKi and ROS inhibitors attenuated HMD-mediated Akt phosphorylation on Ser473 which a substrate of DNA-PK. Akt signalling important in regulating the activation and antigen-presenting ability of DC [591]. Importantly, the study demonstrated that pharmacological inhibition of DNA-PK reduced the induction of allergic reaction, Th2 effector cytokine production and airway inflammation in response to HDM. Although these observations are in contrast with our hypothesis of the role of DNA-PK in regulating IL-23 response, the high levels of DNA-PK in human cells may explain the involvement of this protein kinase in multiple molecular pathways.

DNA-PK inhibition resulted in 10-fold increase in IL-23 secretion and also increased production of IL-1β, IL-6 and IL-10. This suggests that DNA-PK may function in a signalling pathway that regulates multiple cytokines. Although the molecular mechanism has not been proved yet, it is expected that increased levels of IL-1β, IL-6 and IL-23 produced by DNA-PK inhibited would favour differentiation of naïve T cells towards Th17 response. Increased IL-10 production through DNA-PK inhibition is consistent with a report that demonstrated elevated IL-10 secretion by HDM-challenged DNA-PKcs deficient mice [585]. IL-1β induces IL-23p19 expression in human fibroblast like synoviocytes in RA [592]. Thus to exclude the possibility that our observed

increased in IL-23 is not dependent on IL-1β, we used IL-1ra. IL-1ra is a member of the IL-1 family that binds to IL-1 receptors but does not induce any intracellular signalling [593]. The presence of IL-1ra did not alter ATM or DNA-PK mediated enhanced IL-23 level indicating that IL-1β is not involved in our model. One important consideration is that we determined IL-1β level in our DC supernatant by quantifying the total protein concentration by ELISA as opposed to the mature IL-1β molecule. As an important transcription factor, we tried to examine whether NF-κB has an enhanced activity in ATM or DNA-PK inhibited DC compared to only LPS activated DC. Primarily, our confocal microscopy data showed that inhibition of DNA-PK but bot ATM resulted in longer retention of NF-κB p65 in the nucleus. However, this observation should be validated by complementary experiment in future to be confirmed. One of experiments might involve preparing cytoplasmic and nuclear extracts of DC treated with either ATMi or DNA-PKi and looking at the levels of phosphor-NF-κB in the cytoplasm and nucleus compared to LPS matured DC.

In summary, we have identified a novel role of DNA-PK in DC function where it negatively regulates TLR4 induced cytokine production. We showed that LPS induces phosphorylation and activation of DNA-PK. Simultaneous inhibition of ATM and DNA-PK had additive effect on IL-23 production suggesting these two may function through different molecular mechanism. This study identifies a novel immune-regulatory role for DNA-repair systems in DC resulting in the regulation of a series of Th17 associated cytokines.

Chapter 6 Investigation of the involvement of the UPR pathway in ATM or DNA-PK-regulated IL-23 response in DC

6.1 Introduction

The lumen of the endoplasmic reticulum (ER) provides an environment for synthesis, folding, and maturation of all most all secreted and transmembrane proteins [378]. When protein folding is compromised in the ER due to physiological or pathological conditions, the accumulated misfolded or unfolded proteins lead to ER stress. ER stress activates a transcriptional induction pathway termed the unfolded protein response (UPR), which functions mainly to restore normal ER environment [378]. The three ER stress sensors PERK, IRE1 and ATF6 transduce information about the folding status of the ER to the cytosol and nucleus and regulate transcription of genes to restore balance in the ER, induce growth arrest or initiate apoptosis. Activation of PERK results in decreased efficiency in protein synthesis through phosphorylation of translation initiation factor-2α (eIF2α). However, this translational shut down favours ATF4 gene expression which induces transcription of several UPR genes including C/EBP-homologous protein (CHOP) and growth-arrest and DNA damage inducible protein GADD34. CHOP activates apoptosis if the cell fails to resolve ER stress, whereas GADD34 dephosphorylates eIF2α to continue protein synthesis and cell survival [594]. Activated IRE-1 acts an endoribonuclease and splices a 26-bp intron from X-box protein-1 (XBP1) mRNA, producing a transcription factor that triggers

several ER stress chaperone genes [380]. In addition to its well-known functions in ameliorating conditions of cellular stress, the UPR has recently been shown to play roles in regulating immunity [412, 419, 427, 428, 430, 431, 595-597]. We have described in the previous chapters that IL-23 production in DC is markedly increased upon inhibition of ATM kinase or DNA-PK. Here we have investigated the potential involvement of ER stress responses in ATM and DNA-PK regulated IL-23 signalling.

Grp78/Bip is an ER chaperone that binds to unfolded proteins and also activates signal transduction through PERK, IRE1 and ATF6 in response to ER stress. The transcriptional activity of the Bip promoter is increased by 20 fold following treatment with thapsigargin (Tg) which depletes ER calcium stores by blocking the ER calcium ATPase [598]. The most widely used biological marker for the onset of UPR is the transcriptional activation of the Bip promoter. Specifically, Bip promoter activity is regulated by a constitutively expressed multifunctional protein YY1. Despite being expressed constitutively, YY1 has no effect on the basal activity of the Bip promoter. YY1 has been shown to induce activity of the Bip promoter only upon ER stress which is mediated partially through interaction with the nuclear form of ATF6 [599]. In this regard, ATF6 is an ER membrane bound leucine zipper protein family transcription factor which is activated by proteolysis in response to ER stress to induce transcription of the ER chaperone genes [600]. The activated nuclear form of ATF6 interacts with YY1 resulting in Bip promoter induction. Interestingly, the binding and dissociation of Bip is critical for the activation of

ATF6. Furthermore, in unperturbed ER, ATF6 remains as a monomer, dimer or oligomer through the inter- and intramolecular disulphide bonds in the luminal domain. ER stress dependent reduction of ATF6 levels allows the reduced monomer to translocate to the Golgi apparatus for proteolytic cleavage to generate the active nuclear form. The unique activation process of ATF6 compared to that of IRE1 or PERK may explain the different kinetics in the responses of IRE1, PERK and ATF6 dependent pathway to various ER stress inducers [410].

The involvement of ATM in oxidative stress in the brain, testes and thymus has been established [601]. With respect to this, ATM deficiency stimulated ER stress in astrocytes which could be attributed by high levels of the ER stress marker, Bip in ATM-/- astrocytes [517]. Recent observations have described the upregulation of ER stress and UPR in ATM deficient thymocytes and thymic lymphoma cells [237]. In ATM-/- thymocytes and ATM-/- thymic lymphoma cells, there were increased levels of Bip, phospho-eIF2α and XBP-1 compared to ATM+/+ thymocytes. The levels of caspase-3 were also increased in these cells reflecting mitochondria dependent apoptosis induced by ER stress. Oxidative stress has been described to be responsible for stimulating ER stress in ATM deficient thymocytes. This has been shown experimentally as treatment with H_2O_2 increases the levels of ER stress marker proteins, whereas antioxidants alleviate the effects of ER stress [237]. Very recently He at el proposed that ATM inhibits tunicamycin (a drug that induces ER stress by inhibiting protein glycosylation) induced ER stress in embryonic lung fibroblast

cell. This group found that with treatment of tunicamycin, the markers of ER stress are activated in ATM deficient cells but not in ATM proficient cells and ATM also blocks the activation of caspase-12, a hallmark of ER stress mediated apoptosis, by tunicamycin. In the absence of ATM, the key role of caspase-3 has also been confirmed in activating cell death pathway [602].

ER stress plays a critical role in the development and survival of DCs. Mice lacking XBP-1 have reduced number of DC subsets with notably severe defects in the plasmacytoid DC population (pDC) [419]. This can be explained by an increased sensitivity to apoptosis during differentiation of XBP-1 deficient DC progenitors as constitutive activation of UPR and high levels of XBP-1 transcript have been demonstrated in immature DCs [419]. It has recently been published that ER stress and the XBP-1 pathway enhances DC maturation and activation mediated by high mobility group box-1 protein (HMGB1) [603]. HMGB1 is an evolutionarily ancient non-histone DNA-binding protein within the nucleus, and also functions as a late inflammatory cytokine released by macrophages, NK cells and DCs [603]. XBP-1 is also required for the differentiation of B cells into plasma cells [412]. In macrophages, the TLR and IRE1-XBP1 pathways act in concert for optimal and sustained production of proinflammatory cytokines such as IL-6 and TNF. Interestingly, phosphorylation of IRE1 triggered by TLR stimulation is independent of ER stress as demonstrated by the absence of any other ER stress markers. This suggests that ER stress may regulate the intensity and duration of innate immune response, and may promote inflammation [428]. ER stress is also responsible for the production of IFN-β by macrophages through activation of

XBP-1. The involvement of CHOP, one of the ER stress transcription factor has recently been described as an indispensable factor for IL-23 expression by DCs [604].

In this chapter, we tested the hypothesis that ER stress responses are involved in TLR4 dependent IL-23 secretion induced by ATM or DNA-PK inhibition in DC. We investigated the splicing of XBP-1 mRNA and phosphorylation of eIF2α as markers of ER stress upon ATM or DNA-PK inhibition in TLR4 triggered DC. Our initial findings suggested that these molecules may signal through ER stress response as the increase in IL-23p19 transcription coincided with increase in the spliced form of XBP1 mRNA. Similarly we also observed regulation in eIF2α phosphorylation following inhibition of ATM or DNA-PK. Therefore, we further examined the role of UPR pathways regarding ATM/DNA-PK-dependent IL-23 signalling by using selective antagonists of the IRE1 and PERK arms of UPR.

6.2 Results

6.2.1 Expression of the ER stress marker Bip in ATM or DNA-PK inhibited moDC

Since an increase in Bip expression is a classical hallmark of ER stress responses, we examined the expression of Bip in ATMi or DNA-PKi conditioned moDC. As expected, DC treated with tunicamycin (TM) (induces ER stress by inhibiting protein glycosylation) showed marked increase in Bip expression. However, ATMi or DNA-PKi treated moDC did not show any increase in Bip as compared to LPS/IFN-γ stimulated DC. The MEWo cell line was used as a positive control for these studies as tumour cells have been described to have increased levels of Bip activation [605].

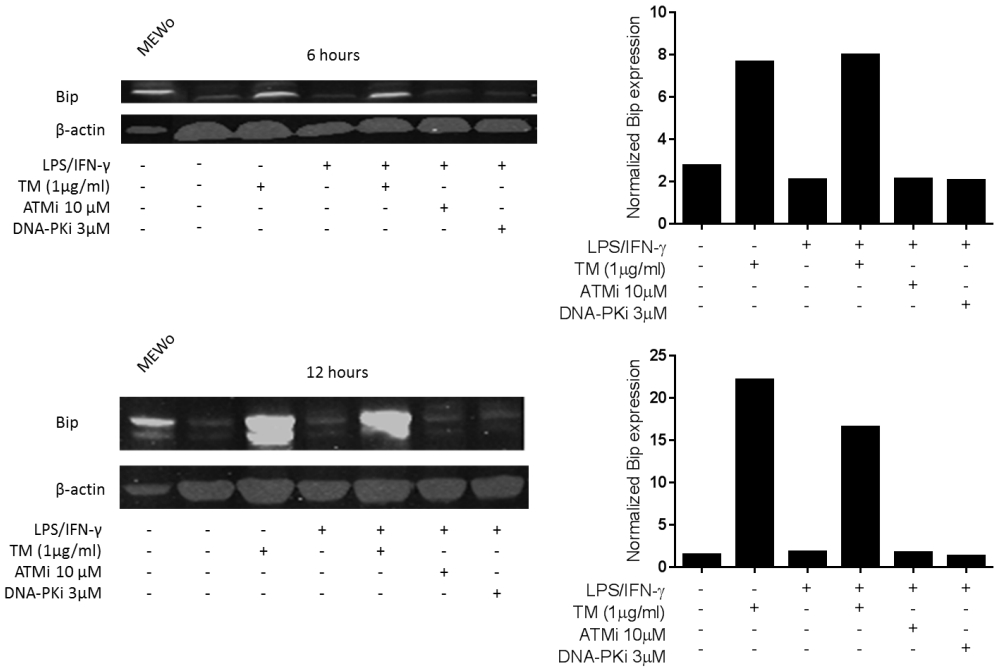


Figure 6.1 Expression of Bip from ATM and DNA-PK inhibited DC

Figures on the right panel represent western blots showing the expression of Bip, one of the ER stress marker for the indicated stimulatory conditions of moDC. moDC were pre-conditioned with 1µg/ml TM, 10µM ATMi, 3µM DNA-PKi or left untreated before activation with LPS/IFN-γ. The cells were then

harvested at 6 hours, and 12 hours. Bip level was determined by 2-colour western blot. Graphs on the right panel show normalized Bip levels in relation to β-actin control from corresponding blots at 6 hours and 12 hours.

6.2.2 Treatment with tunicamycin increases XBP-1s mRNA levels in DC and U937

In order to determine the effect of ATM or DNA-PK inhibition on the IRE1 arm of the UPR, we first validated the effect of TM in activating IRE1 in moDC and monocytic tumour cell line U937. One of the early changes resulting from IRE1 activation is the splicing of XBP-1 mRNA. Thus the XBP-1s mRNA level was determined by qPCR in TM treated moDC and U937 cell line. After 6hr treatment with TM, both moDC and U937 cells showed marked increase in XBP-1s mRNA in all three independent experiments (Figure 6.1 A & B). These observations confirmed that XBP-1 splicing could be used as a marker of activation of ER stress response in our cellular model systems.

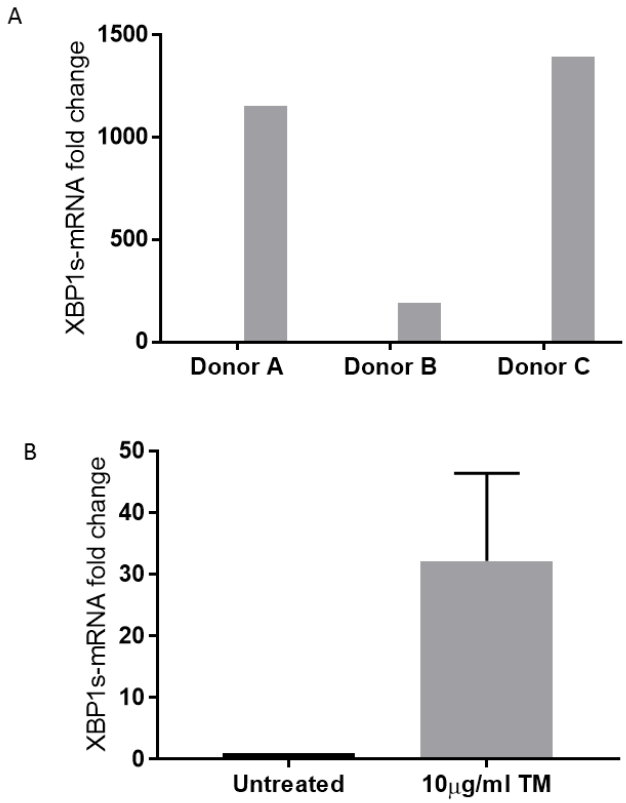


Figure 6.2 Effect of tunicamycin on splicing of XBP-1 mRNA in moDC and U937 cell line

moDC or U937 cells were treated with 10µg/ml TM for 6 hr, cells were lysed, RNA extracted and converted to cDNA. Data show the expression of XBP-1s mRNA as measured by RT-qPCR. Figure 6.2 A represents the fold change in XBP-1s level in moDC and 6.2 B represents the mean of three independent experiments in U937. Black bar shows untreated and grey bar shows TM treated samples.

6.2.3 Splicing of XBP-1 increases with ATM inhibition and synchronizes with increased IL-23 transcription in moDC and U937

Next we examined the involvement of ATM kinase in the production of the spliced form of XBP-1 mRNA in TLR4 triggered moDC and U937 cells. There was a significant increase in XBP-1 mRNA levels in DC following LPS/IFN-γ stimulation. This was maximal at 6 hr and then returned to baseline levels after 20 hr in all the three donors (Figure 6.3 left panel). ATM inhibition resulted in consistently higher rate of splicing compared to LPS induced splicing. Upon ATM inhibition, there was 2-fold increase in XBP-1s transcript level from that of LPS/IFN-γ stimulation alone. However, this also returned to baseline levels after 20 hr. The increase in XBP-1s splicing following ATM inhibition was also observed in the U937 cell line which was differentiated with phorbol myristate acetate (PMA) to acquire LPS responsiveness [606]. However, in U937, we did not observe decreases in splicing even after 20 hr (Figure 6.4 left panel). Importantly, pretreatment with ATMi also markedly elevated the transcription IL-23p19 gene in TLR4 activated DC and U937 (right panels of Figure 6.3 and Figure 6.4). In all the experiments, the change in IL-23p19 transcription was maximal around 9-12 hr post activation which followed the changes in XBP-1 splicing that occurred at 6 hr.

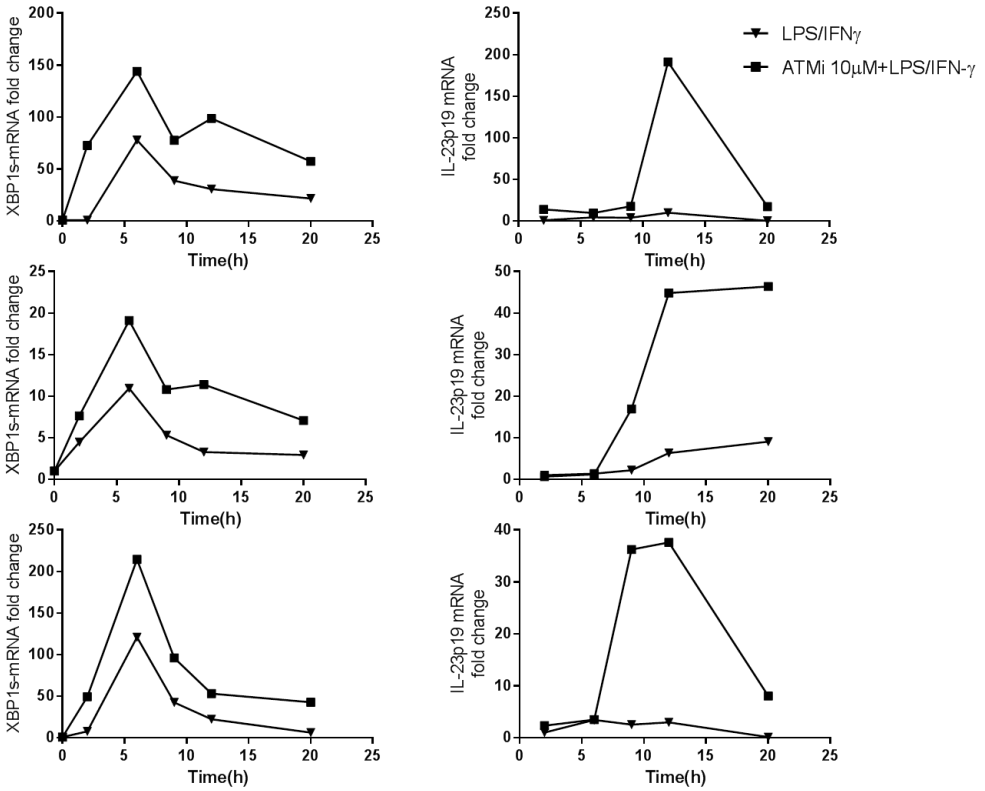


Figure 6.3 Increase in XBP-1s mRNA level and IL-23p19 transcription upon ATM inhibition in TLR4 triggered DC

Cells were lysed and RNA was extracted from iDC, LPS-matured DC (triangle symbol) and ATMi (10μM) pre-treated matured DC (square symbol) at the indicated time points and analysed for XBP-1s and IL-23p19 mRNA by RT-qPCR. Results were calculated as relative sXBP-1/IL-23p19 level normalised to HPRT and plotted as a fold change compared to gene expression by iDC. Data from 3 donors are presented in the left panel for XBP-1s and in right panel the corresponding donor-matched IL-23p19 mRNA.

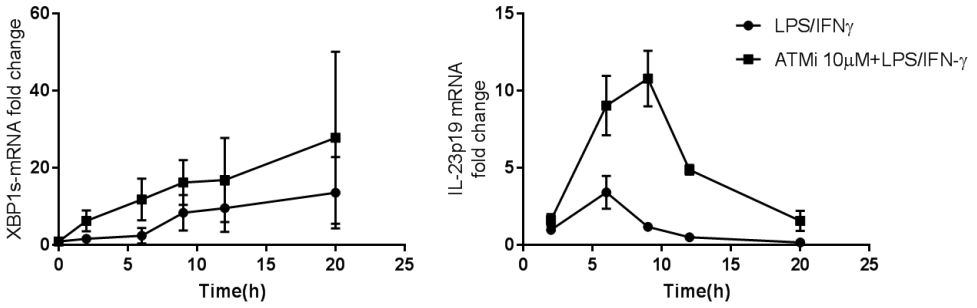


Figure 6.4 Increase in XBP-1s mRNA level and IL-23p19 transcription upon ATM inhibition in TLR4 triggered U937 cells

U937 cells were differentiated with 10ng/ml PMA for 24 hr before stimulation with TLR4 agonist. Then the cells were left untreated or pretreated with ATMi (10μM) for 1hr prior to LPS stimulation. Cell were then lysed and RNA was extracted from untreated, LPS-treated (triangle symbol) and ATMi pre-treated cells (square symbol) at the indicated time points and analysed for XBP-1s and IL-23p19 mRNA by RT-qPCR. Results were calculated as relative sXBP-1/IL-23p19 level normalised to HPRT and plotted as a fold change compared to gene expression by untreated cells. Data from 3 independent experiments are presented in the left panel for XBP-1s and in right panel the corresponding IL-23p19 mRNA.

6.2.4 XBP-1 mRNA splicing is induced upon DNA-PK inhibition and mirrors the increased IL-23 transcription in moDC and U937

The effect of inhibition of DNA-PK was also examined on the expression of XBP-1s transcript. Like ATM inhibition, the presence of DNA-PKi resulted in higher splicing of XBP-1 from TLR4 triggered DC as compared to LPS/IFN-γ stimulation alone. Following DNA-PK inhibition XBP-1s level was increased by 2-fold at 6 hours from that of LPS maturation only except from one donor that did not show any increase (Figure 6.5 left panel). However, in U937 cell line,

there was very little difference in XBP-1 splicing between LPS matured and DNA-PKi treated DC (Figure 6.6 left Panel). Nevertheless, blocking DNA-PK activity increased the transcription of IL-23p19 which was highest at 9-12 hr in moDC and 6-12 hr in U937 (Figure 6.5 and 6.6 right panel).

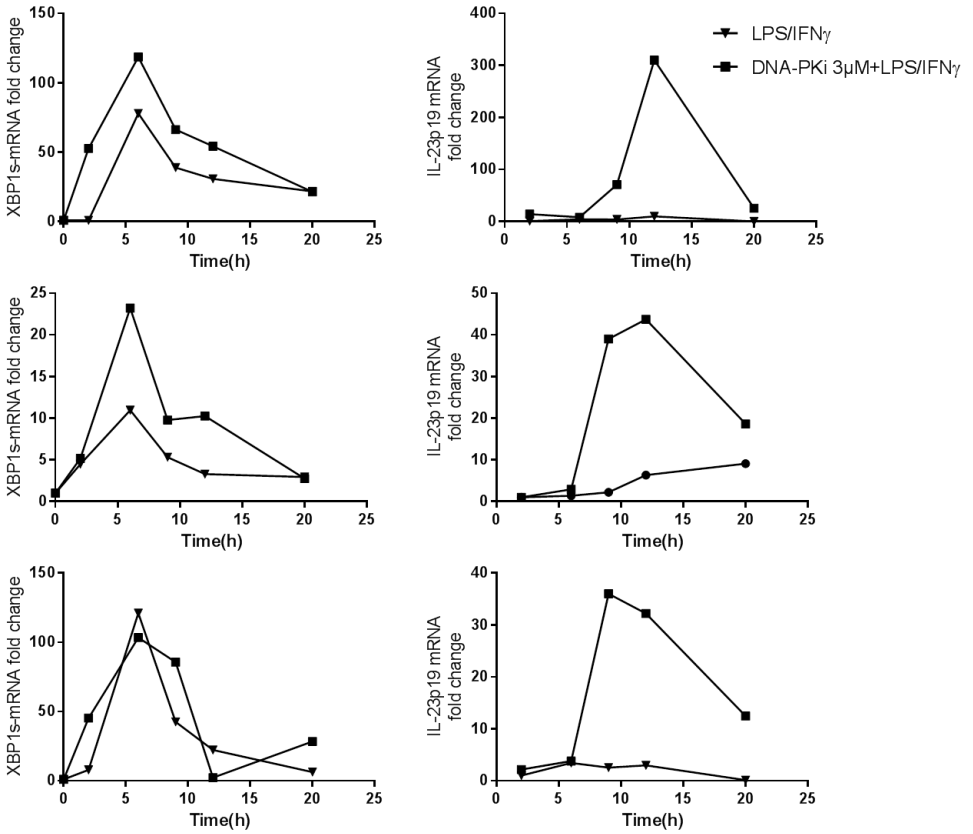


Figure 6.5 Regulation of LPS-dependent XBP-1 splicing and IL-23p19 gene expression by DNA-PK in DC

Cells were lysed and RNA was extracted from iDC, LPS-matured DC (triangle symbol) and DNA-PKi (3μM) pre-treated matured DC (square symbol) at the indicated time points and analysed for XBP-1s and IL-23p19 mRNA by RT-qPCR. Results were calculated as relative sXBP-1/IL-23p19 level normalised to HPRT and plotted as a fold change compared to gene expression by iDC. Data from 3 donors are presented in the left panel for XBP-1s and in right panel the corresponding IL-23p19 mRNA.

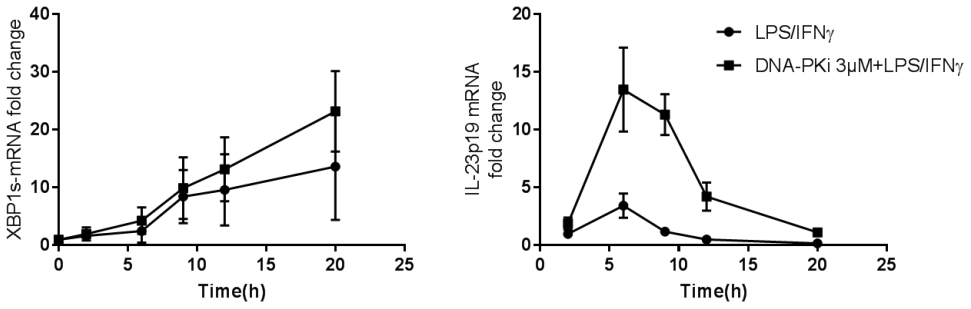


Figure 6.6 Regulation of LPS-dependent XBP-1 splicing and IL-23p19 gene expression by DNA-PK in U937

U937 cells were treated as described before. Cell were then lysed and RNA was extracted from untreated, LPS-treated (triangle symbol) and DNA-PKi pre-treated cells (square symbol) at the indicated time points and analysed for XBP-1s and IL-23p19 mRNA by RT-qPCR. Results were calculated as relative sXBP-1/IL-23p19 level normalised to HPRT and plotted as a fold change compared to gene expression by untreated cells. Data from 3 independent experiments are presented in the left panel for XBP-1s and in right panel the corresponding IL-23p19 mRNA.

6.2.5 IRE1 antagonist inhibits XBP1 splicing induced by ER stress inducers and in TLR4 activated DC

Prior to testing the effect of IRE1 inhibition on IL-23 secretion induced by ATM or DNA-PK inhibition, we initially determined whether the inhibitor could block IRE1 activation that is the ribonuclease activity of activated IRE1. Treatment with TM or TG led to a marked increase in XBP-1 splicing and this was inhibited by pretreatment with an IREi inhibitor in a dose dependent manner (Figure 6.7 A & B). IRE1 inhibition was also able to prevent LPS/IFN-γ induced increase in XBP-1s transcript in all of the donors studied (Figure 6.8).

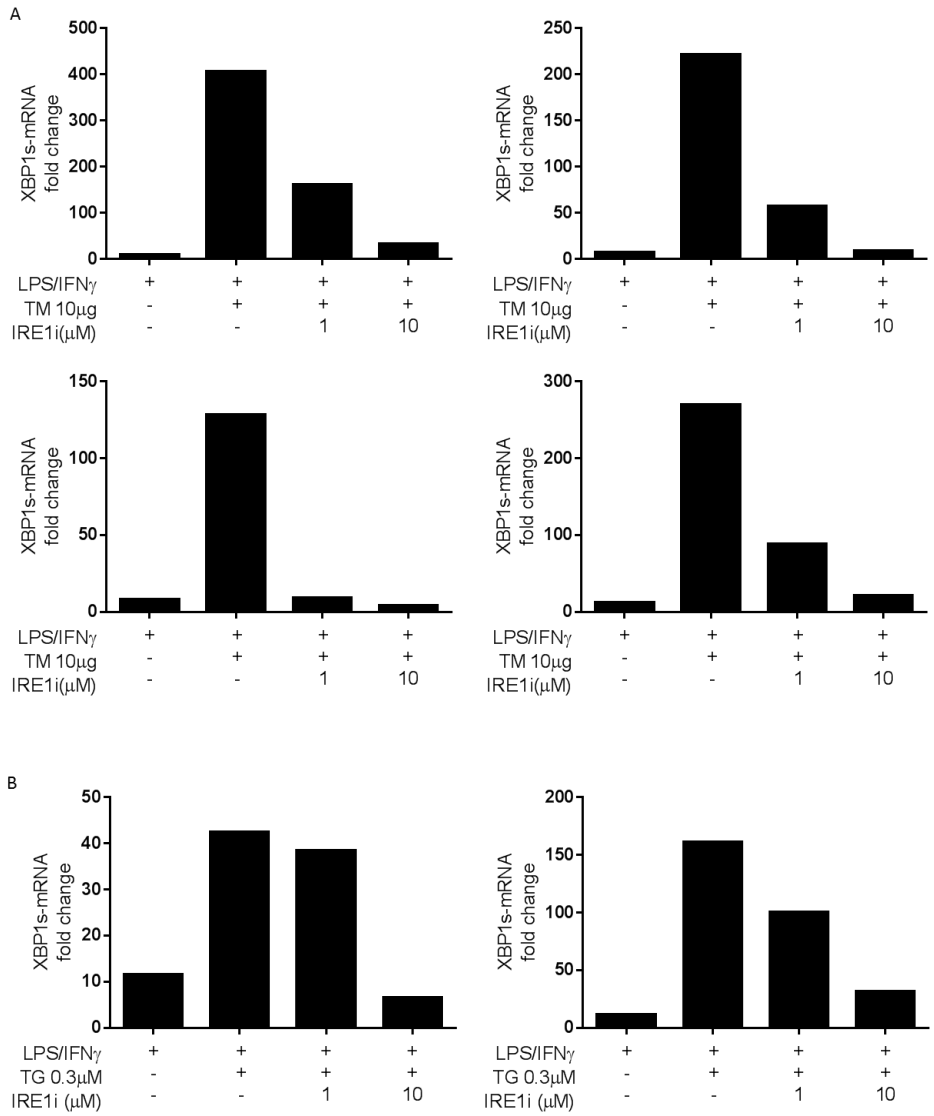


Figure 6.7 Pretreatment with IRE1i blocks TM or TG induced XBP-1s mRNA generation

moDC were treated with 1μM or 10μM IRE1i for 1 hr, followed by treatment with 10μg/ml TM or 0.3μM TG and then activated with LPS/IFN-γ for 6 hr. Cells were then lysed, RNA extracted and RT-qPCR was carried out for XBP-1s gene expression. Results were calculated as relative sXBP-1 level normalised to HPRT and plotted as a fold change compared to gene expression by iDC. Data from 4 donors are presented in figure 6.7 A for TM exposure and from 2 donors in figure 6.7 B for TG exposure.

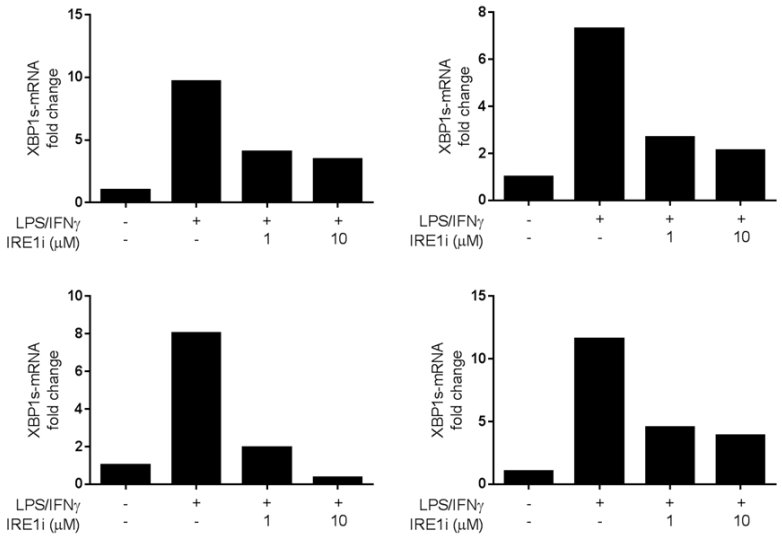


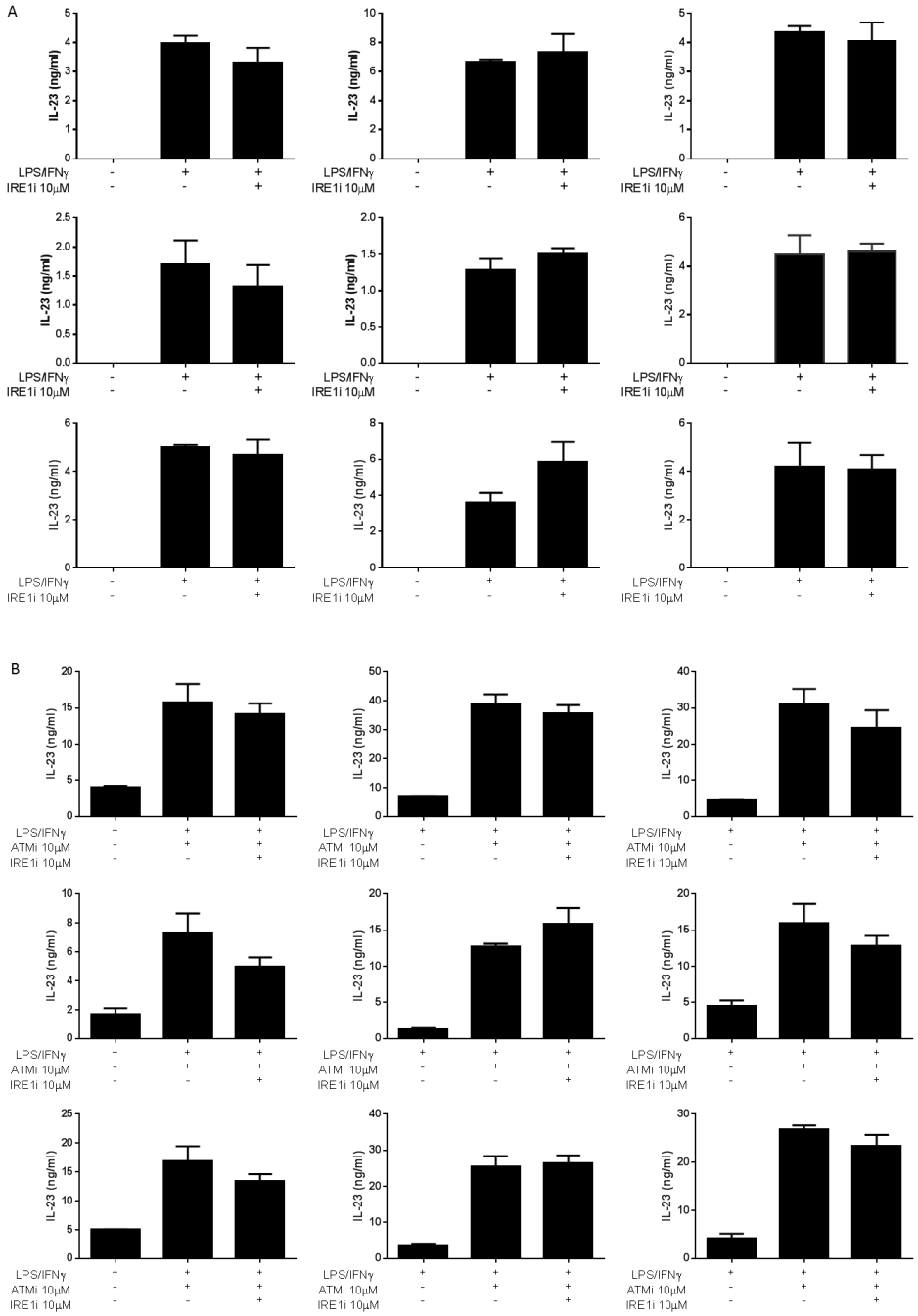
Figure 6.8 Effect of IRE1 inhibition on LPS-dependent splicing of XBP-1 mRNA

To demonstrate the inhibitory effect of IRE1i on LPS-stimulated IRE1 activation, the splicicing of XBP-1 was examined by RT-qPCR. Cells were conditioned with 1μM or 10μM IRE1i for 1 hr prior to activation with LPs/IFN-γ. 6 hr post activation, cells were lysed and RNA was extracted to make cDNA. Results were calculated as relative sXBP-1 level normalised to HPRT and plotted as a fold change compared to gene expression by iDC. Figure 6.8 shows the results from all four donors tested.

6.2.6 IRE1 inhibition has no effect on ATM or DNA-PK inhibited IL-23 regulation

We showed that ATMi or DNA-PKi intensified LPS induced IRE1 activation and potentially increased the signalling output from this pathway. Having demonstrated that the small molecule inhibitor for IRE1 decreased the generation of XBP-1 spliced variant in both LPS and TM-treated DC, we next investigated the impact of the IRE1 pathway on ATM or DNA-PK dependent signalling for IL-23 production. Inhibition of IRE1 did not reduce either LPS-

dependent or ATMi-dependent IL-23 secretion (Figure 6.9 A, B & D) suggesting that the IRE1 pathway does not play a role for IL-23 expression. Although, 5 of 9 donors showed a reduction in DNA-PK regulated IL-23 release upon IRE1 inhibition, the overall effect in 9 donors was insignificant (Figure 6.9 C & D)



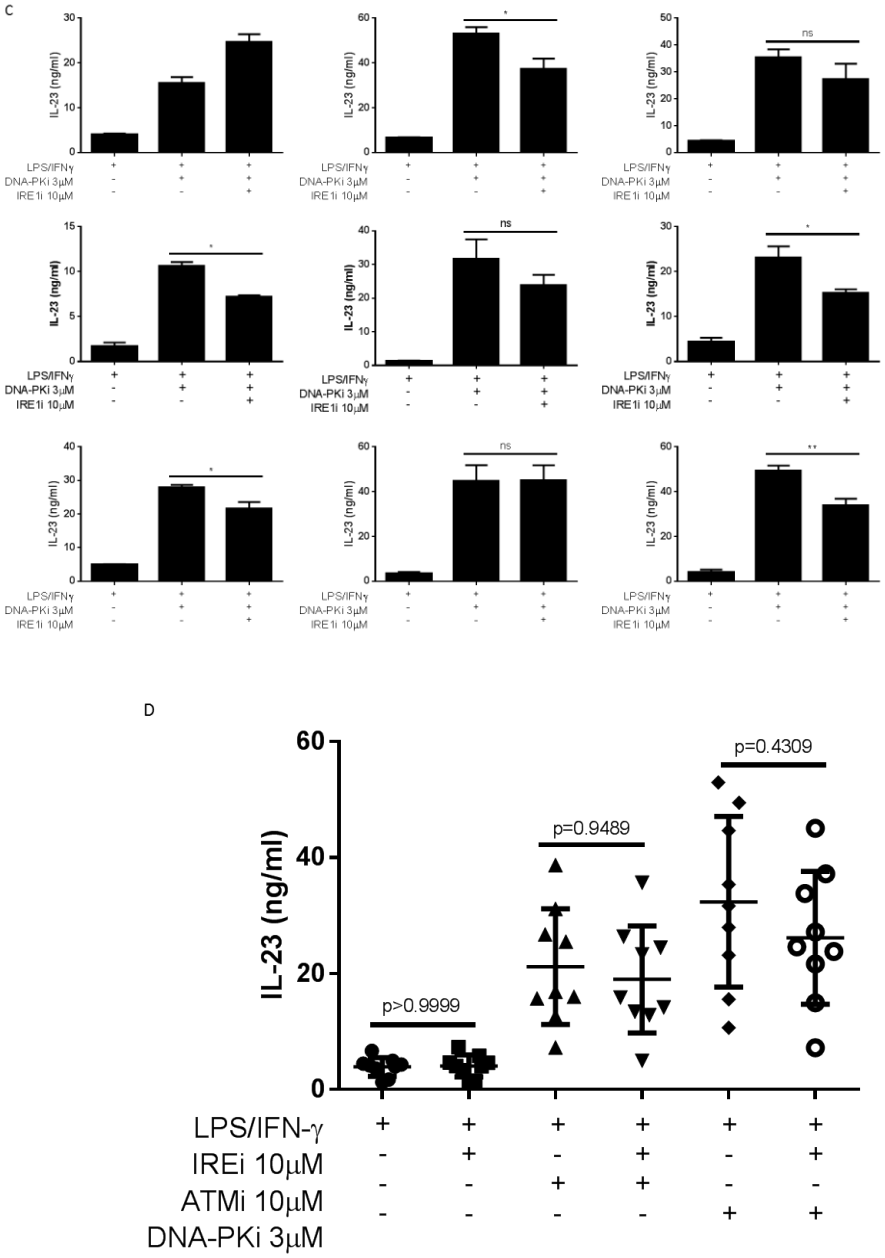


Figure 6.9 ATM or DNA-PK dependent IL-23 production is independent of IRE1

moDC were incubated with 10μM IRE1i for 1 hr, followed by treatment with ATM or DNA-PKi prior to activation with LPS/IFN-γ for 24 hr. supernatant was collect and IL-23 concentration 212was measured by ELISA. Data show the responses from all 9 donors examined. Figure 6.9 A represents the impact of IRE1 inhibition on TLR4-induced, B for ATM-dependent and C for DNA-PK – dependent IL-23 secretion from all the donors. To show the overall effect of

IRE1 inhibition on IL-23 from ATMi or DNA-PKi pretreated matured DC, IL-23 response from 9 independent donors are plotted in as a summary figure in 6.9 D for the indicated conditions. Each donor is indicated by a discrete point. p value in the first graph of Figure C is missing because statistical significance has been drawn based on the inhibitory effect of IREi on IL-23 secretion.

6.2.7 TLR4 stimulation plays a suppressive role in phosphorylation of eIf2α which can be reversed by inhibition of ATM or DNA-PK

The transcription factor, CHOP derived from the PERK pathway of the UPR has previously been shown to play an important role for IL-23 expression by DC [604]. Thus we hypothesized that the PERK pathway might be involved in ATM or DNA-PK regulated IL-23 expression. One of major methods employed for detection of activation of PERK is to measure the level of eIF2α phosphorylation. Expression of the phosphorylated form of the eIf2α protein in resting DC was decreased by exposure to LPS at 2 hr (Figure 6.10 A). Whilst these changes were evident on visual inspection of the blots, their extent was more accurately revealed following image analysis (B). However the presence of ATMi or DNA-PKi prevented the LPS-induced reduction in phospho-eIf2α levels. As expected, TM treatment resulted in substantial phosphorylation of eIF2α which was inhibited in the presence of a small molecule inhibitor for PERK.

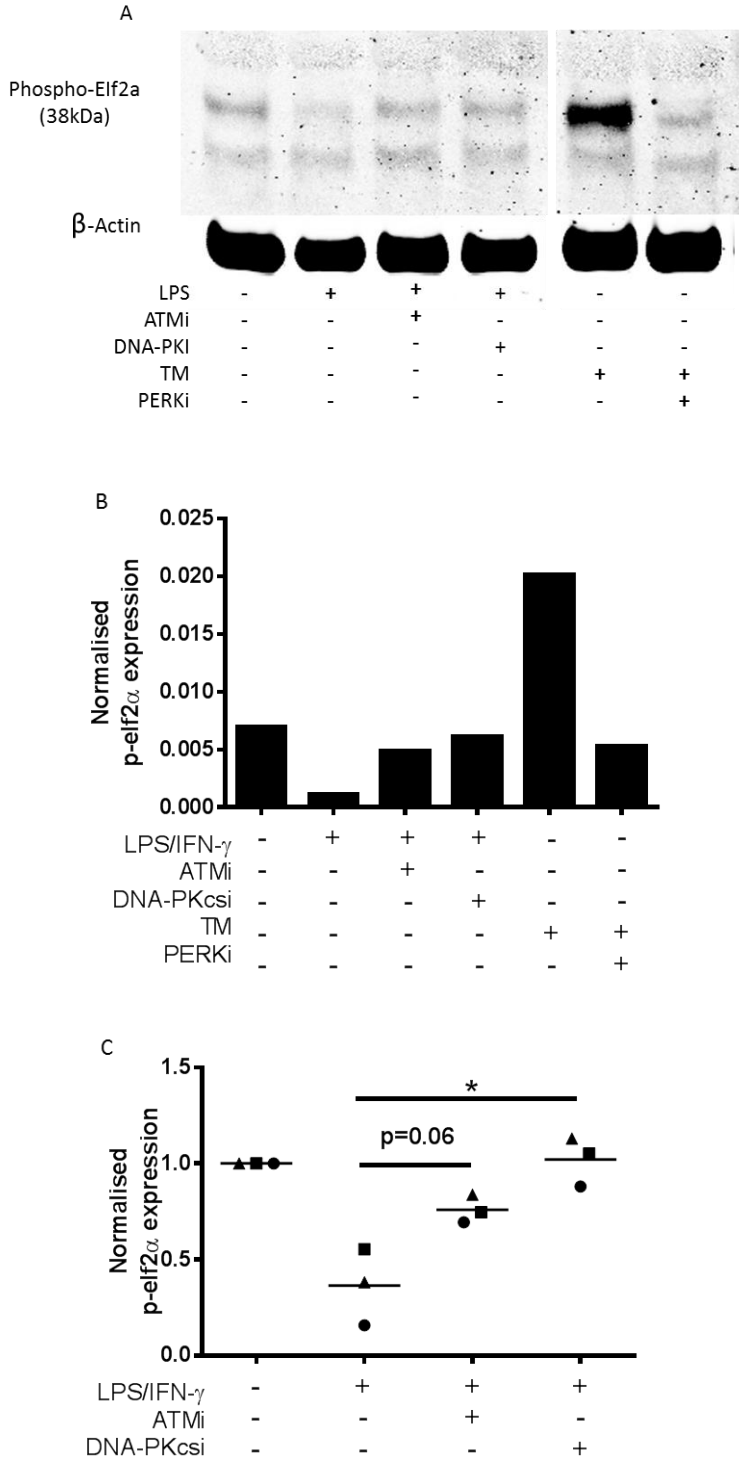


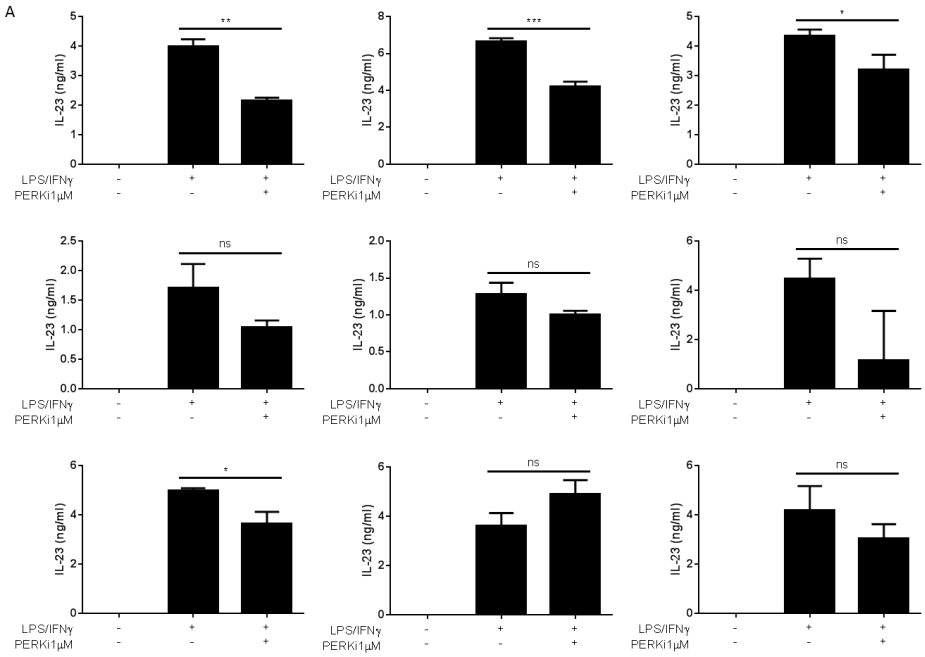
Figure 6.10 Regulation of phosphorylation of eIF2α in DC by ATM and DNA-PK following activation by LPS

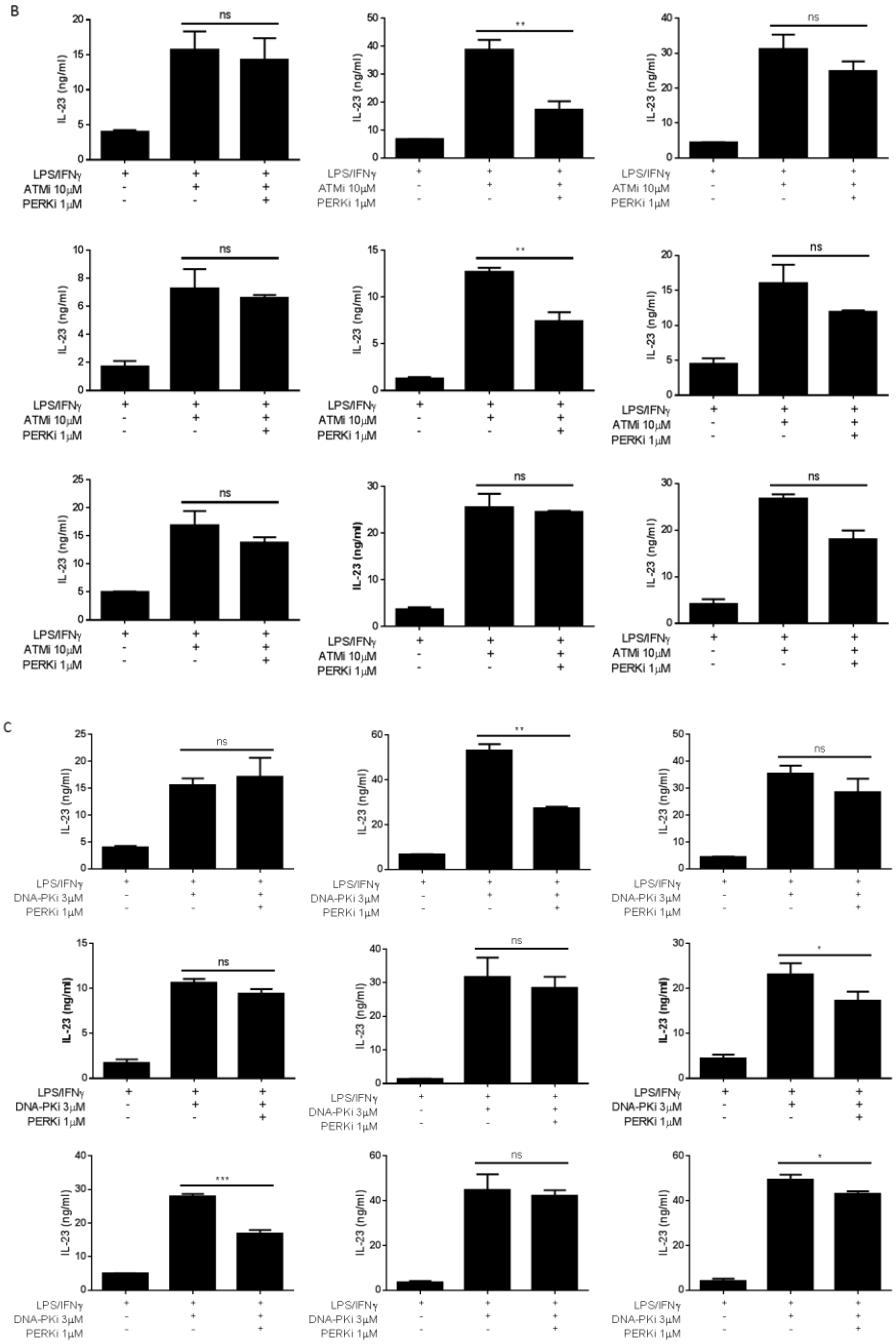
The level of phospho-eIF2α was determined at 2 hr post-activation with LPS for the indicated treatment conditions by western blot (Figure 6.10 A). Figure

6.10 B presents the normalized expression of phospho-eIF2α for the corresponding blot shown in A. A summary graph for phospho-eIF2α level from all three independent experiments has been depicted in figure 6.10 C. All protein values were normalized to the expression of β-actin protein level.

6.2.8 Inhibition of PERK pathway does not affect IL-23 production

As shown above, inhibition of ATM or DNA-PK had an effect on TLR4 mediated phosphorylation of eIF2α. So we speculated that PERK pathway might have a role in IL-23 secretion induced by ATM or DNA-PK. However, inhibition of PERK using a small molecule inhibitor did not influence either ATM or DNA-PK dependent IL-23 secretion.




B
C
IL-23 (ng/ml)
ns
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*
LPS/IFNγ
ATMi 10μM
DNA-PKi 3μM
PERKi 1μM

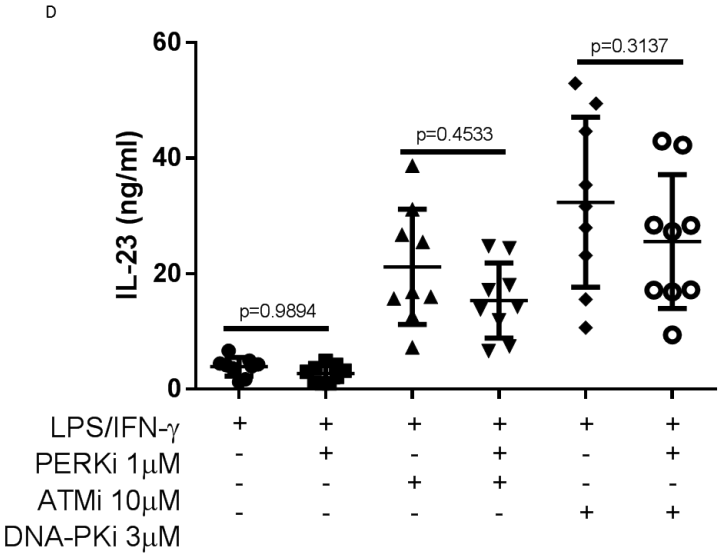


Figure 6.11 Consequence of inhibiting PERK activation on IL-23 signalling mediated by ATM or DNA-PK

moDC were incubated with 1μM PERKi for 1 hr, followed by treatment with ATMi or DNA-PKi prior to activation with LPS/IFN-γ for 24 hr. Supernatant was collect and IL-23 concentration 217was measured by ELISA. Data show the responses from the matched 9 donors in figure 6.9. Figure 6.11 A represents the impact of PERK inhibition on TLR4-induced, B for ATM-dependent and C for DNA-PK –dependent IL-23 secretion from all the donors. To show the overall effect of PERK inhibition on IL-23 from ATMi or DNA-PKi pretreated matured DC, IL-23 response from 9 independent experiments have been plotted in as a summary figure in 6.11 D for the indicated conditions. Each donor is indicated by a discrete spot.

6.3 Discussion

The major stress and damage sensing pathway within cells such as DNA damage repair and ER stress sensing pathways function to restore the normal status or remove the damage cell. However, this is become apparent that these pathways can also influence immunity. In contrast to most other cells, DC and macrophages have a basal level of activation of both their DNA repair and ER stress pathways [419]. This is because these cells are frequently subject to intracellular ROS following microbial infection and thus prone to DNA damage, and are highly secretory thus stressing the protein synthesis machinery. So far we have described important regulatory roles of ATM and DNA-PK in IL-23 signalling in DC. Here we have reported that ATM or DNA-PK inhibition intensifies TLR4 induced IRE1 activation, namely splicing of XBP-1 and also regulates the PERK pathway upon TLR4 stimulation in DC.

The ER stress response pathway (the UPR) is one of the most important regulatory mechanisms that eukaryotic cells have evolved to deal with internal cellular imbalance due to accumulation of unfolded or misfolded proteins. The UPR is a fundamental process with important physiological functions in all cells and ER stress has been associated with variety of diseases. ER stress signalling pathways are also important for the normal differentiation and function of secretory cells such as B cells, pancreatic beta-cells, osteoblasts, and liver cells [607]. ER stress is also involved in differentiation and plasticity of T cell along with the development and maturation of DCs [419, 596, 608]. The link between immunity and UPR is fundamentally important as illustrated by the

evolutionary conservation in Caenorhabditis elegans that uses XBP1 pathways for survival when infected with pathogenic bacteria expressing pore-formin toxin[427, 609]. ER stress has also been described to be associated with the induction of proinflammatory cytokine secretion. In a rat model of spondyloarthritis, ER stress induced by misfolded HLA-B27 resulted in enhanced IL-23 production and subsequent Th17 response. Evidences have demonstrated that inflammation and UPR are interconnected by various ways such as ROS production, release of Ca^{2+} from the ER, activation of the transcription factor NF-κB and the mitogen activated protein kinase (MAPK) known as JNK (JUN N- terminal kinase)[597, 610]. IRE1-XBP1 branch of the UPR has been reported to act in the synergistic enhancement of TLR induced inflammatory responses [427, 431, 597]. Both IRE1 pathway and TLR signalling induce the production of ROS and acute phase proteins and both activate NEMO and TRAF adaptors to trigger NF-κB and JNK which are important inflammatory components of cells. These similarities imply that these signalling pathways may have coevolved common strategies to respond to specific insults [595, 611, 612].

There are some evidences that links ER stress in the process of interaction of ATM with intracellular stress sensing systems. ATM deficiency has been described to be associated with ER stress induction. [237, 613]. ATM expression also blocks tunicamycin induced ER stress[602]. These observations suggest a complex signalling network within the DNA repair pathway, the ER stress response and inflammation. Our results of increased XBP-1s mRNA generation

followed by coordinated increase in IL-23p19 expression in ATMi or DNA-PKi treated cells suggest the regulatory role of these DNA repair proteins on IRE1 pathway of UPR. We also demonstrated that ATM or DNA-PK inhibition reverses the suppressive effect of LPS on phosphorylation of eIF2α which again indicates the association between these pathways. The existence of such a complex relationship may enable the cell to integrate signals from various forms of stress to respond appropriately to specific condition. However, in DC and macrophages, infection with pathogens or danger signals from stressed and damaged cells regulate the balance of different cytokine production. UPR activation is the intracellular counterpart of extracellular danger. Together PRR and UPR activation inform the cell that it harbours a pathogen and these integrate to make decision as to the appropriate cytokine response. As part of this response, ROS are generated to destroy intracellular pathogens with the consequential oxidative damage to DNA where the DNA repair proteins come in play.

In order to further confirm the involvement of ER stress response in IL-23 response in the presence of ATMi or DNA-PKi, we use small molecule inhibitors of the UPR pathway to dissect the individual arm of this signalling pathway. Inhibition of neither IRE1 nor PERK seemed to have any negative effect on IL-23 produced by ATM or DNA-PK-inhibited DC. Our data suggest that ATM and DNA-PK may regulate ER stress response likewise their role in other forms cellular stresses, but UPR pathway might not be the ultimate mechanism through these two DNA repair proteins regulate IL-23. Alternatively, in future

we could investigate the effect simultaneous inhibition of the both arms, as in the absence of one pathway, the other pathway may compensate for IL-23 production.

Goodall and colleagues showed that the UPR derived transcription factor is crucial for IL-23 production by DCs [604]. Expression IL-23p19 in response to TLR stimulation gene was down regulated by knockdown of CHOP even in the absence of ER stress which suggests that TLR signalling itself can activate the ER stress pathway leading to CHOP induction. CHOP has been reported to regulate other cytokine expression. It binds to the promoter of IL-8 following stimulation of T cells with prostaglandin [614]. CHOP has also been shown to regulate IL-6 production. Rather than binding to IL-6 promoter, it enhances IL-6 transcription by trapping an inhibitory isoform of NF-IL-6 [615]. We have previously used CHOP KO C57/BL6 mice to examine the potential role of CHOP in ATM or DNA-PK-mediated IL-23 production. Although, the absence of CHOP impaired TLR-dependent IL-23 secretion, there was no significant difference in IL-23 levels in CHOP KO and WT mice when ATM or DNA-PK was also inhibited. This suggests that our model of IL-23 regulation is independent of CHOP. However, the signalling pathway for IL-23 through ATM or DNA-PK might be different between human and mouse as we have seen differential effect of IR on TLR4 stimulated IL-23.

The reason ER stress favours IL-23 response over IL-12 likely lies in its role as an important driver of immediate/early immunity to pathogens. In particular,

IL-23 rapidly induces IL-17 secretion by local T cells allowing rapid activation of neutrophils and initiation of innate local immune responses. In contrast, IL-12 may have a more important role later in the immune response in terms of Th1 differentiation for dealing with intracellular pathogens and promoting their clearance [616].

In contrast to monocytes, DC and macrophages have a basal level of activation of a number of systems involved with single- and double strand DNA repair [475]. As a result of this, DC and macrophages are more resistant to DNA damage dependent cell death than monocytes. Probably, the resting levels of DNA-PK in DCs functions as an evolutionarily conserved mechanism that functions to confer protection to DNA damage resulting from ROS. The DNA repair defect in monocytes functions to maintain homeostasis in the immune system and attenuates immune response and ROS production by preferential killing of monocytes which are precursors of DCs and macrophages [469]. Thus, by negatively regulating the ER stress response, ATM and DNA-PK may also subject the immune system to an additional level of regulation by preventing a hyperactive inflammatory response.

Chapter 7 Final discussion

This study addressed a novel immune-regulatory function of proteins that are normally recognised for their role in DNA-repair, namely ATM kinase and DNA-PKcs. We characterised a phenotype, namely IL-23 secretion, associated with inhibiting or activing ATM or DNA-PK in antigen presenting cells. Lastly, we made some headway towards understanding the signalling pathways associated with the observed phenotype and showed that whilst inhibition of ATM or DNA-PK give similar IL-23 responses, they do not achieve this through the same pathways. This thesis brings together 3 fundamental facets of leukocyte biology and stress responses, namely DNA-repair, Dendritic Cells, and the signal-3 cytokine Interleukin-23. Appreciating the relationship between these may have important ramifications for our understanding of mammalian biology and therefore for health and disease.

The key findings from these studies were that inhibition of ATM or DNA-PK with highly selective small molecule inhibitors markedly and selectively enhanced IL-23 secretion in DC that were activated with bacterial PAMP. This phenotype was restricted to double-strand DNA-repair pathways as inhibitors of the single-strand DNA-repair protein ATR had no effect. The effect of ATM and DNA-PK was restricted to TLR4. This suggested a response that was restricted to Gram negative bacterial strains (ie that contain LPS), and did not occur with viral associated TLR such as TLR3, 5, 7 or 8. Importantly, our data showed the involvement of CREB activity in ATM mediated IL-23 regulation.

Whilst blockade of ATM or DNA-PK altered ER stress signalling pathways, these events did not directly modulate IL-23 responses and their biological importance remains to be studied. Whilst the underlying molecular mechanisms behind the unconventional cytoplasmic function of two nuclear DNA-repair proteins require further characterisation the fact that they have a prominent and selective effect on IL-23 may justify more immediate investigation into their role in various pathological settings such as infectious disease, cancer or autoimmunity.

Our work originated from the observations made some years ago in which IL-23 production was elevated by the PI-3K inhibitor WM treated DC [486]. One major drawback of the initial study was the lack of specificity of the PI-3K inhibitor employed, namely Wortmannin. By studying a range of off-target candidates for WM, we showed that the IL-23 regulatory role of WM was due to inhibition of ATM or DNA-PK, playing important roles in DC function and cytokine production. The knowledge regarding the functions of ATM or DNA-PK in the context of immune regulation is very limited. Nevertheless, evidence of non-nuclear functions of ATM are now starting to emerge. ATM plays fundamental and essential roles in DC proliferation as DC developed from ATM-null BM show lower proliferation and constitutive apoptosis [617]. It has been reported that reduced and impaired activation of Akt is responsible for lower cell proliferation and that ATM deficiency causes delayed expression of Jak2 and STAT5 [617], which are critical for DC development and function [618, 619]. Importantly, ATM regulates DC biology independent of its DNA

damage function as GM-CSF activated ATM does not phosphorylated SMC1 (a known ATM substrate under conditions of DNA damage) [617]. In our study, the important role of ATM in IL-23 signalling was demonstrated by biological assays using a highly selective ATM kinase inhibitor, KU55933. Whilst small molecule inhibitors are of considerable value to this type of research, it should be noted that they invariably have some off-target effects. Therefore, the inclusion of alternate experimental approaches to confirm such observations would be a valuable addition to this work. Such studies could involve use of siRNA to knockout ATM expression in model cells, such as U937, which also shows increased IL-23p19 expression upon ATM inhibition. Alternatively, ATM has been knocked out in murine models. However, careful consideration should be taken in using ATM-null mice given crucial roles of ATM in DC development [617].

Consistent with our findings, several recent studies have pointed to the role of ATM in suppressing inflammation. ATM negatively regulates the production of IL-8, MIP-1α and IL-6 and delays apoptosis in LPS activated neutrophils [474]. Moreover, inhibition of ATM in peripheral blood monocytes also significantly increases the levels of IL-1β, IL-6 and TNF in response to LPS activation [474]. Although this study does not clearly explain the underlying mechanism, partial involvement of the MAPK signalling pathway has been suggested [474]. Similarly, pharmacological activation of the DNA damage response suppresses the production of proinflammatory cytokines and favours survival in septic mice [620]. ATM activation is partly responsible for this protective effect,

although the authors conclude that this mechanism is lung specific and not mediated by blood cells [620]. Another study reported that ATM deficiency in macrophages augments production of type I interferon [621]. However, there is no evidence of direct role of ATM in interferon regulation. Rather, it has been proposed that unrepaired DNA lesions in A-T cells trigger Stimulator of IFN genes (STING) and induce type I interferon secretion [621]. Reduced ATM kinase activity stimulates innate immune response in glial cells of *Drosophila* which is responsible for neurodegeneration and glial cell death [494]. On the contrary, DNA damage responses, particularly activation ATM and ATR, triggers antigen presenting cell like functions in fibroblasts [622]. Fibroblasts gain the ability to stimulate naïve antigen specific $CD8^+$ T cells through upregulation of the expression of the costimulatory molecules ICAM-1, VCAM-1, ICOSL, MULT-1, RAE1, CD155 and CD112 upon treatment with DNA damaging agents [622]. Similarly, persistent DDR signalling induces increased secretion of inflammatory cytokines such as IL-6 and IL-8, in senescent cells. Initiation and maintenance of this cytokine response occurs through ATM, NBS1 and CHK2 and is independent of p53 activation [623].

DC are the primary antigen presenting cells. They take up antigens for processing in the context of MHCI/II, followed by migration to lymphoid tissues where they facilitate differentiation of naïve T cells into different effector T cells subsets. Because of their importance, studies of such APC can have major impacts in the fields of innate and adaptive immunity. We have shown that in human DC, production of IL-23, IL-1β, IL-10 and IL-6 is enhanced

in response to LPS when DNA-PK is inhibited. In contrast to ATM, limited and controversial data exist describing the role of DNA-PK in DC function. Earlier studies have found that DNA-PK is not required for TNF-α and IL-12 production in response to unmethylated CpG-ODN motifs in $CD11c^{+}$ DC and BMDC from *DNA-PKcs*$^{-/-}$ mice [624, 625]. Rather the involvement of TLR9 and MyD88 have been documented in these studies. However, a recent report shows that the production of CpG-ODN induced IL-6 and IL-12 was reduced in BMDC from *DNA-PKcs* deficient mice [455]. Importantly, CpG-ODN was reported to bind DNA-PKcs and induce its interaction with TRAF6 [455]. Furthermore, DNA-PK inhibition in DC results in impaired antigen presentation and attenuation of Th2 immunity in response house dust mite which activates DNA-PK via a ROS-dependent pathway [585]. All these findings are in contrast to our study. The reasons for this are unclear, but the function of DNA-PK in leukocytes may differ depending on the cell type, microenvironment and activation signal.

Several reports have suggested the involvement of ATM in various cytoplasmic signalling pathways. ATM deficiency results in defective calcium and potassium currents in human fibroblasts and mouse neurons [626-628]. ATM also regulates de novo synthesis of ceramide in the ER in response to DNA damage [629]. Furthermore, the association of ATM with various cytoplasmic metabolic pathways has been revealed by recent proteomic analyses [630-633]. Yang and Kastan [484] reported that exposure of cells to insulin activates ATM which in turn phosphorylates the translation repressor

4EBP1 (eIF4E-binding protein) on Ser-111. Phosphorylation of 4EBP1 leads to the release of eIF4E (a component of the translation initiation complex) and promotes cell growth. Loss of this pathway has been suggested to be associated with the poor growth and insulin resistance in A-T [484]. ATM also regulates insulin mediated glucose uptake by phosphorylating Akt, which plays important roles in cell proliferation, apoptosis, cell cycle control and glucose metabolism. In response to insulin stimulation, ATM is required for increased phosphorylation of Akt at Ser473 and enhanced translocation of the cell surface glucose transporter 4 (GLUT4) complex [634]. Recently ATM has been shown to regulate insulin functions and glucose homeostasis indirectly through a p53-dependent pathway. ATM phosphorylation of p53, followed by p53 mediated regulation of intracellular ROS levels, is an important contributor to the physiological regulation of glucose homeostasis [635].

Several ATM-mediated cytoplasmic pathways are involved in the modulation of oxidative stress. ATM enhances the operations of the pentose phosphate cycle (PPP), which is a major source of the important antioxidant factor, NADPH. By phosphorylating HSP27, ATM favours complex formation between HSP27 and the rate limiting PPP enzyme glucose-6-phosphate dehydrogenase (G6PD) [636]. Another ATM dependent cytoplasmic pathway that controls redox homeostasis is mTOR [637]. In response to increased ROS, ATM mediated repression of mTORC1 (mTOR complex 1) enhances autophagy. This pathway takes place in the cytoplasm where ATM phosphorylates the tumour suppressor protein LKB1 (liver kinase B1), which in turn activates AMPK (AMP-

activated protein kinase). Phosphorylated AMPK then phosphorylates and activates TSC2, a negative regulator of mTORC1 [637]. Interestingly, ATM also suppresses mTORC1 signalling in response to hypoxia, but in this case through phosphorylation of HIF-1α [293]. Thus depending on the activation stimuli, ATM may function through different mechanisms to control the same signalling pathway.

Like ATM, DNA-PK has also been reported to be activated by cellular stresses including ROS and hypoxia [638, 639]. Bonquet and co-workers showed that DNA-PK is activated under hypoxic condition through changes in the higher-order chromatin structure in the absence of DNA lesions [638]. This study shows that in response to hypoxia, activated DNA-PK positively regulates HIF-1α expression, however, the underlying mechanism of this pathway is poorly understood. In addition, DNA-PK is activated by low levels of oxidative stress produced during mitochondrial respiration which in turn phosphorylates and stabilizes p53. The signalling mechanism involved in this pathway is independent of DNA damage response [640]. Furthermore, DNA-PKcs (the catalytic subunit of DNA-PK) plays a positive regulatory role in the selenium-induced oxidative stress [639]. DNA-PK kinase activity contributes to oxidative stress by feeding forward-regulation of ROS during the selenium induced senescence response and leads to sustained activation of ATM and DNA-PK [639]. Importantly, the phosphorylation of DNA-PKcs on Ser2056 and Thr2647 by selenium treatment is dependent on Selenium treatment [639]. Upon IR, DNA-PKcs Ser2056 autophosphorylation is independent of ATM,

whereas phosphorylation on Thr2647 and other sites in the Thr2609 cluster in dependent on ATM activation [641-643]. Similarly, we showed that inhibition of ATM did not affect IR induced phosphorylation of DNA-PK on Ser2056 in the presence or absence of TLR stimulation. However, we observed increased IL-23 production with the concurrent inhibition of ATM and DNA-PK, suggesting there might be a cross-talk between these two pathways. In future, it would be important to undertake studies of the potential regulatory impact of ATM on the phosphorylation of DNA-PK at other residues.

We proposed the involvement of the transcription factor CREB in ATM-mediated IL-23 response. However, to be confirmed, this issue will require extensive molecular addressing by using CREB mutant mice or use of siRNA to knockout CREB expression. At present, there is no clue to the underlying mechanism of DNA-PK dependent cytokine production. Shi et al has shown that the transcriptional activity of CREB is negatively regulate by ATM in response to genotoxic stress. Consistent with this, TLR4 induced IL-23 is negatively regulated by ATM upon IR, possibly by suppressing CREB dependent IL23p19 transcription. It would be good to investigate in future whether similar outcome can be achieved by ATM activation upon treatment with H_2O_2.

Confirmation of our study will add a new dimension to the understanding of IL-23 signalling pathway in DC. Clinical features of A-T, such as, neurodegeneration and chronic inflammation might be associated with

increased IL-23 production in the absence of ATM. Given the important role of IL-23 in the generation of Th17 response, these pathways would allow to manipulate Th17 dependent immunity in health and disease. IL-23 drives the induction of proinflammatory Th17 cells that are associated with autoimmune conditions and cancer progression. Recently IL-10 has been shown to be important in inhibiting Th17 mediated inflammation [644]. Inhibition of DNA-PK increases production of IL-23, IL-1β and IL-6, as well as IL-10. Thus this pathway might be activated to generate a moderate inflammatory immune response that could be beneficial in early stages of cancer.

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