Characterisation of Regulatory Type 1 (Tr1) cells in human peripheral blood

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TO MY FAMILY

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Abbreviations:

APC	Antigen presenting cell
Ahr	Aryl hydrocarbon receptor
BSA	Bovine serum albumin
bZIP	Basic Leucine Zipper Domain
CD	Cluster of differentiation
CD55	Decay accelerating factor (DAF)
CD97Fc	CD97 fusion protein
Cells/ml	Cells per millilitre
CTV	Cell Trace Violet dye
CO_2	Carbon dioxide
CPM	Counts per minute
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte-associated protein 4
DC	Dendritic cell
ELISA	Enzyme linked immunosorbent assay
Foxp3	Forkhead box P3
GATA-3	GATA binding protein 3
GPI	Glycosylphosphatidylinositol
GvHD	Graft – versus – host disease
ICAM-1	Intercellular adhesion molecule - 1
ICOS	Inducible co-stimulatory molecule
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
iTreg	Inducible T regulatory cell
iNKT cells	Invariant Natural killer T cells
kDa	kilo Dalton
LAG-3	Lymphocyte activation gene – 3

LAP	Latency Associated peptides
mAbs	Monoclonal antibodies
MAIT	*Mucosal associated invariant T cells*
MFI	Median fluorescence intensity
MHC	Major histocompatibility complex
nTreg	Naturally occurring regulatory T cells
ng/ml	Nanograms per millilitre
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PD-1	Programmed cell death protein 1
PE	Phycoerythrin
PHA	Phyto haemagglutinin
PMA	Phorbol 12-myristate 13-acetate
rh	Recombinant human
ROR	Retinoid-related orphan receptor
SCID	Severe combined immunodeficiency
SD	Standard deviation
STAT	Signal transducer and activator of transcription
TCR	T cell receptor
TGF-β	Transforming growth factor beta
TNF	Tumour necrosis factor
Th	Helper T cell
Tr1	T regulatory type 1 cells
TCM	Central memory T cells
TEM	Effector memory T cells
Teff	Effector T cells
IU/ml	International Units per millilitre
µg/ml	Micrograms per millilitre
µM	Micromolar

Abstract

Regulatory type 1 (Tr1) cells have been described from a wide range of *in vitro* studies. There is a broad consensus of phenotype, based on cytokine production, of IL-10^{+}IFN^{-}IL-4^{-}. However, there is a wide range of reports of other phenotypic markers, dependent on *in vitro* stimulatory conditions. It is not clear whether this represents the phenotype of Tr1 cells *in vivo*/*ex vivo.* Our group previously reported that costimulation of naïve CD4^{+} T cells via CD55 results in the differentiation of a discrete Tr1 cell population (~0.5-5%) that are IL-10^{hi} IFN-γ^{-} IL-4^{-}, that express Tr1 markers CD49b, LAG-3 and CD226 - a phenotype that the classical CD28:CD80/86 costimulation failed to induce *in vitro*. If the CD55 derived Tr1 cells are a differentiated population then they should be identifiable in the peripheral blood CD4^{+} T cell pool. Therefore, the main aim of this study was to examine the peripheral blood CD4^{+} T cell pool of healthy human donors for the existence of a differentiated population of IL-10 producing cells and to characterise these IL-10^{+} cells for the phenotypic markers and transcription factors commonly associated with Tr1 cells.

It was found that CD3/CD55 costimulation preferentially stimulated the small (≤5%) IL-10^{hi} Tr1-like cells in human peripheral blood. The cytokine profile of these cells showed that they were negative for IL-4 and produced no IFN-γ but produced high levels of IL-10. The differentiation status of these IL-10^{hi} Tr1-like cell population revealed

that they were predominantly effector memory (TEM) cells – indicating that once differentiated from naïve precursors, Tr1-like cells maintain a memory pool in the periphery.

Further analysis of phenotypic markers revealed expression of the markers associated with previously defined Tr1 cells, including CD49b, CTLA-4, LAG-3. Notably, these cells showed high expression of CD226, PD-1, c-MAF and granzyme B, compared to previous definitions. These data support the hypothesis that Tr1 cells are a small, differentiated population of cells present in peripheral blood memory pool. It also confirms that CD55 costimulation is an effective activator of Tr1 cells. Preliminary findings suggest that inhibitors of p38MAPK/MK2 pathway inhibited IL-10 secretion by Tr1-like cells, but these did not influence IFN-γ production by Th1 cells which suggests that these pathways could be inhibited in immunosuppressive conditions or replenished in autoimmune conditions. This study has potential impact in the treatment of certain cancers where Tr1 cells are thought mediate immunosuppression and in Multiple Sclerosis where they are thought to be deficient.

The current study supports the existence of Tr1 cells as a differentiated population in human peripheral blood. These cells were predominantly effector memory cells preferentially stimulated via CD55 and were described as $CD4^{+}IL\text{-}10^{hi}IFN\text{-}\gamma^{-}$, $IL\text{-}10^{hi}IL\text{-}4^{-}$, $IL\text{-}10^{hi}CD226^{hi}$, $IL\text{-}10^{hi}PD\text{-}1^{hi}$, $IL\text{-}10^{hi}LAG\text{-}3^{hi}$, $IL\text{-}10^{hi}c\text{-}MAF^{hi}$, and $IL\text{-}10^{hi}Granz\text{-}B^{hi}$.

Chapter 1
Introduction

1.1: Background

The immune system helps protect us from micro-organisms like bacteria, fungi and infectious agents, in a bid to keep us healthy. The immune system can be broadly separated into two arms; innate *(non-specific)* or adaptive *(specific)* both mediated by white blood cells (leukocytes)(Herwald & Egesten, 2017; Vesely, Kershaw, Schreiber, & Smyth, 2011).

Innate immunity:

The innate through its immune cells (macrophages, neutrophils, natural killer cells, dendritic cells, basophils, eosinophils, etc) serves as the body's first line of defence and initiates adaptive immune response only when overwhelmed by pathogens(Gasteiger et al., 2017; Medzhitov & Janeway, 2000). These innate immune cells sense the presence of potential pathogens through germ-line receptors collectively known as 'Pattern Recognition Receptors' (PRR), which instinctively engage classes of molecule (Pathogen-associated Molecular Pattern Receptors, PAMP) expressed by many types of micro-organisms (bacteria, fungi, and/or viruses) but not produced by mammalian hosts (Fritz, Le Bourhis, Magalhaes, & Philpott, 2008). PRRs include Toll-like receptors (TLR), retinoic acid inducible gene (RIG)-like receptors (RLR) and NOD

like receptors (NLR), which recognize PAMPs characteristic of broad classes of pathogens(Mogensen, 2009).

Recognition of infectious PAMP by PRR initiates signals that result in cytokine and chemokine production that kill the infected cells. PRR also function in quickly recognizing host-derived molecules produced only by dead or injured host cells, in a bid to clearing them and allowing for organ repair without necessarily evoking adaptive immune responses (Elliott, Siddique, & Weinstock, 2014). Most innate immune cells respond to PRR signals releasing chemokines and cytokines that mediate immune responses. Additionally, the mechanism of action for the Natural Killer (NK) cells is based on 'the missing self-hypothesis' where mature NK cells do not kill normal cells that strongly express 'self' MHC class I which engages the NK cell inhibitory receptors like killer-cell immunoglobulin-like receptor (KIR) but rather proceeds to attack cells that fail to express the NK inhibitory receptors(Brown, Dobbie, Alakoskela, Davis, & Davis, 2012; Elliott et al., 2014; Karre, Ljunggren, Piontek, & Kiessling, 1986). Perforins and granzymes produced from NK-cell granules destroy infected cells by inducing apoptosis (K. D. Stone, Prussin, & Metcalfe, 2010). NK cells also produce interferon-gamma (IFN-γ), which helps to mobilize antigen presenting cells (APCs) and promote the development of effective anti-viral immunity, a process that also activates the adaptive immunity(J. S. Marshall, Warrington, Watson, & Kim, 2018).

Adaptive immunity:

Adaptive immune responses are mediated mainly by the B cells (antibody responses-humoral) and T cells (cell mediated responses) which are highly specialized, systemic cells directed at specific pathogens (Bonilla & Oettgen, 2010; Siffrin, Brandt, Herz, & Zipp, 2007). One of the key features of adaptive immune system is that, once the primary response is over and infection is terminated, it creates a repertoire of memory T cells (immunological memory) such that, upon re-infection these memory cells, unlike their naïve counterparts, would have less requirements for activation resulting in a more rapid effector response and quicker pathogen clearance (Pennock et al., 2013). Therefore, in the 'self/non self' model, the adaptive immune system functions in (i) Recognizing specific 'non-self' antigens from 'self' antigens during antigen presentation (Jiang & Chess, 2009), (ii) generating immune responses against specific pathogens or pathogen-infected cells, (iii) generating a repertoire of immunological memory of B and T cells (Akkaya, Kwak, & Pierce, 2020). Other models of immunosurveillance include the Danger Model (Walter Land & Messmer, 2012; Matzinger, 2012) or the Injury Hypothesis (W. Land et al., 1994) which posit that antigen presenting cells respond to danger signals from stressed or injured cells in order to initiate an immune response.

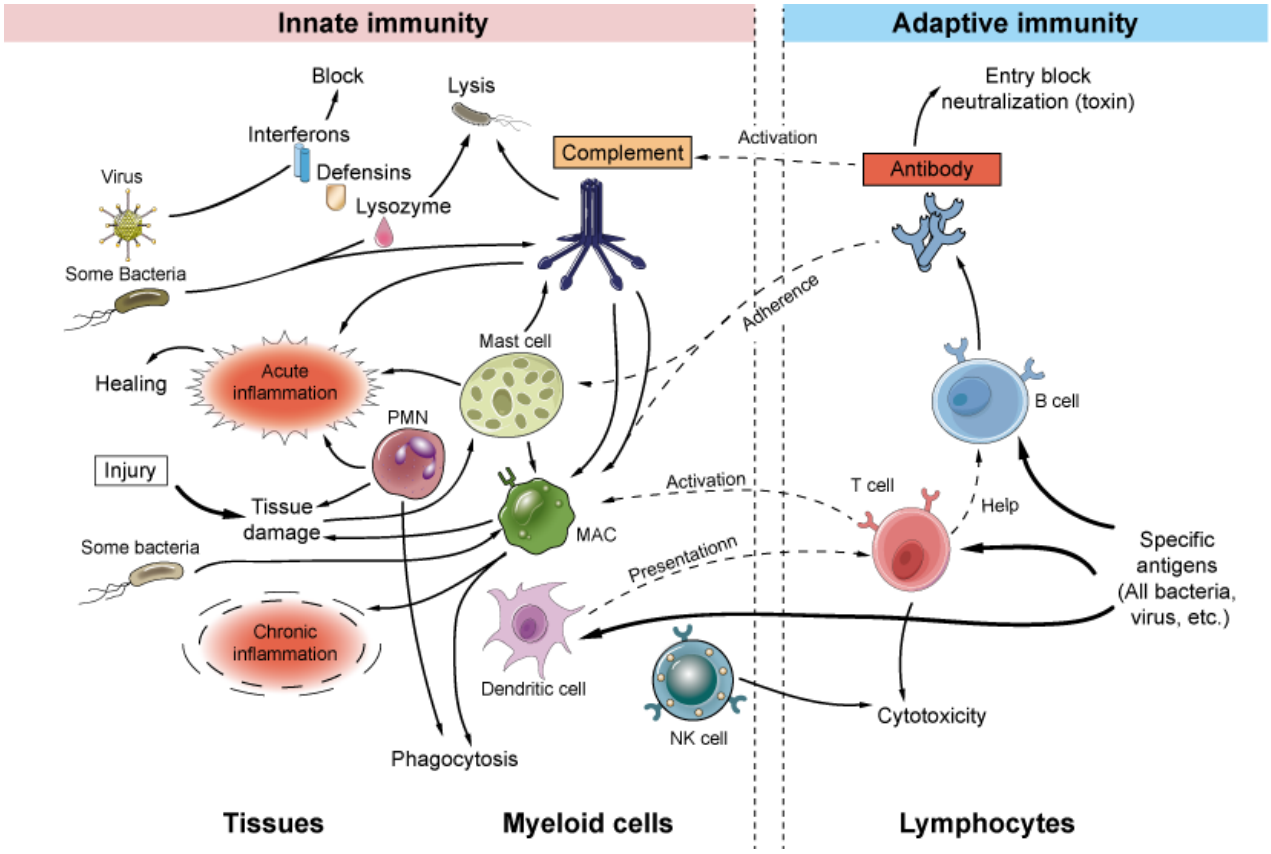


Figure 1.1: Mechanisms of Innate and adaptive immunity

Innate (left); adaptive (right) with each composed of both cellular (lower half) and humoral (upper half) elements. Adaptive immunity functions by interacting with the innate immunity.
(Source: CD creative Diagnostics® SinoBiological)

Table 1.1: Summary of Innate vs Adaptive Immune system

Characteristics	Innate Immunity	Adaptive Immunity
Line of defence	First	Second
Timeline	Immediate: minutes or hours (0 to 96 hours)	Long term: days (>96 hours)
Specificity	Specific for PAMPs associated with general pathogens	Highly specific!
Major Cell Types	Macrophages, Neutrophils, Natural Killer Cells, Dendritic Cells, Basophils, Eosinophils	T cells, B cells, and other antigen presenting cells (APCs)
Key Components	Antimicrobial peptides and proteins, such as toxic granules	Antibodies, cytokines
Self vs. Nonself Discrimination	Innate immunity is based on self vs nonself discrimination	Not as good as the innate immune system. Defects result in autoimmune diseases
Immunological Memory	None	Robust memory response
Diversity and Customization	Limited: Receptors used are standard and only recognize antigen patterns. No new receptors are made to adapt the immune response	Highly diverse: can be customized by genetic recombination to recognize epitopes and antigenic determinants.

1.2: T Lymphocytes (Cells):

T lymphocytes originate from a common lymphoid hematopoietic stem cell then leaves the bone marrow for the thymus for maturation, selection and export to the periphery (G. Anderson, Moore, Owen, & Jenkinson, 1996). The thymus provides a suitable microenvironment with specific combination of stromal cells, cytokines, and chemokines to generate functional T cells from T cell precursors (Gameiro, Nagib, & Verinaud, 2010). They are crucially necessary for cellular responses to antigens that culminate in adaptive immunity and the rearrangement of the T cell receptor (TCR) gene together with thymic selection represents crucial steps in T cell maturation and function.

1.2.1: Selection of conventional T cells in the thymus:

The thymus is a primary lymphoid organ located in the upper anterior thorax, just above the heart. Its lobules are clearly differentiated into regions- the outer cortex and an inner medulla with each region endowed with distinct thymic cells subsets, creating microenvironments uniquely specialized to support positive or negative selection of thymocytes. The thymus is vital for T-cell development and immune responses (Boehm & Bleul, 2007; Fink, 2013). αβ T cells are the majority of T cells, however approximately 5% of cells bear the γδ TCR during thymic selection. Developing thymocytes interact with the thymus stromal (non-haematopoietic) cells and undergo several

processes in distinct regions of the thymus(Kondo, Ohigashi, & Takahama, 2018).

Thymic T cell precursors undergo several selection processes, including positive selection, negative selection and agonist selection; to become a functional and self-tolerant T cell repertoire (Kurd & Robey, 2016).

Positive Selection of T cells:

T cells are positively selected when they display weak tonic self-reactivity when exposed to self-peptide but are negatively selected (deleted) once they display strong affinity to self-peptide. In achieving this, several hormones, cytokines and stromal factors aid the precursor T lymphocytes progress through several phenotypically distinct phases viz: double negative (DN1 to DN4), double positive(DP) and single positive(SP), characterized by the expression of CD3, CD4 and CD8 co-receptors on T-cell surface membrane (Blackburn & Manley, 2004; Xing & Hogquist, 2012). During DN phase, cells that lack expression of CD44, but express CD25 (DN3) undergo beta-selection which selects for cells that have successfully rearranged their TCR-β chain locus. Successful rearrangement of TCR β-chain leads to further differentiation and cells that do not undergo beta-selection die by apoptosis (Nitta & Suzuki, 2016; H. Takaba & H. Takayanagi, 2017). Following successful TCRβ gene rearrangement and pre-TCR signalling, $CD4^{+}CD8^{+}$ DP thymocytes migrate to the thymic cortex and rearrange their TCRα locus (Ladi, Yin, Chtanova, & Robey, 2006; Love & Bhandoola, 2011; Takahama, 2006).

T cells bind to intrathymic antigen peptides presented by major histocompatibility complex (MHC) class I & II molecules on the surface of dendritic cells (DCs) and thymic epithelial cells (TECs)(Belizario, Brandao, Rossato, & Peron, 2016). Therefore, DP thymocytes that experience αβTCR signals in response to antigen peptides presented by MHC class I, can undergo positive selection, leading to maturation and commitment to the CD8 lineage (i.e. $TCR^{+}CD3^{+}CD8^{+}$) whereas DP thymocyte response to MHC class II molecules are also positively selected resulting in commitment to the CD4 (i.e. $TCR^{+}CD3^{+}CD4^{+}$) lineage(Starr, Jameson, & Hogquist, 2003). Positively selected thymocytes express chemokine receptor CCR7 and migrate from the thymic cortex to the medulla where a subpopulation of medullary thymic epithelial cells (mTECs) express the CCR7 ligand such as CCL21 (Love & Bhandoola, 2011; Takahama, 2006).

Negative Selection of T cells:

Medullary thymocytes therefore undergo negative selection that kills by apoptosis, thymocytes that express TCRs with high affinity for self-peptide MHC complexes (autoreactive cell clones). Negative selection saves self-reactive clones with suppressive (regulatory T cells) activity expressing CD25 and Forkhead box P3(Foxp3), natural Treg cells(Hsieh, Lee, & Lio, 2012; Sakaguchi, Yamaguchi, Nomura, & Ono, 2008), as well as other unconventional T cells populations (Klein, Kyewski, Allen, & Hogquist, 2014). Consequent upon negative selection,

relatively small number (less than 5%) SP thymocytes become mature functional T cells and thereafter leave the thymus to constitute the $CD4^+$ and $CD8^+$ population in the peripheral pool (Hogquist, Xing, Hsu, & Shapiro, 2015).

Agonist selection:

Agonistic selection occurs based on the ability of T cells to recognize self-antigen–MHC complexes, with the resultant unconventional T cells that exert regulatory immune functions saved (Oh-Hora et al., 2013; Stritesky, Jameson, & Hogquist, 2012). These cells include $Foxp3^+$ regulatory T (Treg) cells, invariant natural killer T (iNKT) cells and $TCR\alpha\beta^+$ $CD8\alpha\alpha^+$ intestinal intraepithelial lymphocytes (IELs)(Hsieh et al., 2012; Lambolez, Kronenberg, & Cheroutre, 2007).

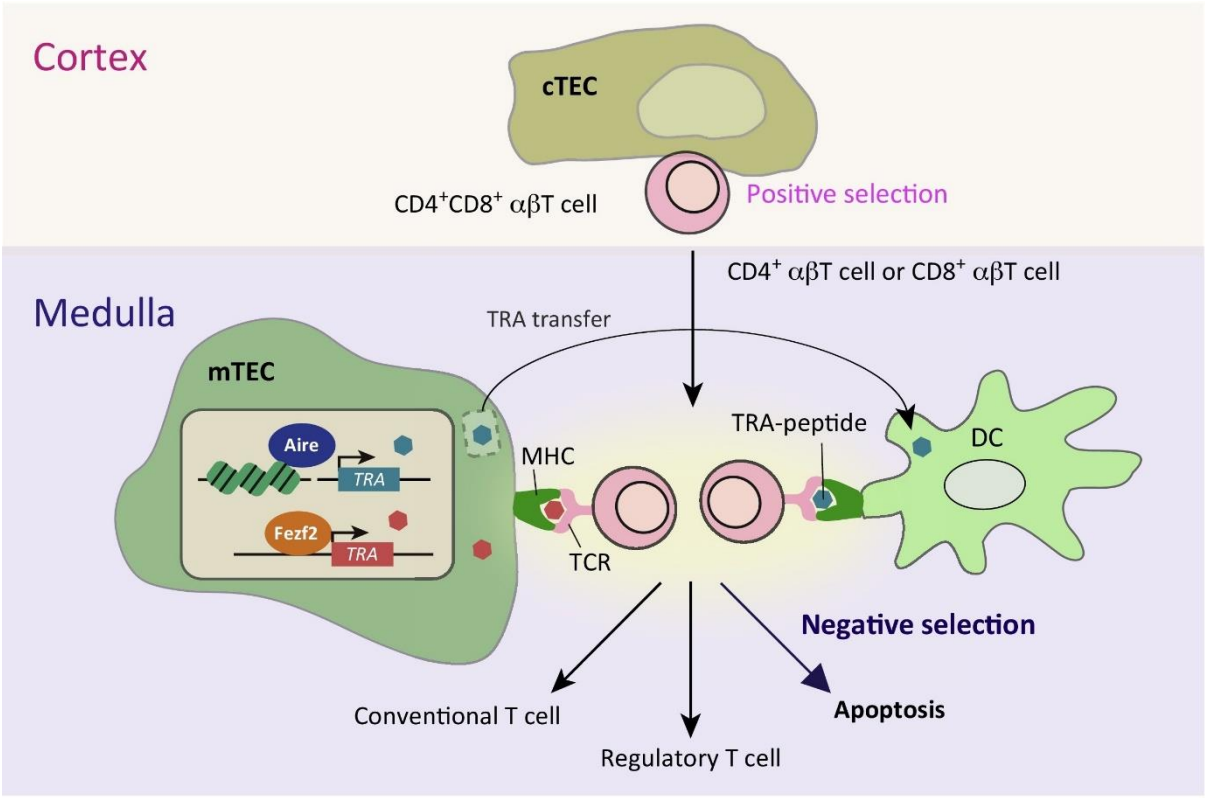


Figure 1.2: T Cell Selection and Tissue-Restricted Antigen (TRA) Expression in the Thymus

Bone marrow derived thymocyte progenitors migrate into the cortex and interact with cortical thymic epithelial cells (cTECs). Following interactions with self-peptide major histocompatibility complex (MHC) molecule complexes, CD4 or CD8 single positive T cells get positively selected, migrate into the thymic medulla and interact with medullary thymic epithelial cells (mTECs), which present the peptide of tissue-restricted antigen (TRA) in the context of MHC, leading to elimination of the most autoreactive T cells (negative selection), while some differentiate into Treg cells in agonist selection (Adapted from Takaba, H. and H. Takayanagi, *The Mechanisms of T Cell Selection in the Thymus.* Trends in Immunology, 2017)(Hiroyuki Takaba & Hiroshi Takayanagi, 2017).

1.2.2: Unconventional T cells:

Apart from conventional T cells which express αβ TCRs during thymic development, several functionally distinct unconventional T cell lineages also emerge from thymic selection. Unconventional T cells can also develop extrathymically and functions in recognising non-peptide antigens. They include CD1-restricted T cells which recognise lipids, MHC-related 1 (MR1)-restricted T cells which recognise vitamin B metabolites and γδ T cells which recognise phosphoantigens (Godfrey, Uldrich, McCluskey, Rossjohn, & Moody, 2015). Unconventional T cells express TCRs that are of limited diversity and they are often described as the 'bridge' between innate and adaptive immunity, the most studied of which are invariant natural killer T (iNKT) cells, mucosal associated invariant T (MAIT) cells and γδ T cells (Dashtsoodol et al., 2017; Lambolez et al., 2006; Pellicci, Koay, & Berzins, 2020).

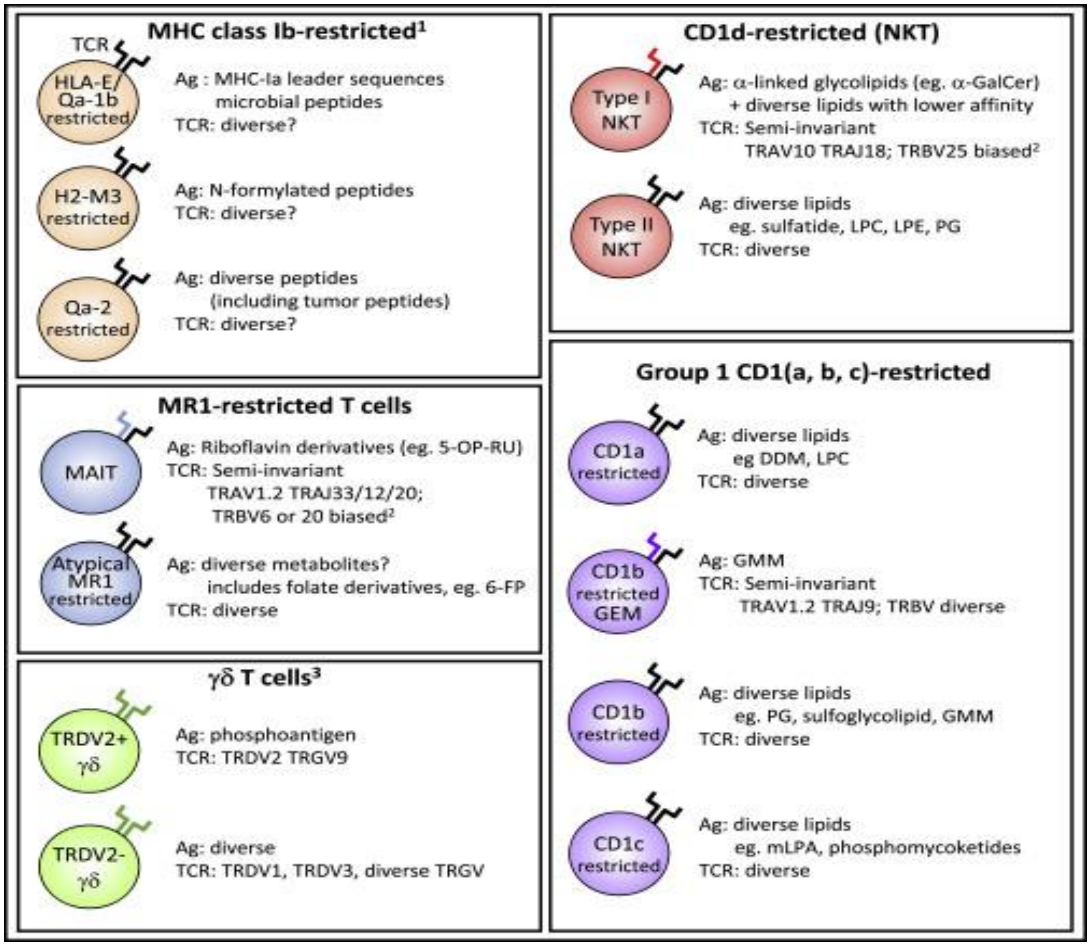


Figure 1.3: Types of unconventional T Cells

Restriction elements for different groups of unconventional T cells. 5-OP-RU, 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil; 6-FP, 6-formylpterin; Ag, antigen; a-GalCer, a-galactosylceramide; DDM, dideoxymycobactin; GEM, germline-encoded mycolyl lipid-reactive; GMM, glucose monomycolate; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; mLPA, methyl lysophosphatidic acid; MAIT, mucosal-associated invariant T; NKT, natural killer T; PG, phosphatidylglycerol; PM, phosphomycoketide; ? = insufficient or very limited data; 1, H2-M3 and Qa-2 restricted T cells are mouse only; 2, human TCR sequences; 3, only human γδ T cells shown (Adapted from Godfrey, D.I., et al., *Unconventional T Cell Targets for Cancer Immunotherapy.* Immunity, 2018) (Godfrey, Le Nours, Andrews, Uldrich, & Rossjohn, 2018).

Invariant Natural Killer T (iNKT) cells:

The majority of the iNKT cells are formed during the double positive ($CD4^+CD8^+$) agonist selection process and are characterized by their semi-invariant TCR comprised of an invariant Vα14Jα18 TCRα chain in mouse (Vα24Jα18 in human) and TCRβ chains with a bias for Vβ8, Vβ7, and Vβ2 (Eberl & Littman, 2003; Lantz & Bendelac, 1994; D. G. Wei, Curran, Savage, Teyton, & Bendelac, 2006). These T cells possess TCRs that are CD1d-restricted and express NK cell molecules such as NK1.1/CD161 (Bendelac et al., 1995; Godfrey, MacDonald, Kronenberg, Smyth, & Van Kaer, 2004). Based on prototypical cytokine and transcription factor signatures, NKT cells can be functionally subdivided into NKT1 (expressing IFNγ and T-bet), NKT2 (expressing IL-4, IL-13 and GATA-3) and NKT17 (expressing IL-17 and RORγt) (Das, Sant'Angelo, & Nichols, 2010; Lee, Holzapfel, Zhu, Jameson, & Hogquist, 2013; Sklarz et al., 2017; Winter & Krueger, 2019). iNKT cells bridge the gap between innate and adaptive immunity by rapidly secreting cytokines and lysing lipid antigen targets following TCR recognition.

Mucosal Associated Invariant T (MAIT) cells:

MAIT cells, just like the iNKT cells are formed in the thymic cortex during the double positive ($CD4^+CD8^+$) thymocyte stage expressing TCR with specificity for complexes of MR1 with 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU)(Salou, Legoux, & Lantz, 2021). MAIT are evolutionarily conserved T cells, expressing a TCR repertoire with

limited diversity in humans, mice, rats, sheep, and pigs(Goldfinch et al., 2010; Xiao et al., 2019) . MAIT cells are activated via the MAIT TCR-MR1 axis by bacteria and yeast through T cell receptor (TCR)-mediated recognition of metabolites derived from the vitamin B2 biosynthetic pathway, even though the physiological and pathophysiological roles of MAIT cells remain unclear(Goldfinch et al., 2010; Howson et al., 2018; Le Bourhis et al., 2010; Napier, Adams, Gold, & Lewinsohn, 2015). MAIT cells are also activated by viruses(Loh et al., 2016). They have been characterised for effector functions related to the expression of the master transcription factor promyelocytic leukaemia zinc finger (PLZF) (Salou et al., 2019; Savage et al., 2008) and they provide mucosal barrier protection by promoting tissue repair (Constantinides et al., 2019; Hinks et al., 2019). They are also implicated in several autoimmune disorders (Godfrey, Koay, McCluskey, & Gherardin, 2019), including diabetes (Rouxel et al., 2017) and in cancer (Gherardin et al., 2018).

Gamma-delta (γδ) T cells:

γδ T cells are a group of unconventional T cells composed of γ and δ chains with diverse structural and functional heterogeneity and are thought to be a bridge between the innate and adaptive immunity(Holtmeier & Kabelitz, 2005; Melandri et al., 2018). γδ T cells display properties similar to NKs cells of innate immunity by expressing NK receptor (NKG2D) and showing cytotoxic effects on stressed and abnormal cells including viral infected and tumour cells(Wu et al., 2014)

and a specific TCR can serve as a PRR (Morita, Mariuzza, & Brenner, 2000). On the other hand, they are considered as a part of the adaptive immune system in that they rearrange TCR genes in target-response processes which leads to a memory phenotype. This is a distinctive feature of adaptive immunity (Melandri et al., 2018). Although γδ T cells do not require MHC presentation of peptide epitopes (although some recognise MHC class 1b molecules), they can distinguish "foreign" or transformed cells from healthy self-cells by using activating and inhibitory killer Ig-like receptors (Paul & Lal, 2016). They demonstrate cytotoxicity via the granzyme-perforin axis and antibody-dependent cellular cytotoxicity by expressing CD16 (Couzi et al., 2012). γδ T cells mediate effector functions by producing effector cytokines including TNF-α, IFN-γ, and IL-17(Ramstead & Jutila, 2012). They prime T cells at the tumour site by presenting antigens(Brandes, Willimann, & Moser, 2005), and interact with B cells to promote immunoglobulin (Ig) class switching. Besides effector functions, γδ T cells play regulatory and suppressive role in the tumour microenvironment by expressing transcription factors FoxP3 and Helios in addition to secreting immunoregulatory cytokines including IL-4, IL-10 and TGF-β. They also mediate interactions between CD86 on APC and CTLA-4 on γδ T cells (Paul & Lal, 2016).

Following development in the thymus the naïve T cells enter the peripheral circulation. These migrate through secondary lymphoid organs (Lymph nodes) and encounter antigen presented by professional antigen presenting cells. Naive cells that recognise MHC-peptide complex are activated and enter a differentiation programme. This is dependent on several factors including the type of costimulation and also the cytokines produced by the dendritic cells and helper T cells in the local environment.

1.3: T cell Activation/ Antigen Presentation

Naïve T cells are difficult to activate and require the activation that comes from the professional APCs. For T cell activation, differentiation and replication to occur, T cells must be presented antigens by antigen presenting cells (APCs) in the context of major histocompatibility complex (MHC). These APCs also express costimulatory molecules CD80 or CD86 (B7.1 or B7.2) which interact with CD28 on T cells (Jonathan H. Esensten, Ynes A. Helou, Gaurav Chopra, Arthur Weiss, & Jeffrey A. Bluestone, 2016). APCs process antigens into peptides and present them together with class I or class II MHC molecules on the cell surface where they interact with appropriate TCRs (den Haan, Arens, & van Zelm, 2014; Kambayashi & Laufer, 2014). There are three professional APCs in the immune system including dendritic cells (DCs), macrophages, and B cells (Kambayashi & Laufer, 2014), with few nonprofessional APCs, including thymic epithelial cells and

vascular endothelial cells, which function in antigen presentation for only brief periods (Alexandropoulos & Danzl, 2012; Haverson, Singha, Stokes, & Bailey, 2000; Pober, Merola, Liu, & Manes, 2017; Santambrogio, Berendam, & Engelhard, 2019; Wosen, Mukhopadhyay, Macaubas, & Mellins, 2018). Essentially, for T cells to effectively respond to a particular antigen, it must receive three different signals; Signal 1 being antigen presentation to the T cell where TCRs bind strongly to antigen-MHC (Major Histocompatibility Complex) on an APC(Inman, Frigola, Dong, & Kwon, 2007; Mirshahidi, Huang, & Sadegh-Nasseri, 2001). Signal 2 involves costimulation and coinhibition of the T cell (L. Chen & Flies, 2013; W. Liu, Almo, & Zang, 2016), where costimulatory molecules on the APC bind to their receptors on T cells (Lenschow, Walunas, & Bluestone, 1996). Costimulation promotes cellular activation and proliferation (Croft, 2003; Shahinian et al., 1993). Costimulatory molecules enhance the avidity of TCR-MHC interactions thereby resulting in T cell activation. Cytokine secretion characterises signal 3 (Inman et al., 2007), where permissive biochemical environment is provided for rapid T cell division and differentiation (Schluns & Lefrancois, 2003). Inadequacies in any of the three signals, could result in apoptosis or T cell anergy (a hypo responsive state of T cell) (Driessens, Kline, & Gajewski, 2009; Schwartz, 2003).

Dendritic cells:

Dendritic cells (DCs) are derived from hematopoietic bone marrow progenitor cells with the broadest antigen presentation capabilities necessary for naive T cells activation and polarization into effector cells (Kambayashi & Laufer, 2014). They present antigens to both helper and cytotoxic T cells and mediate cross presentation of exogenous antigens, linking the innate and adaptive immunity(den Haan et al., 2014; Shortman & Liu, 2002). DCs are considered the most efficient APCs capable of initiating, coordinating, and regulating adaptive immune responses(Corinti et al., 2000; Eiz-Vesper & Schmetzer, 2020; Steinman & Witmer, 1978). They are broadly divided into $CD123^{+}CD11c^{-}$ plasmacytoid DC (pDC) and $CD123^{-}CD11c^{+}$ classical or myeloid DCs (cDC) (Boltjes & van Wijk, 2014; Merad, Sathe, Helft, Miller, & Mortha, 2013; O'Keeffe, Mok, & Radford, 2015; Patente et al., 2019) which play crucial role in peripheral tolerance and prevention of autoimmune diseases(Mbongue, Nicholas, Firek, & Langridge, 2014). Prior to encountering foreign antigen, immature dendritic cells (iDCs) express very low levels of MHC class II and costimulatory molecules on the cell surface and are therefore poorly immunogenic. However, repetitive stimulation of T cells with human iDCs can convert naïve T cells to antigen (Ag)-specific anergic Treg cells (M. Comi, G. Amodio, & S. Gregori, 2018; Jonuleit, Schmitt, Schuler, Knop, & Enk, 2000; Levings et al., 2005; Raker, Domogalla, & Steinbrink, 2015). Furthermore, maturation of DCs in the presence of exogenous IL-10

results in IL-10 producing DCs (DC-10) that are phenotypically and functionally stable after stimulation and are capable of inducing Ag-specific IL-10 producing regulatory Type 1 (Tr1) cells in an IL-10 dependent mechanism (Amodio & Gregori, 2012; M. Comi et al., 2018; Comi et al., 2020). Therefore, some studies report that *in vitro* priming of T cells by iDCs or stimulation with tolerogenic DCs drive the differentiation of Tr1 cells. However, other studies have reported that costimulation via CD55 drive the differentiation of naïve T cells to into Tr1 cells (Capasso et al., 2006a; Sutavani et al., 2013).

1.3.1: Costimulation:

After receiving signal 1 (i.e. binding to antigen-MHC complex on APC), naïve T cells must receive a Co-stimulatory (confirmatory) signal order to avoid anergy or apoptosis (Inman et al., 2007; Ogawa & Abe, 2019). Signals for co-stimulation or co-inhibition are delivered by co-stimulatory or co-inhibitory molecules largely composed of the immunoglobulin superfamily (IgSF) and tumor necrosis factor/receptor superfamily (TNFSF/TNFRSF); and further subdivided into specific families based on the amino acid sequence, protein structure and function(L. Chen & Flies, 2013). These molecules play a crucial role in regulating T cell activation, proliferation, differentiation into effector subsets and T cell survival (L. Chen & Flies, 2013). Positive signals are transmitted by co-stimulatory receptors in order to enhance adaptive immunity by increasing IL-2 production by dendritic cells and by

promoting cell division and clonal expansion, protecting cells from apoptosis/anergy, and generating long-lived memory in T cells (Kohlmeier & Benedict, 2003). Whereas co-inhibitory molecules transduce signals for downregulation of T cell responses (W. Liu et al., 2016; Zang & Allison, 2007). The production of IL-2 is essential to both proliferation and differentiation of naïve T cells into effector phenotypes (Yashiro et al., 1998). However, not all co-stimulatory molecules are able to stimulate IL-2 gene transcription for IL-2 production though in some cases, IL-2 is produced but at levels insufficient to drive naïve T cell proliferation and differentiation (Yashiro et al., 1998). CD28 and 4-1BB have been well characterized for their role in driving naïve T cell proliferation and differentiation due to their ability to stimulate high levels of IL-2 production whereas, other co-stimulatory molecules (e.g. CD55/CD97) play major roles in modulating effector functions of T cells rather than naïve responses (Croft, 2003; Shuford et al., 1997; Yashiro et al., 1998). Co-stimulatory signal alone is not enough for T cell activation, rather it requires a simultaneous TCR engagement. For instance, CD55/CD97 engagement alone does not result in upregulation of activation markers CD69 and CD25; instead, it simultaneously enhances TCR-MHC interactions, resulting in T cell activation (Capasso et al., 2006a; Sutavani et al., 2013).

Receptor	T cell type	Priming	Cell growth	T_H cell differentiation	Effector function	Survival	Memory
CD28	$CD4^+$	(+)	(+)	ND	ND	(+)	(+)
	$CD8^+$	(+)	(+)	ND	ND	(+)	(+)
ICOS	$CD4^+$	ND	(+)	(+)	(+)	(+)	(+)
	$CD8^+$	ND	ND	ND	ND	(+)	(+)
CTLA4	$CD4^+$	ND	(–)	ND	(–)	(–)	(–)
	$CD8^+$	ND	(–)	ND	(–)	(–)	(–)
PD1	$CD4^+$	ND	(–)	ND	(–)	(–)	
	$CD8^+$	ND	(–)	ND	(–)	(–)	(–)
BTLA	$CD4^+$	ND	ND	ND	(–)	(–)	(+/–)
	$CD8^+$	(–)	(–)	ND	(–)	(–)	(+/–)
HVEM	$CD4^+$	(+)	(+)	ND	(+)	(+)	(+)
	$CD8^+$	(+)	(+)	ND	(+)	ND	(+)
CD27	$CD4^+$	(+)	(+)	(+)	(+)	(+)	(+)
	$CD8^+$	(+)	(+)	ND	ND	(+)	(+)
4-1BB	$CD4^+$	ND	(+)	ND	(+)	(+)	(+)
	$CD8^+$	ND	(+)	ND	(+)	(+)	(+)
OX40	$CD4^+$	ND	(+)	(+)	(+)	(+)	(+)
	$CD8^+$	ND	(+)	ND	(+)	(+)	(+)
DR3	$CD4^+$	ND	(+)	(+)	(+)	ND	(+)
	$CD8^+$	ND	(+)	ND	ND	ND	ND
GITR	$CD4^+$	ND	(+)	ND	ND	ND	ND
	$CD8^+$	ND	(+)	ND	(+)	ND	ND
CD30	$CD4^+$	ND	(+)	(+)	ND	ND	ND
	$CD8^+$	ND	(+)	ND	ND	(+)	(+)
SLAM	$CD4^+$	ND	ND	(+)	(+)	ND	ND
	$CD8^+$	ND	ND	ND	(+)	ND	ND
CD2	$CD4^+$	(+)	(+)	ND	(+)	ND	(+)
	$CD8^+$	(+)	(+)	ND	(+)	ND	(+)
2B4	$CD4^+$	ND	ND	ND	ND	ND	ND
	$CD8^+$	ND	(+)	ND	(+/–)	ND	ND
TIM1	$CD4^+$	ND	(+/–)	(+/–)	(+)	ND	ND
	$CD8^+$	ND	ND	ND	ND	ND	ND
TIM2	$CD4^+$	ND	ND	(+)	(+)	ND	ND
	$CD8^+$	ND	ND	ND	ND	ND	ND
TIM3	$CD4^+$	ND	(–)	(–)	(–)	ND	(–)
	$CD8^+$	ND	(–)	ND	(–)	ND	(–)
TIGIT	$CD4^+$	ND	(–)	ND	(–)	ND	ND
	$CD8^+$	ND	ND	ND	ND	ND	ND
CD226	$CD4^+$	ND	ND	ND	ND	ND	ND
	$CD8^+$	ND	(+)	(+)	(+)	ND	ND
CD160	$CD4^+$	ND	(–)	ND	(–)	(–)	(–)
	$CD8^+$	ND	(–)	ND	(–)	ND	(–)
LAG3	$CD4^+$	ND	(–)	ND	(–)	ND	ND
	$CD8^+$	ND	(–)	ND	(–)	ND	ND
LAIR1	$CD4^+$	ND	(–)	ND	(–)	ND	ND
	$CD8^+$	ND	(–)	ND	(–)	ND	ND
B7-1	$CD4^+$	ND	(–)	ND	(–)	ND	ND
	$CD8^+$	ND	(–)	ND	(–)	ND	ND
B7-H1	$CD4^+$	ND	(–)	ND	(–)	ND	ND
	$CD8^+$	ND	(–)	ND	(–)	ND	ND

Figure 1.4: List of costimulatory and coinhibitory receptor function in stages of T cell differentiation

‘(+)’ positive effect, ‘(−)’ negative effect, and ‘ND’ effect not known. BTLA, B and T lymphocyte attenuator; DR3, death receptor 3; GITR, glucocorticoid-induced TNFR-related protein; HVEM, herpes virus entry mediator; LAIR1, leukocyte-associated immunoglobulin-like receptor 1; SLAM, signalling lymphocytic activation molecule; TIGIT, T cell immunoreceptor with immunoglobulin and ITIM domains; TIM, type I transmembrane immunoglobulin and mucin (Adapted from Chen, L. and D.B. Flies, *Molecular mechanisms of T cell co-stimulation and co-inhibition.* Nat Rev Immunol, 2013). (L. Chen & Flies, 2013).

Scholarly literatures abound regarding the CD28 and B7 families. They belong to the IgSF and are among the best described co-stimulatory and co-inhibitory molecules (Chattopadhyay et al., 2009). B7-1(CD80) and B7-2(CD86) serve as the ligands for co-stimulatory receptor CD28 while CTLA-4 serves as a co-inhibitory receptor (Zang & Allison, 2007) as shown in Figure 1.5.

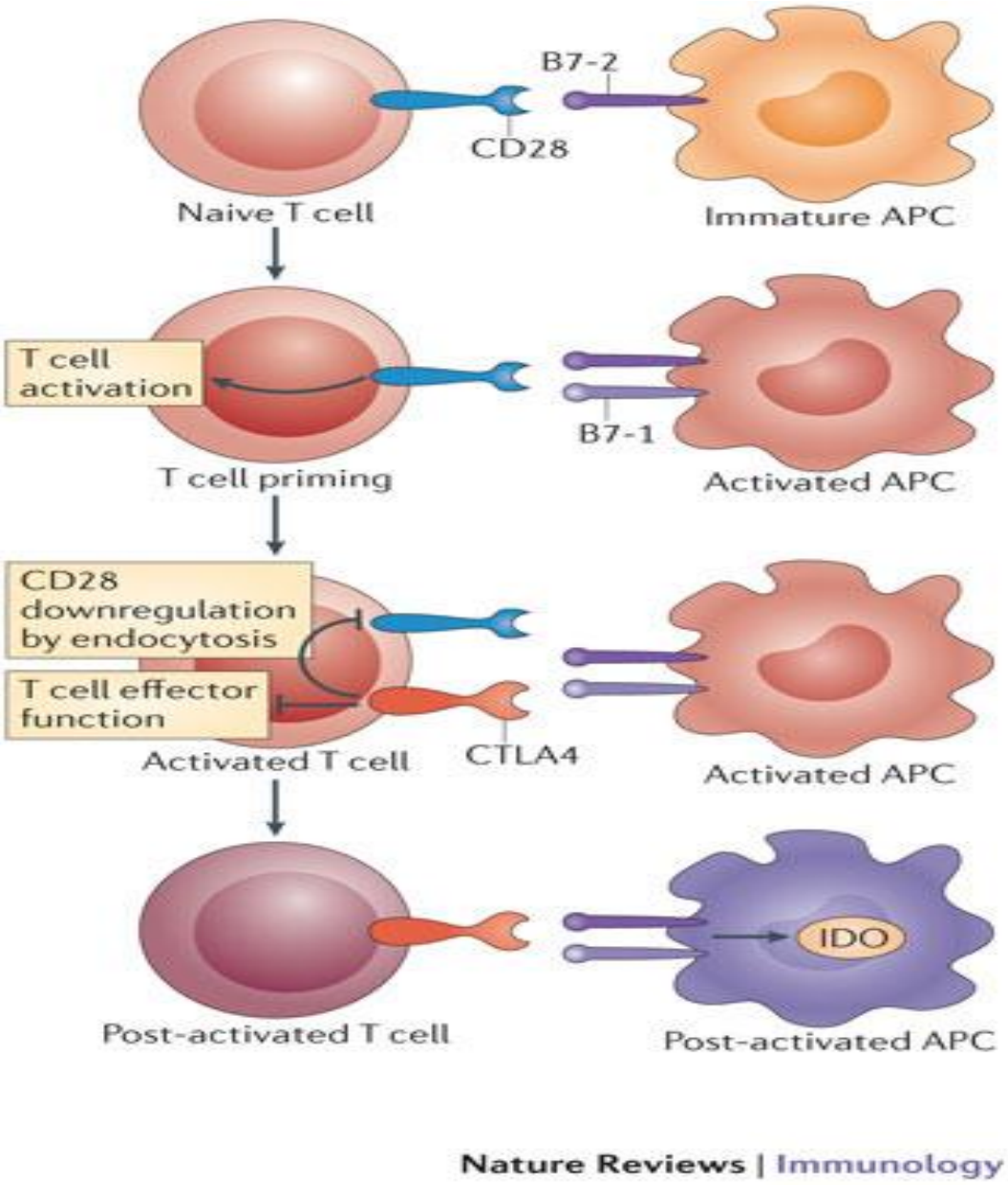


Figure 1.5: The CD28:B7 costimulation paradigm

Following antigen presentation by antigen presenting cells (APCs) in the context of MHC to naïve T cells, the co-stimulatory receptor CD28 binds to its ligands, B7-1, and B7-2 resulting in cell activation and upregulation of cytotoxic T lymphocyte antigen 4 (CTLA-4). This leads to the downregulation of CD28 expression by endocytosis as CTLA-4 binds to ligands B7-1 and B7-2. (Adapted from Chen, L. and D.B. Flies, *Molecular mechanisms of T cell co-stimulation and co-inhibition.* Nat Rev Immunol, 2013). (L. Chen & Flies, 2013)**.**

The costimulatory receptor, CD28 is constitutively expressed on naïve $CD4^+$ and $CD8^+$ T cell surfaces providing the required co-stimulatory signal for T cell activation, proliferation, and differentiation; whereas induction of CTLA-4 occurs following T cell activation aimed at suppressing T cell response (Rudd, Taylor, & Schneider, 2009). More recently, PD-1and PD-L1/PD-L2 co-inhibitory pathways have been reportedly linked to the regulation of adaptive immunity (Freeman et al., 2000).

1.3.2: Members of the CD28 family (ICOS, PD-1 and CTLA-4)

ICOS:

ICOS (inducible co-stimulator) is a receptor expressed only on activated T cells, after either the TCR binds to MHC-antigen complex or CD28 binds to B7(Coyle et al., 2000). ICOS binds to its ligand ICOSL (also known as B7h, GL50, B7RP-1, LICOS, ICOS-L, B7-H2, KIAA0653, CD275), which is expressed constitutively on lymphoid tissues (Inman et al., 2007; S. Wang et al., 2000). ICOS is not involved in the immediate T cell IL-2 release but modulates differentiation of T helper cells subtypes by promoting release of IL-4, IL-5, IL-10, IFN-γ, and TNF-α, regulated by CD28 (Hutloff et al., 1999).

PD-1:

PD-1 (programmed death 1) is a co-inhibitory receptor induced following T cell activation. It's a member of CD28 family containing an immunoreceptor tyrosine-based inhibitory motif (ITIM) expressed on

thymocytes, B cells, monocytes, endothelial cells and Natural killer cells (Agata et al., 1996; Hutloff et al., 1999). PD-1 and its ligands, PD-L1 (B7-H1), PD-L2 (B7-DC) (Karre et al., 1986; Yashiro et al., 1998) are well described check-points to T cell mediated immunity(Y. Dong, Sun, & Zhang, 2016), although some investigations suggest some co-stimulatory roles for PD-1(H. Dong, Zhu, Tamada, & Chen, 1999). Co-inhibitory receptors deliver signals that play decisive roles in the maintenance of peripheral tolerance and prevention of autoimmunity; but impairs viral and tumour immunity, thereby promoting chronic infection and tumour progression (Bardhan, Anagnostou, & Boussiotis, 2016; Nishimura, Nose, Hiai, Minato, & Honjo, 1999). Whereas PD-L1 is induced on T cells, B cells and macrophages in response to inflammatory cytokines expression (e.g. IFN-γ) (Loke & Allison, 2003), PD-L2, which has a higher affinity for PD-1 receptor than PD-L1, is expressed on dendritic cells and macrophages following induction by IL-4 secretion (X. Zhang et al., 2004). Responses by T helper 1 cells favour the expression of PD-L1 in the sense that, PD-L1 induces apoptosis and is involved in inflammatory responses and autoimmunity (Ansari et al., 2003; H. Dong et al., 2002), while T helper 2 cells responses favour the expression on PD-L2 (Inman et al., 2007).

CTLA-4:

CTLA-4 (Cytotoxic T-Lymphocyte Antigen-4) is a member of the IgSF and is a CD28 co-inhibitory homologue, induced following T cell

activation (W. Liu et al., 2016). It binds to its ligands, B7-1 with 10-fold and B7-2 with 100-fold higher affinity (Collins et al., 2002). CTLA-4 is composed of a single extracellular IgV domain followed by a stalk region and a cytoplasmic tail (W. Liu et al., 2016). It is worthy to note that Tregs constitutively express CTLA-4 following the upregulation of CTLA-4 expression in Foxp3 dependent manner(Sansom & Walker, 2006).

Another large family of co-stimulatory/co-inhibitory molecules is the tumour necrosis factor/receptor superfamily (TNFSF/TNFRSF). Members of which include CD27/CD70, CD40/CD40L, OX-40/OX-40L, HVEM/LIGHT, 4-1BB/4-1BBL and GITR/GITRL (Driessens et al., 2009; Inman et al., 2007; Watts, 2005). Therefore, members of the TNFSF/TNFRSF act in a similar fashion to the IgSF, enhancing T cell proliferation, differentiation, and regulation through release of cytokines.

1.3.3: CD55 as costimulatory receptor in T cell immunity:

CD55 (Decay accelerating factor) is a 70-kDa single chain complement regulatory protein with four extracellular short consensus repeat (SCR) domains (Bergelson et al., 1994). CD55 is linked to the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor within the lipid rafts which is rich in (GPI)-linked proteins and signalling molecules including the Src Family kinases (SFKs) (Fra, Williamson, Simons, & Parton, 1994; Lublin & Atkinson, 1989; Simons & Ikonen, 1997). One function of CD55 is to protect cells from complement-mediated lysis and

induces the decay of the C3 and C5 convertases, preventing C3b deposition and downstream assembly of the complement membrane attack (MAC) complex (Noris & Remuzzi, 2013; Xie, Jane-Wit, & Pober, 2020). More recently, CD55 has been reported to bind to CD97, specifically EGF 1,2,5 isoform (epidermal growth factor-seven-span transmembrane (EGF-TM7), leading to T cell activation (H. H. Lin et al., 2001). CD97, earlier identified as early activation marker of T cells has recently been described as the cellular ligand to CD55(Abbott et al., 2007). The CD55-CD97 receptor-ligand pair has been shown to be a potent costimulatory signal to human T cells, and together with TCR engagement via CD3 stimulation result in the induction of IL-10 producing Tr1 cells from naïve T cells (Spendlove & Sutavani, 2010; Sutavani et al., 2013; Tosello, Mary, Amiot, Bernard, & Mary, 1998).

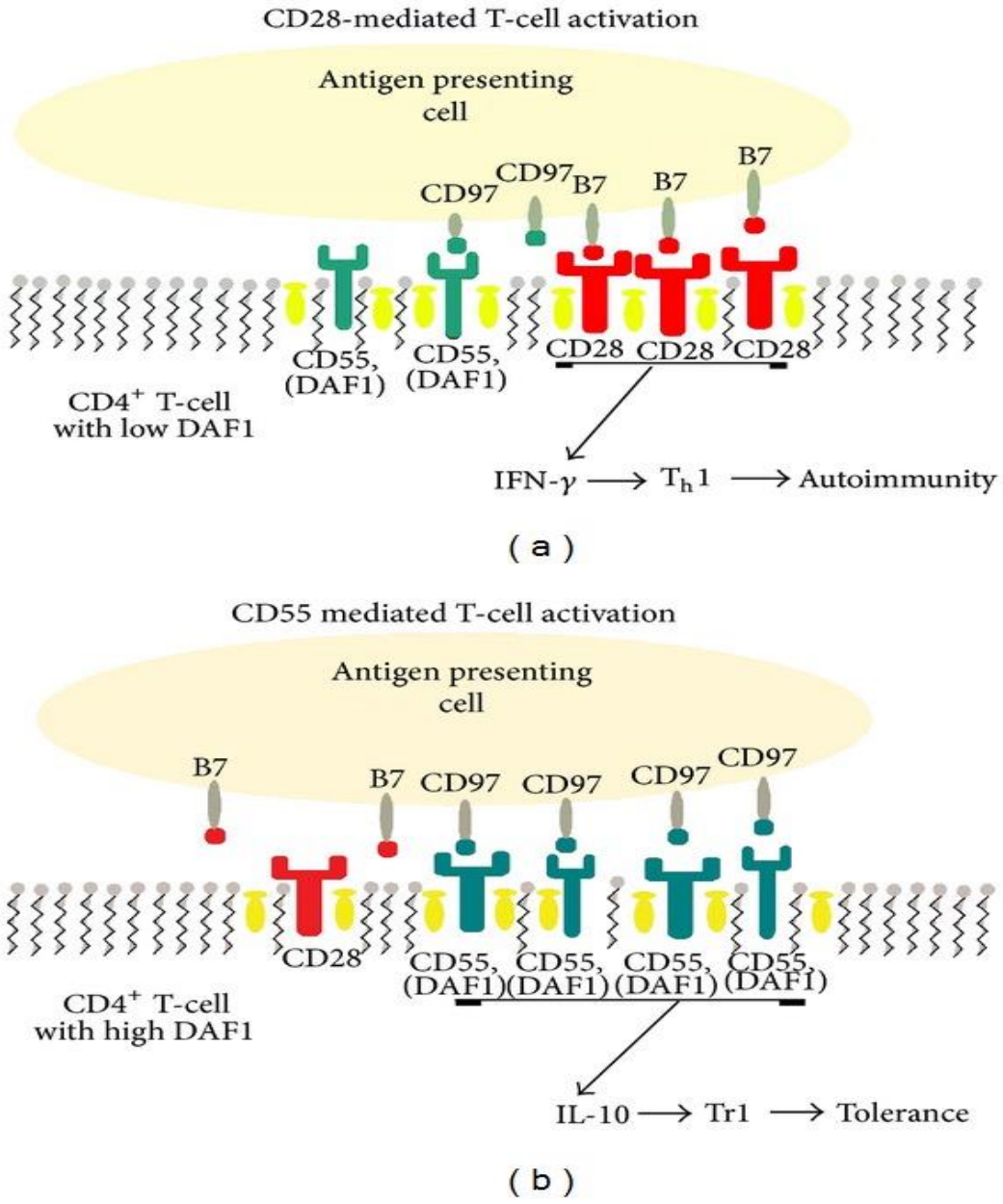


Figure 1.6: CD55 costimulation compared

(a) proinflammatory (i.e., IFN-*γ*) cytokine production mediating a Th1 response following CD28 mediated T cell activation (b) CD97-CD55 interactions leading to IL-10 production, a T regulatory cell phenotype and immune tolerance (Adapted from Toomey, C.B., et.al., *The role of decay accelerating factor in environmentally induced and idiopathic systemic autoimmune disease.* Autoimmune diseases, 2014). (Toomey, Cauvi, & Pollard, 2014).

1.3.4: T cells phenotypes :

Naïve T Cells :

Mature T Cells manifest little functional activity until they encounter a specific antigen that interacts with an antigen receptor on their cell surface. Lymphocytes that have not yet been activated by antigens are known as naïve lymphocytes and they are often designated $CD3^{+}CD4/8^{+}CD45RA^{+}CD45RO^{-}CD62L^{+}CCR7^{+}$ (Larbi & Fulop, 2014). Upon antigen encounter or stimulation by a monoclonal antibody, naïve T cells differentiates and expands into effector, memory as well as regulatory (Tr1) populations (Ellis, Reneer, Velez-Ortega, McCool, & Marti, 2012; Sutavani et al., 2013).

Effector T Cells:

T cells acquire effector functions, 4-5 days after naïve T-cell activation. They differentiate into effector T-cells having the ability to synthesize molecules required to exert helper or cytotoxic functions upon contact with their specific antigens, without further need for co-stimulation (Gudmundsdottir, Wells, & Turka, 1999; London, Lodge, & Abbas, 2000). The intrathymic differentiation of thymocytes into specialized effector T cell populations require specific transcription factors including Th-POK (T-helper-inducing POZ/Kruppel-like factor), GATA3 (GATA-binding protein 3), and RUNXs (Runt-related transcription factor)(Egawa, 2009; He, Park, & Kappes, 2010; Luckey et al., 2014).

MHC class I restricted T cells ($CD8^+$) exert cytotoxic functions and upregulate Runx3 gene, as they undergo CD8 lineage differentiation, whereas MHC class II restricted T-cells ($CD4^+$) exert helper functions and upregulate Th-POK gene during CD4 lineage differentiation(He et al., 2010; Luckey et al., 2014).

(a) $CD8^+$ T cells:

All CD8 T cells differentiate into $CD8^+$ cytotoxic T cells, which kill their target cells, providing defence against intracellular pathogens, especially viruses (Andreasen, Christensen, Marker, & Thomsen, 2000). The EOMES has been well documented as the transcription factor for effector $CD8^+$ T cells (Pearce et al., 2003). The co-stimulatory requirements for naïve CD8 T cells are, by far, more than those for naïve CD4 T cells, possibly due to the highly destructive effector actions of $CD8^+$ cytotoxic T cells (Blazevic, Trubey, & Shearer, 2001).

(b) $CD4^+$ T Cells

During contact with antigens presented by antigen presenting cells, $CD4^+$ T cells become costimulated, expand and differentiate into functionally distinct effector T cells subsets including T_H1 (T helper 1), T_H2, T_H17, T_H9, T_{FH} (T Follicular helper), TH 22 and T_{reg} (Regulatory T Cells) (Basu, Hatton, & Weaver, 2013; Bluestone & Abbas, 2003; C. King, 2009; Veldhoen et al., 2008). These effector T-cells are characterized by different signature cytokines and transcription factors as shown in the diagram (Figure 2) below.

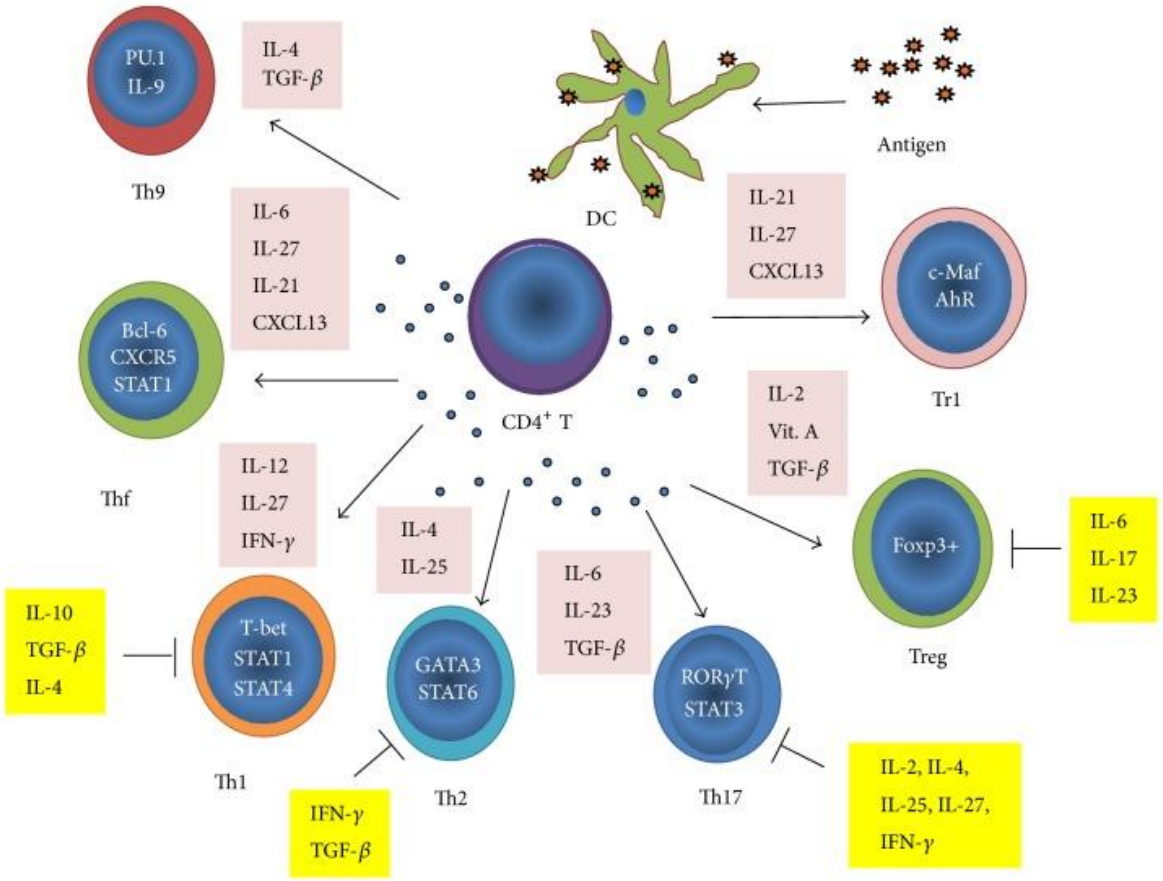


Figure 1.7: Cytokines and transcription factors controlling CD4⁺ T cell differentiation.

Following stimulation by APCs, naïve CD4⁺ T cells proliferate and differentiate into at least seven effector cell subsets each induced by a signature pattern of cytokines (pink box), transcription factors (blue, nucleus) and regulated by distinct cytokines (yellow box). (Belizario, J.E., et al., *Thymic and Post thymic Regulation of Naive CD4(+) T-Cell Lineage Fates in Humans and Mice Models.* Mediators Inflamm, 2016) (Belizario et al., 2016).

1.3.5: Effector cells subtypes:

T_H1 Cells:

T helper 1 (T_H1) cells are characterized by the production of IL-2, IL-18, IFN-γ, and TNF-β (lymphotoxin) with T-bet and STAT1 serving as the master regulator controlling transcriptional programming of Th1 differentiation and expansion (Reiner, Sallusto, & Lanzavecchia, 2007; Sakaguchi & Powrie, 2007; L. Zhou, Chong, & Littman, 2009). T_H1 cells are involved in cell-mediated immune responses with particular role in eradicating infections by microbes that can survive or replicate within macrophages (e.g., viruses, protozoans, and intracellular bacteria).

(II) T_H2 Cells:

T helper 2 (T_H2) cells have master specific transcription factors GATA3 (GATA-binding protein 3) and STAT6 with IL-4 serving as the most important cytokine for the induction of the transcription factors and T_H2 differentiation. T_H2 cells help to control retroviral infections as well as infections by extracellular parasites such as helminths and nematodes, by promoting the secretion of I_gG1 and I_gE by B-cells(Reiner et al., 2007), thereby promoting immediate-type hypersensitivity reactions- "humoral immunity". They have also been shown to play important roles in the development of allergic inflammation (Romagnani, Maggi, Liotta, Cosmi, & Annunziato, 2009).

(III) T_H17 Cells:

T helper 17 (T_H17) cells are primarily respond to extracellular bacteria and fungi and amplify neutrophilic responses targeted at such pathogens(Littman & Rudensky, 2010). Transcription factors, RORγt (retinoic acid receptor-related orphan receptor gamma t) and STAT3 regulate T_H17 differentiation while IL-6 and TGF-β, promote development (W. Chen et al., 2003; Gorelik & Flavell, 2000) whereas IFN-γ and IL-4 suppress early differentiation (Annunziato et al., 2007; O'Shea et al., 2009). T_H17 cells promote the production of I_gG2 and I_gG3 antibodies by B-cells as well as secrete IL-17 and IL-22 which are important in activating barrier epithelial cells in the gastrointestinal, respiratory and urogenital tracts and the skin, to produce antimicrobial peptides against microbes(Littman & Rudensky, 2010).

(IV) T_H9 Cells:

T helper 9 (T_H9) cells have just been recently described and are renown for high IL-9 production. T_H9 cells have the highest expression of IL-9 when compared to a variety of other cells, such as mast cells, NKT cells, T_H2, T_H17 and $T_{regs,}$ known for IL-9 expression(Noelle & Nowak, 2010). Differentiation of T_H9 cells requires the integration of the signalling pathways for IL-2, IL-4 and TGF-β with STAT 5 (signal transducer and activator of transcription 5), STAT 6 (signal transducer and activator of transcription 6), PU.1 and IRF4 (Interferon response factor 4) serving and the major transcription factors that regulate their

differentiation(Rojas-Zuleta & Vasquez, 2016). GATA 3 is also implicated as one of the transcription factors in T_H9 cells differentiation, though expressed in a lesser number (Dardalhon et al., 2008). T_H9 cells are involved in immunity against parasites, cancer and allergic diseases(Rojas-Zuleta & Vasquez, 2016).

(V) T_{FH} Cells:

T follicular helper (T_{FH}) cells are a specialized $CD4^+$ T-cells subset that promote germinal centre responses, thereby contributing to the eradication of most classes of pathogens (Wali, Sahoo, Puri, Alekseev, & Nurieva, 2016). The costimulatory molecules involved in T_{FH} cells activation pathway include CD28 and ICOS (Inducible T-cell costimulatory)(Linterman et al., 2009), SAP (the cytoplasmic adaptor protein SLAM-associated protein)(Cannons, Tangye, & Schwartzberg, 2011), BTLA (B- and T- lymphocyte attenuator)(Murphy & Murphy, 2010), CTLA-4 (Cytotoxic T-lymphocyte-Associated Protein-4)(Alegre, Frauwirth, & Thompson, 2001), and PD-1 (Programmed Death ligand-1)(Good-Jacobson et al., 2010). The list of transcription factors that regulate T_{FH} cells differentiation is on the increase with BCL-6 (B-Cell lymphoma 6 protein), c-MAF, BATF, IRF-4, STATS, ASCL-2 (Achaete-scute homologue-2), BLIMP-1, Foxo1 and FoxP1 (Bollig et al., 2012; X. Liu, Nurieva, & Dong, 2013; E. L. Stone et al., 2015; Wali et al., 2016). T_{FH} cells share production of some of the same lineage-defining cytokines of T_H1, T_H2 and T_H17 in a bid to induce the development and

antibody isotype switching in germinal centres into nodes where final B cell maturation occurs(Belizario et al., 2016). Cytokines of highest expression by T_{FH} cells include IL-21(Nurieva et al., 2008), IL-4(I. L. King & Mohrs, 2009), IFN-γ and IL-17(H. D. Marshall et al., 2011). Therefore, T_{FH} cells appear to be domiciled in the lymphoid tissues, providing help to the B-cells.

(VI) T_H 22 Cells

T_H 22 cells are characterized by high production of pro-inflammatory cytokine IL-22 and tumour necrosis factor-α (TNF-α) and are implicated in pathogenesis of autoimmune disorder (Azizi, Yazdani, & Mirshafiey, 2015). They are distinct from TH17 and TH1 for not producing IL-17 or IFN-γ, but express aryl hydrocarbon receptor (AHR) as the key transcription factor (Mübeccel Akdis, Palomares, van de Veen, van Splunter, & Akdis, 2012).

1.4: Natural regulatory T (nTreg) Cells:

Discovered in 1969 as suppressor T cells in mice (Nishizuka & Sakakura, 1969) and later reported as CD25 positive activated T cells that mediate self-tolerance (Sakaguchi, Sakaguchi, Asano, Itoh, & Toda, 1995), Treg cells are now known to modulate the immune system, maintain tolerance to self-antigens and prevent autoimmune diseases(Romano, Fanelli, Albany, Giganti, & Lombardi, 2019; Sharabi et al., 2018; Vandenbark & Offner, 2008). They are CD4 positive and highly express IL-2Rα (CD25), with FoxP3 as the master transcription factor (Fontenot,

Gavin, & Rudensky, 2003). FoxP3 is the master regulator for Treg cells in mice(Cortez et al., 2020), however, it is believed that there is another master regulator in humans that controls FoxP3 expression, hence it being inducible in other T cell subsets in humans(Deng et al., 2019; Thornton & Shevach, 2019).

The mechanism by which $CD4^+CD25^+FoxP3^+$ Treg cells mediate immune suppression is via cell to-cell contact- dependent mechanism and the secretion of soluble cytokines(Field, Kulhankova, & Nasr, 2007; Hou, Wang, Zhou, & Li, 2021; Kondělková et al., 2010; Thornton & Shevach, 1998). They participate in all cell-mediated immune responses, directly affecting T_H1,T_H2,T_H17,CTL, and B-cells(Broere, Apasov, Sitkovsky, & van Eden, 2011). Natural Treg (nTreg) cells proceed from agonist selection in the thymus (Kurd & Robey, 2016) and are well known for cell surface expression of $CD4^+CD25^{hi}CD127^{low}$, OX40/CD134, CD27, CTLA-4, CD62-L, and TGF-β (Hsieh et al., 2012).

Several studies have shown that $FoxP3^+$ regulatory T cells can be induced in the periphery from $CD4^+$ $Foxp3^-$ conventional T cells (M. Kanamori, H. Nakatsukasa, M. Okada, Q. Lu, & A. Yoshimura, 2016; Mitsuhiro Kanamori, Hiroko Nakatsukasa, Masahiro Okada, Qianjin Lu, & Akihiko Yoshimura, 2016; Schmitt & Williams, 2013; Yu et al., 2021), in which case they are termed inducible Tregs (iTreg) cells. iTreg cells emerge from $CD4^+CD25^-$ naive responder T cells through strong TCR costimulation and transforming growth factor β (TGF-β) signalling in the

presence of interleukin 2 (IL-2) and all-trans retinoic acid (ATRA)(Schmidt, Éliás, Joshi, & Tegnér, 2016; C.-M. Sun et al., 2007; X. Sun et al., 2018). IL-2 is crucial for FoxP3 induction while ATRA enhances FOXP3 expression. Although both iTreg and nTreg cells mediate immune homeostasis, iTreg cells supplement the nTreg cells in part by expanding antigen receptor diversity within regulatory responses(Haribhai et al., 2011). Treg cells control autoimmunity but also limit the immune system's response to tumours (Sakaguchi, 2005).

1.5: Other regulatory T cells:

CD39$^+$ Treg Cells:

CD39 phosphohydrolyses ATP or ADP to yield AMP, which is further hydrolysed by CD73 to anti-inflammatory adenosine (Deaglio et al., 2007). Initially described as a B lymphocyte activation marker (Maliszewski et al., 1994), CD39 has now been reportedly expressed on the surface of human and murine naïve T reg cells(G. Borsellino et al., 2007; Deaglio et al., 2007; Raczkowski et al., 2018) , on Tregs memory cells (Dwyer et al., 2010), CD39$^+$ Treg cells with Th17 associated surface markers(Bai & Robson, 2015; Magid-Bernstein & Rohowsky-Kochan, 2017), γδ T cells (Gruenbacher et al., 2016) as well as on Breg cells (Figueiró et al., 2016). CD39 is constitutively expressed by Foxp3$^+$ Treg cells (Giovanna Borsellino et al., 2007) and suppression of Th1 and Th17 responses in an adenosine-dependent manner has been well documented for CD39$^+$ Treg cells(Fletcher et al., 2009). Moreover, CD39$^+$ Treg cells also express CD25, activation markers, co-inhibitory

molecules, and suppressive cytokines but lower levels of CD127 compared with $CD39^-$ Treg cells (J. Gu et al., 2017).

$CD27^+$ Treg Cells:

Treg cells control and restrict the availability of costimulatory molecules to other T cells but they depend on many of the same costimulatory signals for their own stability, since it provides important signalling for Treg cells activation, survival, expansion, and acquisition of effector functions upon antigen recognition (Golovina et al., 2008; J. B. Wing, Tay, & Sakaguchi, 2019; K. Wing et al., 2008). Ligation of CD27 on T cells with CD70 on APCs provides costimulatory signals for T cell activation and $CD4^+$ T cell subsets differentiation (Hintzen et al., 1995; Soares et al., 2007). CD27/CD70 costimulation is also reported to be essential in generating antigen-specific T cell immunity by enhancing the survival of activated T cells (Denoeud & Moser, 2011; Hendriks et al., 2000; Welten et al., 2013). Although thymic development of conventional $CD4^+$ and $CD8^+$ T cells appears independent of CD27 under steady-state conditions. Recent studies have shown that interactions between CD27 on developing T cells and CD70 on mTEC and DCs in the thymic medulla rescues Treg cells from apoptosis during clonal deletion and promotes their development (Arroyo Hornero et al., 2020; Coquet et al., 2013). Furthermore, mouse studies reveal that CD27 co-stimulation increases the abundance of regulatory T cells and reduces atherosclerosis in hyperlipidaemic mice (Winkels et al., 2017).

These observations suggest that the CD27/CD70 pathway potentiates the homeostasis and inhibitory function of Tregs (Bowakim et al., 2018) and CD27 expression correlated with regulatory activity could be used to purify Treg cells from the cell lines expanded from $CD4^+$ $CD25^+$ cell (Duggleby, Shaw, Jarvis, Kaur, & Gaston, 2007).

T helper 3 (Th3) cells:

Th3 cells were first found in mesenteric lymph node $CD4^+$ T cells in oral tolerance research in mice as single cell clones producing TGF-β1 after oral administration of self-antigen (Y. Chen, Kuchroo, Inobe, Hafler, & Weiner, 1994). They have been described both in mice and human as $CD4^+CD25^-CD69^+FoxP3^-LAP^+$ regulatory T cells (Chien & Chiang, 2017b) which mediate oral tolerance by secreting high levels of anti-inflammatory cytokine, transforming growth factor beta (TGF-β) (Howard L. Weiner, Andre Pires da Cunha, Francisco Quintana, & Henry Wu, 2011).

Unlike the well characterised Treg cells, Th3 cells do not express FoxP3 (Chien & Chiang, 2017b). They provide help for Immunoglobulin A (IgA) and are triggered in an antigen-specific mechanism but mediate bystander suppression of Th1 and Th2 responses in a non-antigen specific mechanism(Weiner, 2001b). Th3 cells can be induced from naïve $CD4^+$ T cells through TCR signalling by TGF-β in the presence of IL-10 and IL-4 (Jørgensen, Persson, & Hviid, 2019; Peterson, 2012) and thus can mediate suppression in the periphery in healthy individuals in a

TGF-β1 and IL-10-dependent manner (Chien & Chiang, 2017a; Gandhi et al., 2010). Th3 cells also maintain peripheral tolerance by driving the differentiation of Ag-specific $Foxp3^+$ Treg cells (Carrier, Yuan, Kuchroo, & Weiner, 2007).

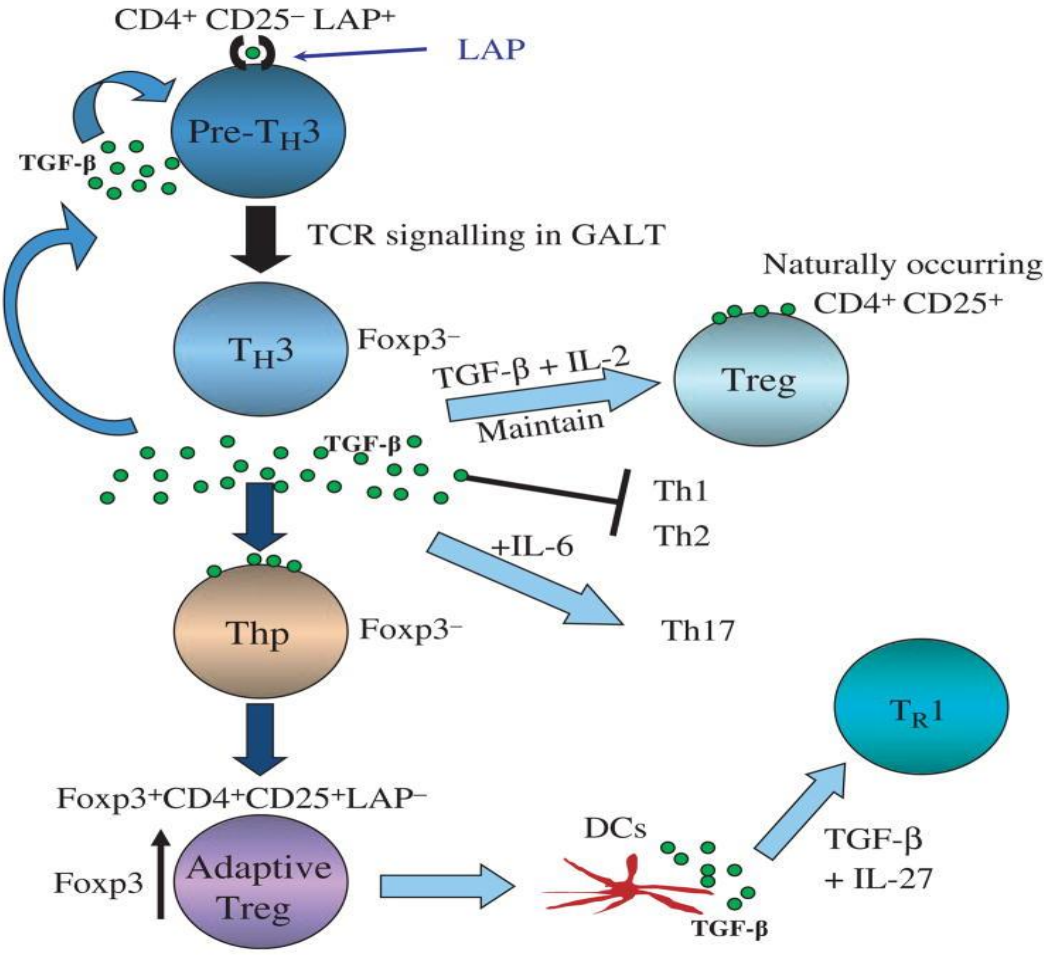


Figure 1.8: Cascade of induction of Th3 cells in oral tolerance

Th3 cells are $CD4^{+}CD25^{-}$Fox-$p3^{-}LAP^{+}$ cells triggered by oral antigens in the gut to secrete TGF-β which acts to maintain nTreg cells ($CD4^{+}CD25^{+}Foxp3^{+}$) while suppressing Th1 and Th2 responses. TGF-β from Th3 cells also acts on $CD4^{+}Foxp3^{-}$ cells and converts them to iTreg cells, which are $Foxp3^{+}CD25^{+}LAP^{-}$. (Adapter from Weiner, H.L., et al., *Oral tolerance.* Immunol Rev, 2011).(H. L. Weiner, A. P. da Cunha, F. Quintana, & H. Wu, 2011).

1.6: T Regulatory type 1 (Tr1) cells:

Roncarolo et al., reported the first study which led to the discovery of Tr1 cells (Roncarolo et al., 1988). They showed that a patient with Severe Combined Immunodeficiency (SCID) did not develop graft-versus-host disease (GvHD) during allogenic human leukocyte antigen (HLA) mismatched hematopoietic stem cell transplantation (HSCT) in the absence of immunosuppressive therapy (Roncarolo et al., 1988). Later, Bacchetta et al., reported that IL-10 producing $CD4^+$ T cells were able to prevent and downregulate undesired immune responses to pathogenic and non-pathogenic antigens which correlated with high IL-10 concentration *in vivo* and induction of IL-10 producing hypo-responsive $CD4^+$ T cells (Bacchetta et al., 1994). Following these observations as well as further observations by Groux et al., these IL-10 producing $CD4^+$ T cells with regulatory activity were named Tr1 cells (Groux et al., 1997).

Therefore, Tr1 cells were reported to be induced by chronic exposure to antigen in the presence of IL-10, suggesting they were not a differentiated population (Roncarolo, Gregori, Bacchetta, & Battaglia, 2014; Roncarolo, Gregori, Bacchetta, Battaglia, & Gagliani, 2018). However, other mechanisms have been reported for the induction of Tr1 cells, including costimulation of naïve $CD4^+$ T cells via CD55 (Sutavani et al., 2013), CD46 (Kemper et al., 2003) among others. IL-10^+TGF-β^+IL-5^+IL-4^-IL-2^{low} IFN-γ^{low}, has been reported as a unique cytokine

profile for Tr1 cells (Bacchetta et al., 2002; Roncarolo et al., 2018; Hanyu Zeng, Rong Zhang, Boquan Jin, & Lihua Chen, 2015) and they do not constitutively express the transcription factor, Foxp3, but upon activation, FoxP3 can be upregulated transiently to levels not as high as those observed for $CD4^{+}Foxp3^{+}$ regulatory T cells (Silvia Gregori & Roncarolo, 2018). Tr1 cells arise from naïve T cells precursors (*in vitro and in vivo*) upon stimulation and they play a key role in regulating adaptive immune responses both in mice and humans, mainly by secreting high levels of IL-10 (Y. Song, Wang, Chen, & Fang, 2021) and Granzyme B, which kills myeloid cells (Grazia Andolfi et al., 2012).

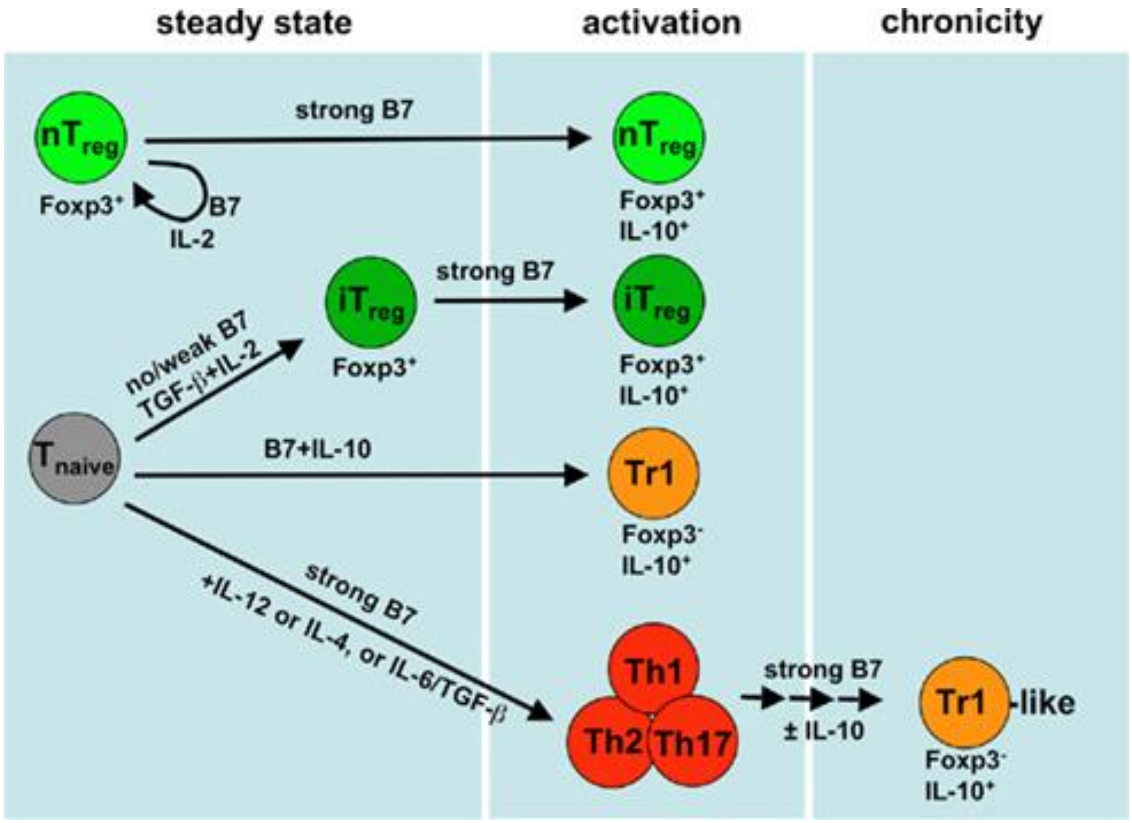


Figure 1.9: Generation and maintenance of different Treg subsets and their co-stimulation requirements

Generation of Tr1 cells from naive T cells requires costimulation and thymus derived Foxp3$^+$ nTreg cells require IL-2 and B7 (CD80/CD86) costimulation to persist. The conversion of iTreg cells from naive T cells into the Foxp3$^+$ subtype prefers the absence of B7 molecules but requires the presence of TGF-β and IL-2. Strong B7 costimulation favours the activation of both Foxp3$^+$ nTreg cells and Foxp3$^+$ iTreg cells such as provided by mature DCs. Arrows show events that require TCR signals in addition (Adapted from Pletinckx, K., et al., *Role of dendritic cell maturity/costimulation for generation, homeostasis, and suppressive activity of regulatory T cells.* Front Immunol, 2011) (Pletinckx, Dohler, Pavlovic, & Lutz, 2011).

1.6.1: Biomarkers for Tr1 Cells:

Ever since their discovery, Tr1 cells have not been fully characterized due to lack of specific biomarkers, thereby limiting their study and clinical applications.

Table 1.2 Markers commonly associated with Tr1 cells in human

Markers	**References**
CD49b (α2 integrin)	Gagliani et al., 2013
Programmed Death 1 (PD-1)	Sharpe, Wherry, Ahmed, & Freeman, 2007
Latency Associated Peptide (LAP)	Scurr et al., 2014a
CD226 (DNAX Accessory Molecule-1DNAM-1)	J. Chen & X. Liu, 2009
Lymphocyte Activation Gene 3 (LAG-3)	Gagliani et al., 2013, Roncarolo, Gregori, Bacchetta, Battaglia, & Gagliani, 2018
Cytotoxic T lymphocyte-antigen-4 (CTLA-4)	Hosseini, Gharibi, Marofi, Babaloo, & Baradaran, 2020; Mitsuiki, Schwab, & Grimbacher, 2019
T-cell Immunoglobulin and Mucin-domain containing-3 (TIM-3)	White & Wraith, 2016

Different groups have put forward various markers including CD49b, LAG-3; a CD4 homolog that binds with high affinity to MHC class II molecules) and CD226. Others have reported TIM-3, LAP, ICOS (Inducible T cell co-stimulator), a receptor expressed only on activated

T cells, after either the TCR binds to MHC-antigen complex or CD28 binds to B7, and PD-1, but none of these biomarkers are exclusive to Tr1 cells as they are also expressed by other cell types (J. Chen & X. Liu, 2009; Gagliani et al., 2013; Gandhi et al., 2010; Magnani et al., 2011; Okamura, Fujio, Sumitomo, & Yamamoto, 2012; Roncarolo, Bacchetta, Bordignon, Narula, & Levings, 2001). While Maria-Grazia Roncarolo's group reported that IL-10 producing cells that co-expressed of CD49b and LAG-3 with CD226 were regarded as Tr1 cells(J. Chen & X. Liu, 2009), and Scurr et al., in his paper reported $LAP^{+}FOP3^{-}$ Tr1 cells as being the most potent immunosuppressive TILs in Colorectal cancer(Scurr et al., 2014a), David C. Wraith's group considered TIM 3 as one of the possible markers, but concluded that none of these markers could be considered ideal candidates as cells surface biomarkers for Tr1-like cells(White & Wraith, 2016)

1.6.2: Transcription Factors commonly associated with Tr1 cells

Tr1 cells have not been fully characterised as a unique master regulator is yet to be identified. However, many studies have identified transcription factors associated with regulatory T cell functions play important role in the regulation of Tr1 cells, including c-MAF (Xu et al., 2009), aryl hydrocarbon receptor (AhR) (Apetoh et al., 2010), FoxP3 (Bending et al., 2018), among others. Broad overview of potential

transcription factors is given and specific details on Tr1 markers are captured in Chapter 6.

c-MAF, a basic leucine zipper (bZIP) transcription factor is a member of the AP-1 superfamily (Imbratta, Hussein, Andris, & Verdeil, 2020). Initially characterized as transcription factor for Th2 cells, the role of c-MAF in immune regulation has been reported among many immune cell types including. innate immune cells (Parker et al., 2020), γδ T cells (Zuberbuehler et al., 2019) and B cells(M. Liu et al., 2018). c-MAF is reported to transactivate IL-10 promoter(Xu et al., 2009) and interacts with aryl hydrocarbon receptor (AhR) to promote the secretion of IL-10 and IL-21 (Apetoh et al., 2010), and is involved in Tr1 differentiation (Barroso, Mahler, Fonseca-Castro, & Quintana, 2021; Imbratta et al., 2020).

Foxp3 expression is used to identify nTreg cells in mice (Hori, Nomura, & Sakaguchi, 2003) and in humans (W. Liu et al., 2006; Miyara et al., 2009), expression of which is reported to be crucial for Treg cells stability and function and mutation of the FoxP3 gene mutation has been reported to cause autoimmune diseases (Bennett et al., 2001; Colamatteo et al., 2020). Upon activation of T cells, expression of FoxP3 could represent a negative immune regulation, however, in humans FoxP3 expression alone is not sufficient to establish a Treg cell phenotype as non-Treg cells also transiently express FoxP3(Sugimoto et al., 2006). The transcriptional events following FoxP3 expression

stabilizes FoxP3 gene, consolidates Treg cell differentiation and function (Bending et al., 2018) which therefore indicates that transient expression of Foxp3 by Tr1 cells is not a prerequisite for suppressive functions(W. Lin et al., 2007; J. Wang, Ioan-Facsinay, van der Voort, Huizinga, & Toes, 2007).

T-box transcription factor TBX21 (T-bet) is a Th1 master transcription factor for Th1 cells and is thought to inhibit the polarization of other $CD4^+$ T cell subsets such as Th2 or Th17 cells(E. S. Hwang, Szabo, Schwartzberg, & Glimcher, 2005). Th1 differentiation is driven by IL-12, which promotes the expression of IFN-γ and T Bet; whose expression correlates with IFN-γ expression in both Th1 and NK cells (Szabo et al., 2000). During acute viral infection, T-bet is reported to promote cell proliferation and inhibit apoptosis in differentiated T Follicular Helper T (TFH)(P. Wang et al., 2019). Initially thought to be expressed exclusively by Th1 cells, T Bet is now reported to be extensively expressed by both innate and adaptive immune cells during type 1 immune response (Kallies & Good-Jacobson, 2017) as well as regulatory T cells (Levine et al., 2017).

The Retinoic acid receptor-related orphan Receptor gamma t (RoRγ(t)) is the master transcription factor for IL-17 producing cells. It plays an important role in the development, maintenance, and function of Th17 cells (Imura et al., 2019). RoRγ (t) is a useful target in treating autoimmune conditions associated with Th17 gene mutations and

inhibits the apoptosis of undifferentiated T cells (Kurebayashi et al., 2000). It's regulatory role in thymopoiesis reduces apoptosis of thymocytes while promoting their differentiation into Th17 cells(Ivanov et al., 2006). However, although predominantly pro-inflammatory in nature, RoRγ (t) is reportedly expressed by a population of regulatory T cells in the colon (Hegazy & Powrie, 2015).

GATA-3 is a master regulator selectively induced early in Th2 development (Zheng & Flavell, 1997). It is highly expressed in naive, freshly activated T cells, induced by IL-4 through Stat6, and is inhibited by IFN-γ and IL-12 through Stat1 and Stat4 (Ouyang et al., 1998). GATA-3 expression autoactivates the endogenous GATA-3 gene which stabilizes the Th2 phenotype and increases the expression of Th2 cytokines(Ouyang et al., 2000). It controls Th2 development by inhibiting Th1 differentiation through the repression of the IL-12 receptor beta2-chain expression and augmenting its own expression via positive feedback autoregulation (M. Zhou & Ouyang, 2003).

Granzyme/perforin pathway represents the major mechanism by which cells of the innate (Natural killer cells) and adaptive (e.g cytotoxic T lymphocytes) immune systems mediate the killing of intracellular pathogens and tumour cells(Kam, Hudig, & Powers, 2000; Lieberman & Stroup, 2003). Tr1 cells have been reported to express granzyme B and its killing ability is not MHC/TCR-dependent; rather perforin-dependent (William J. Grossman et al., 2004; W. J. Grossman, J. W. Verbsky, B. L.

Tollefsen, et al., 2004). Recent studies show that Tr1 cells prevent the activation of antigen specific T cells by eliminating APCs of myeloid origin through the secretion of granzyme B and perforin (Magnani et al., 2011). Furthermore, Tr1 cells have been shown to kill myeloid APCs by expressing CD226 and granzyme B(S. Gregori, K. S. Goudy, & M. G. Roncarolo, 2012).

1.7: T cell plasticity:

$CD4^+$ T cells also play critical roles in the pathogenesis of autoimmunity, asthma, allergy and in cancer (Angkasekwinai & Dong, 2021; Borst, Ahrends, Bąbała, Melief, & Kastenmüller, 2018; Brandt & Hedrich, 2018; Khan & Ghazanfar, 2018; Seumois et al., 2020). The concept of distinct T helper (Th) cell lineages has been a useful paradigm for the conceptualization of $CD4^+$ T cell differentiation. For instance, T helper cells were initially classed into Type 1 T helper cells (Th1) and type 2 helper T cells (Th2) based on cytokine profile and secreted proteins. While Th1 cells produced IL-2, IFN-γ and GM-CSF, in response to APCs; Th2 cells produced IL-4 (Mosmann, Cherwinski, Bond, Giedlin, & Coffman, 1986). It was thought that selective cytokine production induced by overexpression of lineage specific 'master transcription factors' was important for the proper elimination of microbial pathogens, intracellular microbes for Th1 cells and helminths for Th2 cells (Abbas, Murphy, & Sher, 1996; C. Dong & Flavell, 2000). This concept of stable, terminally differentiated Th cells made sense. However, there is increasing body of evidence that differentiated

$CD4^+$ T cell populations can alter their phenotype depending on environmental cues or epigenetic modifications, hence the idea of potential lineage plasticity of T cell subsets (Cosmi, Maggi, Santarlasci, Liotta, & Annunziato, 2014; Künzli et al., 2020; J. J. O'Shea & W. E. Paul, 2010). The prospect of lineage commitment versus flexible programs for T cells has direct implications for disease pathogenesis and success of therapeutic interventions in autoimmune conditions, allergic inflammatory disorders, and cancers. Interestingly, several studies have emerged to suggest that Tr1 cells may represent a developmental endpoint of several T helper cell differentiation pathways and not a separate lineage of $CD4^+$ T cells (Ankathatti Munegowda, Xu, Freywald, & Xiang, 2012; Cano-Gamez et al., 2020; Gagliani et al., 2015; McGinty, Brittain, & Kenna, 2020; Vasanthakumar & Kallies, 2013). These are *in vitro* observations and whether these occur *in vivo* remains unclear.

1.8: Homeostatic regulation of T cell responses:

Balance of the effector T cells (Teff) and regulatory T cells (Treg) determines peripheral self-tolerance. Self-antigens are thought to control the balance between the effector and regulatory T cells indicating that imbalance in the Teff and Treg ratio would result in several immune disorders including autoimmunity, allergy, microbial immunity, tumour immunity, GvHD, etc.,(Gratz et al., 2014; Sakaguchi, 2005). For instance, immune over-reaction (sustained inflammatory responses by Teff) could result in auto-immune diseases like multiple sclerosis, Type1 diabetes, rheumatoid arthritis, etc., while immune under reaction (sustained anti-inflammatory responses by Tregs) result in cancer and microbial immunity as depicted in figure 1:10 below. It is becoming more obvious that immune regulation occurs in the peripheral tissues and not the lymphoid organs, thereby underscoring the importance of the balance in Teff and Treg (C. R. Li, Deiro, Godebu, & Bradley, 2011; Rosenblum, Gratz, Paw, & Abbas, 2012)

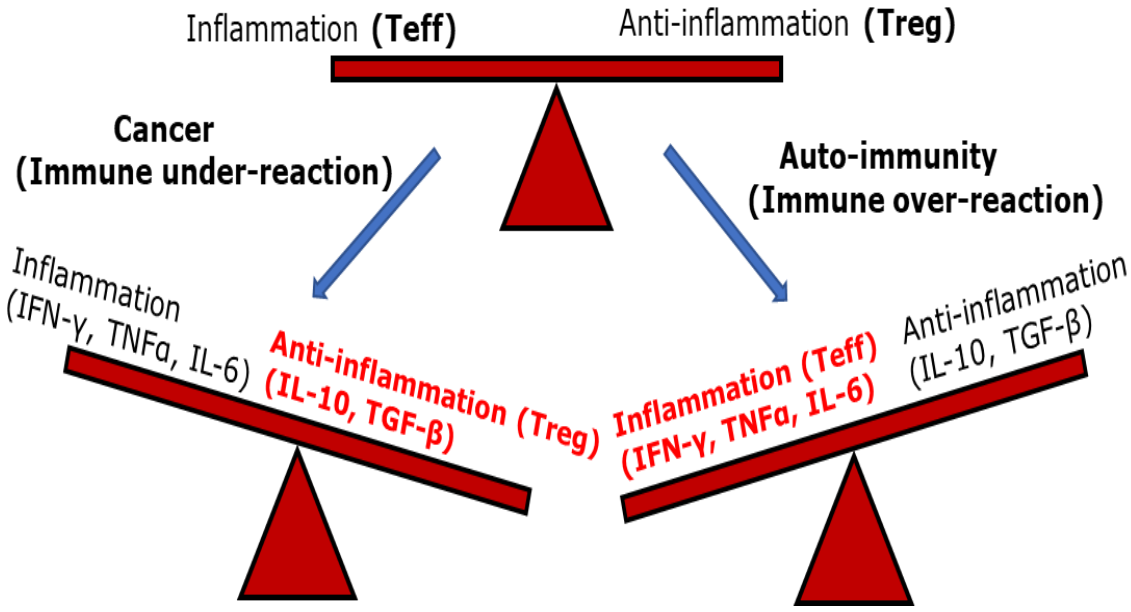


Figure 1.10: **Balanced immune system=optimal effectiveness**

The immune system exists in a state of balance between pro-inflammatory cells that attack invading pathogens and regulatory cells that prevent autoimmunity. Both inflammatory and anti-inflammatory reactions are normal components of the same immune response, which co-ordinately fight infections while preventing immune pathology. In some cancers (Immune under-reaction) the immune response becomes more anti-inflammatory due to secretion of suppressive cytokines including IL-10 and TGF-β while in autoimmune conditions (immune overreactions), the balance is tipped towards a more pro-inflammatory response by effector cytokines including IFN-γ, TNFα and IL-6.

1.9: Memory T Cells:

One of the characteristic features of the adaptive immune system is immunological memory thus, memory T cells are very essential in peripheral immunity against specific infections. They are long-lived memory cells that may be re-stimulated following antigen presentation by APCs but are maintained independent of costimulation or possess minimal costimulatory requirements (Gasper, Tejera, & Suresh, 2014; B. V. Kumar, Connors, & Farber, 2018; Soon, Engel, Lee, & Haque, 2019). Memory T cells can traffic more rapidly through secondary lymphoid organs and peripheral tissues, areas poorly accessed by naïve or effector T cells(Pennock et al., 2013). Memory T cells are specific for a particular antigen and are functionally enhanced to respond more quickly and effectively to re-infection by a specific antigen (Farber, Netea, Radbruch, Rajewsky, & Zinkernagel, 2016). Memory T-cells (for both CD4 and CD8) are described based on phenotype and their functional and trafficking capabilities as Central memory (T_{CM}) or Effector memory (T_{EM}) (Geginat, Lanzavecchia, & Sallusto, 2003; Jameson & Masopust, 2009; Opata & Stephens, 2013; Wherry et al., 2007).

T Central Memory (TCM):

T_{CM} cells are $CD3^{+}CD62L^{+}CCR7^{+}CD45RA^{-}CD45RO^{+,}$ expressing high levels of IL-7 receptor (CD127), high levels of CD44 (adhesion marker)

and display high proliferative potential upon antigen reencounter and home to secondary lymphoid organs.

T Effector Memory (TEM):

T_{EM} cells are $CD3^{+}CD62L^{-}CCR7^{-}CD45RA^{+}CD45RO^{-}$, expressing low levels of CD127 with fast cytokines (granzyme B and IFN-γ) production and home to inflamed tissues(Pennock et al., 2013). Taken together, T_{CM} and T_{EM} cells are regarded as the 'first responders' at the site of reinfection by an antigen.

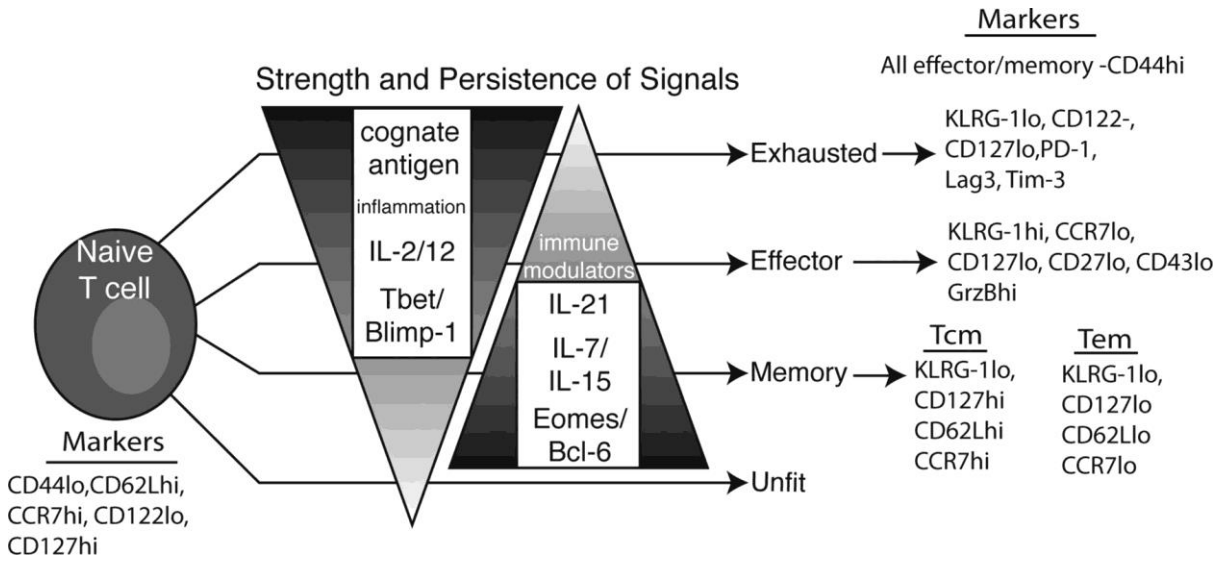


Figure 1.11: Different phenotypes of memory T cells in response to stimulation

Several environmental queues including cytokine milieu, influence the transcriptional events within T cells. Generally, IL-7/15/21 and Eomes/Bcl-6 are considered to favour memory generation. In contrast, IL-2/12 and T-bet/Blimp-1 favour terminal differentiation and effector cells. Exhaustion can result when T cells experience these factors too intensely or for too long. A proper balancing of all these factors will lead to long-lived, protective memory. Cellular markers are given on the right. PD-1, programmed cell death-1; Tim-3, T cell Ig mucin-3; KLRG-1, killer cell lectin-like receptor subfamily G member 1; LAG-3, lymphocyte activation gene 3 (Adapted from Pennock, N.D., et al., *T cell responses: naive to memory and everything in between.* Adv Physiol Educ, 2013) (Pennock et al., 2013).

1.10: Relationship of CD55 signalling to T cell activation and IL-10 secretion:

Activation of naive T cells occurs through stimulation of their TCRs by antigen/MHC complexes expressed by antigen-presenting cells or by receptor crosslinking. CD3 triggering transmits signals through immunoreceptor tyrosine-based activation motifs (ITAMs) (Letourneur & Klausner, 1992; Samelson, Patel, Weissman, Harford, & Klausner, 1986; Sugie, Jeon, & Grey, 2004) and TCR engagement triggers the activation and recruitment of protein tyrosine kinases (PTKs), including Src family kinases (SFKs), Lck and Fyn and the Syk family PTK zeta chain of TCR-associated protein 70 (ZAP-70), leading to the activation of Linker for activation of T cells (LAT) (Parolini, Sargiacomo, Lisanti, & Peschle, 1996; W. Zhang, Sloan-Lancaster, Kitchen, Trible, & Samelson, 1998). These events are amplified by signalling via environmental cues, CD55 or other costimulatory receptors, resulting in T cell activation and acquisition of effectors functions, including cytokine secretion(J. R. Hwang, Byeon, Kim, & Park, 2020; Parolini et al., 1996).

CD55, a GPI-anchored protein, has been reported to transduce signals into human T cells (L. S. Davis, Patel, Atkinson, & Lipsky, 1988b). Despite the lack of intracellular domains, cross-linking of glycosylphosphatidylinositol (GPI)-anchored proteins extracellularly, brings the SFKs together intracellularly, leading to phosphorylation of protein substrates and the triggering of signalling cascades within the

microdomains of the lipid rafts (Horejsi et al., 1999; Stefanová, Horejsí, Ansotegui, Knapp, & Stockinger, 1991). Lipid rafts function as multimolecular platforms where GPI- anchored proteins as well as scaffold proteins including caveolins and flotillins (Michel & Bakovic, 2007) interact to develop different physiological functions and therefore serve as efficient platforms for rapid signal transduction(Allen, Halverson-Tamboli, & Rasenick, 2007; Dart, 2010; Mesa-Herrera, Taoro-González, Valdés-Baizabal, Diaz, & Marín, 2019; Pike, 2003).

Therefore, CD3/CD55 costimulation of T cells leads to the phosphorylation of the protein tyrosine kinases $p56^{lck}$ and $p59^{fyn}$ and subsequently, ZAP-70 and the activation of other downstream signalling molecules leading to the production of effector cytokines, including interleukin 10 (IL-10) (Shenoy-Scaria et al., 1992a). Capasso, M., et al. reported that costimulation of human $CD4^+$ T cells was mediated by CD97 (Capasso et al., 2006b) and Sutavani, R.V., et al., reported that CD55 costimulation of naïve $CD4^+$ T cells induced a discret population of high IL-10 producing (Tr1) cells (Sutavani et al., 2013). However, the downstream signalling events that regulate IL-10 secretion by Tr1-like cells via CD55 signalling remains unknown.

1.11: Hypothesis:

Current definitions of Tr1 cells are based on a range of *in vitro* manipulations that induce IL-10 production from T cells. This raises the question of whether Tr1 cells are a differentiated population or are T cells responding to environmental cues. The study hypothesis was that Tr1 cells exist in human peripheral blood as a distinct and discrete population that respond specifically in producing only IL-10.

Aims:

The aim of this thesis was to examine the peripheral blood $CD4^+$ T cell pool for the existence of IL-10 producing cells. These will be characterised for the differentiation markers associated with well-defined T cell populations. The $IL\text{-}10^+$ cells will also be characterised for the markers associated with previously reported Tr1 cells and further characterised for transcription factors commonly associated with defined phenotypes.

Chapter 2

Materials and Methods

2.1: Ethical approval and Blood Donors

Blood donors recruited for this study provided written informed consent and blood was taken in accordance with the Nottingham Regional Ethics Committee approval for study ID: NB 161-1711; Title of Study: Using human blood from normal donors for the study of immune responses.

2.2: T cells isolation

2.2.1: Isolation of Peripheral Blood Mononuclear Cells (PBMCs) from whole blood

Cells isolated from fresh blood were used for this study! Blood was taken from the peripheral circulation of donors into heparinised tubes to prevent blood clotting. Isolation of PBMCs was done by sterile-filtered Histopaque®-1077 (Product code: 10771, Sigma Aldrich) density gradient centrifugation following an optimised isolation protocol. Blood was diluted with sterile Phosphate Buffer Saline (PBS; Product code: D8537- Sigma Aldrich) in a 2:1 ratio and 35mL of the diluted blood layered on 15mL of Histopaque®-1077 in a 50mL conical tube. Centrifugation was done at 800g for 25 minutes at 21^0C, with acceleration set at 9 and deceleration, 1. The PBMC layer (white layer at the interface of Histopaque and plasma) was collected using a sterile pipette and cells were resuspended in excess volume of sterile, room temperature PBS and washed twice at 300g for 10 minutes.

After discarding supernatant from the second wash, cells pellet was disrupted, and cells resuspended in cold MACs buffer (DPBS + 2mM EDTA+ 1% Fetal Bovine Serum). Number of isolated PBMCs was determined using haemocytometer as described below (2.2.2). PBMCs were either used for isolation of naïve $CD4^+$ T cells (described in 2.2.3) or total $CD4^+$ T cells 2.2.4.

2.2.2: Cell count

Cell number was determined by sampling 10µl of cell suspension and mixed with an equal volume of trypan blue stain, to discriminate between live and dead cells. Cells were transferred onto the haemocytometer chamber and only live cells in four quadrants were counted. Total cell count was determined as per the equation below.

Total cell count = $(\text{Total number of cells counted})/4)$ X Dilution factor for Trypan Blue X 10^4 X Volume of cell suspension

2.2.3: Isolation of $CD4^+$ T cells

Positive selection with CD4 microbeads (Human; Product code:130-045-101; Miltenyi Biotec) was used to isolate total $CD4^+$ T cells from PBMCs, following recommended protocol.

Briefly, PBMC count was determined and CD4 microbeads (20µl microbeads per 1 x 10^7 cells in 80µl cold MACS buffer) were added to the cell suspension, mixed gently, and incubated for 15 minutes at 4^0C.Thereafter, cells were washed with cold MACS buffer (1 – 2 ml per 1 x 10^7 cells) by centrifugation at 300g for 10 minutes at room temperature. During centrifugation, a cell isolation column (MS or LS column depending on expected cell number) was prepared by placing column unto a magnet and rinsing with cold MACS buffer (MS column=500µl, LS=3mL). After the wash supernatant was discarded, cell pellet disrupted, and cells resuspended in cold MACS buffer (1 x 10^8 cells per 500µl MACS buffer). Cell suspension was applied to the column and the column was rinsed three times, each time after the column was empty, using the recommended volume of cold MACS buffer (MS=500µl; LS=3 mL). The microbead-conjugated antibody bound $CD4^+$ T cells (target cells) would remain in the column while and the unbound cells (non-target) cells would flow through. After the third wash and once column was clear of fluid, the column was removed from the magnetic field, 5 mL cold MACS buffer added and $CD4^+$ T cells eluted into a 15mL tube using a plunger. Enriched cells were washed at

300g for 10minutes and resuspended in T cell medium. The total number of isolated $CD4^+$ T cells was determined as described (in 2.2.2). A small sample of cells was used for purity assessment on the Flow cytometer for co-expression of CD3 and CD4 and only cells that were ≥97% pure were used for all experiments.

2.2.4: Isolation of naïve $CD4^+$ T cells

Naïve $CD4^+$ T cells were isolated from PBMCs by negative selection using the Naïve $CD4^+$ T Cell Isolation Kit II, human (Product code: 130-094-131, Miltenyi Biotec) and following the recommended protocol.

PBMC count was determined, and cells were mixed with the antibody – biotin cocktail that binds to all cell types in the PBMC pool except for the naïve $CD4^+$ $CD45RA^+$ cells (10µl cocktail per 1 x 10^7 cells in 40µl cold MACS buffer). Cells were incubated at 4°C for 5 minutes then 30µL of cold MACS buffer per 10^7 cells was added and 20µL per 10^7 cells of microbead cocktail II was added to cells and incubated for 10 minutes at 4°C. While cells were incubating, cell isolation column (MS or LS column depending on the number of expected cells) was prepared by placing column unto a magnet and rinsing with cold MACS buffer (MS column=500µl, LS=3mL). Cell suspension was put through the column and rinsed three times with appropriate volume of MACS buffer. The flow through from the column was collected containing unlabelled, enriched $CD45RA^+CD45RO^-$ naïve $CD4^+$ T cells. The cells were washed

with PBS at 300g for 10 minutes and resuspended in T cell medium and cultured. A small portion of cell suspension was collected for cell count and purity of isolated cells was done by Flow Cytometry for marker expression $CD3^+CD4^+CD45RA^+CD45RO^-$ and only cells that were ≥97% pure were used for all experiments.

2.3: Cell culture and stimulation

2.3.1: T Cell culture medium

T Cell Medium: RPMI-1640 + 10% heat inactivated FBS + 1% Non-essential Amino acid + 1% L-Glutamine + 1% Penicillin-Streptomycin + 1% Sodium pyruvate + 2% HEPES buffer (all from Sigma-Aldrich), was used in all cell culture experiments. The T Cell Medium was supplemented with 20IU/ml rhIL-2 (Chiron) in all cell cultures experiments.

2.3.2: Pre-coating cell culture plates with stimulatory antibodies

Cells were cultured in either high binding 96-well plate (Corning®-3361) or 48-well plate (Corning® - 3548), pre-coated with stimulatory antibodies, lid sealed with parafilm and left overnight at 4°C.

The stimulatory antibodies were anti-CD3 antibody (Clone–OKT3, Mouse IgG2a, in-house), anti-CD55 antibody (Clone:791T/36, Mouse IgG2b, in-house) and Anti-CD28 antibody (either Clone: CD28.2, Mouse IgG1, BD Biosciences or Clone: YTH913.2, Rat IgG2b, in-house) and CD97Fc (in house). Optimal concentration of antibody was determined by

antibody titration. Anti-CD3 antibody was used at sub-optimal concentration of 1μg/mL, while both anti-CD55 and anti-CD28 antibody was used at 5μg/mL while 2μg/mL of CD97Fc was used. All monoclonal antibodies (mAbs)/fusion protein were diluted in sterile, room temperature PBS for coating plates and final volume of mAbs in PBS was 200μl/well for a 96 well plate and 1mL/well for a 48 well plate. The PBS and any unbound mAbs were aspirated off immediately before adding on cells suspension.

2.3.3: Cell cultures

Cells were resuspended at 1 x 10^6 cells/mL in TCM containing 20IU/mL rhIL-2. In all cell culture experiments, 1 x 10^5 cells in 200μl cell suspension/well in 96 well plate and at 5 x 10^5 cells in 1mL cell suspension/well in 48 well plate, was used.

2.3.4: T cells stimulation:

Following isolation from PBMCs, T cells were rested overnight in TCM supplemented with 20IU/ml rhIL-2 at 37°C, 5% CO_2 in the incubator. The following day, cells were stimulated with a combination of plate-bound antibodies: anti-CD3 alone, anti-CD3/anti-CD28, anti-CD3/anti-CD55 or anti-CD3/CD97Fc. For experiments involving Phorbol 12-myristate 13-acetate (PMA: P8139, Sigma-Aldrich) and Ionomycin (407952-IMG, Sigma-Aldrich), 100ng/ml and 2μg/ml respectively, was added directly to cell culture for the stimulation of T cells.

Naïve $CD4^+$ T cells were stimulated with plate – bound anti-CD3/anti-CD55 or anti-CD3/anti-CD28 antibodies for 72 hours (three days) at 37°C, 5% CO_2 in the incubator. On Day 3, cytokine secretion assay (2.9.2) was performed to evaluate cytokine production as well as characterisation of cells phenotype.

Total $CD4^+$ T cells were stimulated with plate–bound anti-CD3 alone, anti-CD3/anti-CD55, anti-CD3/anti-CD28, anti-CD3/CD97Fc or PMA/I for 24 hours at 37°C, 5% CO_2 in the incubator. Usually, for experiments involving protein kinase inhibitor (PKI), $CD4^+$ T cells were pre-treated with optimised concentration of the respective PKI for 30 minutes before being stimulated (in the presence of PKI) for either 24 or 72 hours (in the case of CellTrace Violet proliferation assay. 2.6.2) at 37°C, 5% CO_2 in the incubator. After stimulation, cells were evaluated for cytokines as well as markers expression.

2.4: Generation of CD97 fusion protein

2.4.1: Transfection of plasmid DNA:

Expi293F™ Cells were used for transient transfection following an amended protocol for ExpiFectamine™ 293 Transfection Kit (Cat no: A14525, Gibco™).

On the day before transfection, number of viable cells was determined, and 2 x 10^6 viable cells/mL were seeded in Expi293™ Expression

Medium (Cat. no. A14351, Gibco™) and incubated at 37°C in a humidified atmosphere of 8% CO_2 on an orbital shaker rotating at 125 rpm (32g). On the day of transfection, the number of viable cells was determined by trypan blue dye exclusion method (2.2.2) and transfection experiment was performed with cells having ≥95% viability. In this experiment, 2 x 10^8 viable cells were suspended in 100mL Expi293™ Expression Medium for a 100mL transfection. Cell suspension was added to a sterile, disposable 250mL (product code, CLS431144 Corning®) Erlenmeyer shaker flask and then brought up to 85mL by adding fresh, pre-warmed Expi293™ Expression Medium and cells were then returned to the incubator.

CD97 plasmid DNA for transfection was clean, sterile, and free from phenol and sodium chloride. For a 100mL transfection, CD97 plasmid was prepared as follows. 100µg of plasmid DNA was diluted in Opti-MEM® I Reduced Serum Medium (Cat. no. 31985-062, Gibco™) to a total volume of 5mL and was mixed gently. 270µL of ExpiFectamine™ 293 Reagent was diluted in Opti-MEM® I medium to a total volume of 5mL. The setup was mixed gently and incubated for 5 minutes at room temperature (longer incubation times may result in decreased activity). After 5 minute of incubation, diluted CD97 plasmid DNA was added to the diluted ExpiFectamine™ 293 Reagent to obtain a total volume of 10mL. The setup was mixed gently, then incubated for 30 minutes at room temperature to allow the DNAExpiFectamine™ 293 Reagent complex to form. Thereafter, 10mL of the DNA-ExpiFectamine™ 293

Reagent complex was added to cells. Cells were incubated for 20 hours in a 37°C incubator with a humidified atmosphere of 8% CO_2 in air on an orbital shaker rotating at 125 rpm. After 20 hours incubation, 500µL of ExpiFectamine™ 293 Transfection Enhancer 1 was pre-mixed with 5mL of ExpiFectamine™ 293 Transfection Enhancer 2 and added to the flask containing cells to give the final volume of approximately 100mL in 250mL flask.

After 7 days post-transfection, the flask containing cells was taken out of the incubator, and its content emptied into 50mL falcon tubes and centrifuged at 2500 rpm for 10 mins (2x). Supernatant was decanted into Whatman filter paper in a glass funnel inserted into a collection flask, which was then filter-sterilized using 'Stericup' and 'Sterifilter' aided by a vacuum plug. The sterile supernatant was assayed for CD97 fusion protein.

2.4.2: Purification

CD97 fusion protein was purified by Fast protein liquid chromatography (FPLC), Protein G column and sample eluted at absorbance A_{280}. Sample was pooled, neutralized and dialysed against 10mM Tris-HCl (pH 8.0) buffer overnight at room temperature. Further dialysis was done in 2 litres of 150mM NaCl, 10mM $CaCl_2$ and 10mM Tris HCl buffer for seven hours.

2.4.3: Validation:

Samples were run on a standard 10% SDS-PAGE gel under either reducing or non-reducing conditions and stained with Coomassie blue dye to determine approximate size and purity of the recovered protein.

N/B: Mireille Vankemmelbeke provided technical assistance and Thomas Kirk helped on the laboratory bench during the generation of the CD97 fusion protein.

2.5: Protein Kinase inhibitors (PKIs):

2.5.1: Source:

Protein kinase inhibitors used in cell culture; InSolution™ RSK Inhibitor, SL-0101 – Calbiochem (559292, Sigma-Aldrich), MK2 inhibitor PF-3644022 (PZ0188**,** Sigma-Aldrich), MSK-1 inhibitor SB-747651A dihydrochloride (Cat. No. 4630, Tocris Bioscience), MEK inhibitor PD-184352 (Cat. No. 4237, Tocris Bioscience), and p38 MAPK inhibitor VX-745 (Cat. No. 3915, Tocris Bioscience). p38 MAPK inhibitors BIRB-0796 (Doramapimod) and SB/GW – 681323 (Dilmapimod) were kindly provided by Dr Andrew Jackson of the Biodiscovery Institute, University of Nottingham. All PKIs were diluted to stock using Dimethyl Sulfoxide (DMSO, CAS No: 67685, sigma Aldrich).

2.5.2: Serial Dilution:

PKIs were diluted to stock (10mM) in sterile DMSO and stored at -20°C in 200µl aliquots. Working concentration of PKIs were freshly prepared on the day of experiments and immediately added to cells. For the assays, 100µM PKI was prepared in 1mL by adding 10µL of stock (10mM) to 990 µL T cell medium. Two times (2x) working concentration of 10µM was prepared in 1mL by adding 200µL of 100µM new stock to 800µL T Cell Medium, 100µL to 900 TCM (for 5 µM) and 60µL to 940µL (for 3µM) followed by 1:10 serial dilution to lower concentrations (1, 0.5 and 0.3 µM) after which, 100µL PKI was added to 100µL cell suspension for the respective conditions.

Treatment of $CD4^+$ T cells with PKIs was used to evaluate the possible pathways of IL-10 production by Tr1-like cells following costimulation with anti-CD3/anti-CD55.

2.6: Cell proliferation assays

2.6.1: Tritiated thymidine uptake

Naïve or total $CD4^+$ T cells at 1 x 10^5 cells/well in 96-well plate, were stimulated with the various plate-bound costimulatory pairs for 72 hours at 37°C, 5% CO_2 in the incubator. Cells were pulsed with 0.5 µCi of [^{3}H] thymidine (Cat No: 17130601, Amersham Biosciences) for the final 15 hours, harvested on a 96 well plate Harvester (Filtermate 196; Packard; PerkinElmer) and the incorporated radioactivity was measured using a TopCount (Packard; PerkinElmer).

2.6.2: Labelling of $CD4^+$ T cells with CellTrace™ Violet (CTV) dye

$CD4^+$ T cells were stained with CTV dye (C34557, ThermoFisher Scientific) and proliferation measured by flow cytometry following costimulation of cells for 72 hours (3 days). CTV stock solution was prepared by adding 20µL of sterile DMSO per vial and gently mixing to obtain 5mM. CTV stock solution was then diluted into 20mL of PBS (warmed to 37°C) to obtain a 5 µM working concentration. The

experimental set up was covered with foil to protected it from direct sunlight. $CD4^+$ T cells were washed at 300g for 10 minutes with excess sterile PBS per 1.0 x 10^6 cells to remove any FBS present in cell isolation buffer. After the wash, supernatant was discarded, cell pellet disrupted, and cells resuspended in 1mL CTV per 1.0 x 10^6 cells.

This was mixed gently and incubated at 37°C (in water bath or incubator) in the dark for 20 minutes and tubes inverted at least every 5 mins. 40 mL of pre-warmed T Cell Medium was added to cells to quench unbound dye. Cells set up was then incubated in the dark for 5 mins. After incubation, cells were washed at 300g for 10 minutes, supernatant discarded, cell pellet disrupted, and cells resuspended in pre-warmed T Cell Medium. An aliquot of cell suspension was taken for Flowcytometry analysis to ascertain the uniformity of labelling. Cell count was determined, and appropriate number of cells used for the various proliferation experiments.

2.7: Capturing of cell culture images

Cell culture images were taken after 72 hours of cell stimulation. Images were captured at room temperature using the 10X objective lens of a Nikon Eclipse TS100 microscope (Nikon Instruments Europe B.V.), with a Nikon Digital Sight DS-Fi1 camera.

2.8: Flow cytometry

2.8.1: Extracellular staining

Extracellular staining was performed with fluorophore-conjugated anti-human antibodies matched against corresponding isotype control antibodies (Tables 2.1 and 2.2). Optimal antibody concentration was determined by antibody titration to obtain best staining with minimal background. In order to detect nonspecific staining, isotype binding controls were used in this study. However, Fluorescence Minus One (FMO) control could have helped set the upper gating boundaries for positive populations in the multicolour experiments. During optimisation for cytokine secretion assay with CD4 T cells, Fc receptor blocking was performed to block unwanted binding of antibodies. However, FcR blocking did not make any difference compared to test conditions without a blocking step as background non-specific binding were generally very low.

For staining other than by Cytokine Secretion Assay (CSA) or CTV, 1 x 10^5 cells/ sample were stained with relevant antibody combinations. Matched cell samples were used for isotype control. Cells were washed once with MACS buffer by centrifugation at 300g for 10 minutes. Cells were stained on ice with pre-determined optimum antibody dilutions in a final volume of 200µl and incubated at 4^0C for 15 minutes. Unbound antibody was washed off with excess MACS buffer by centrifugation at 300g for 10 minutes, after which cells were resuspended in 200µl of FACS buffer (PBS

+ 1% BSA) for immediate acquisition. Alternatively, cells were fixed with 200µl of 4% paraformaldehyde in PBS, wrapped in foil and stored at 4^0C for acquisition later and data were acquired on MACSQuant-10 (Miltenyi Biotec).

Extracellular staining involving markers associated with Tr1 cells was performed simultaneously with Cytokine Secretion Assay (CSA), in which case $5.0x10^5$ cells/sample was used. Cells were harvested into 15mL tubes after 24 hours of stimulation and CSA was performed following the standard protocol (described in 2.9.1) until the step where the detection antibody was added to cells to determine cytokine production. At this stage, the antibodies specific for various cell surface markers were added along with the detection antibodies for CSA and cells incubated at 4°C for 15 minutes. After incubation, cells were washed with MACS buffer by centrifugation at 300g for 10 minutes, resuspended in 200µl of FACS buffer for immediate acquisition or fixed with 200µl of 4% paraformaldehyde in PBS, wrapped in foil and stored at 4^0C for acquisition on a MACSQuant-10 later. Panels for extracellular staining are shown in Tables 2.1 and 2.2.

Table 2.1 Panel for surface marker staining

(Compensated for four colour flow cytometry staining)

Marker	**clone**	**Fluorochrome**	**Manufacturer**
CD62L	145/15	VioBlue	Miltenyi Biotec
CD45RO	UCHL1	PE	Miltenyi Biotec
IL-10	CSA kit	APC	Miltenyi Biotec
IFN-γ	CSA kit	FITC	Miltenyi Biotec
CD3	BW264/56	VioBlue	Miltenyi Biotec
CD4	VIT4	APC	Miltenyi Biotec
CD45RA	T6D11	FITC	Miltenyi Biotec
Isotype control (CD62L)	Mouse IgG1k (IS5-21F5)	VioBlue	Miltenyi Biotec
Isotype control (CD45RO)	Mouse IgG2ak (S43.10)	PE	Miltenyi Biotec
Isotype control (CD3)	Mouse IgG2ak (S43.10)	VioBlue	Miltenyi Biotec
Isotype control (CD4)	Mouse IgG2ak (S43.10)	APC	Miltenyi Biotec
Isotype control (CD45RA)	Mouse IgG2b (IS6-11E5.11)	FITC	Miltenyi Biotec

Table 2.2 Panel for surface marker staining

(Two colour flow cytometry with IL-10 APC/marker)

Marker	**clone**	**Fluorochrome**	**Manufacturer**
CD49b	AK7	FITC	BioLegend
PD-1	PD1.3.1.3	PE Vio-770	Miltenyi Biotec
Anti-LAP	CH6-17E5.1	VioBright™FITC	Miltenyi Biotec
CD226	DX11	PE	Miltenyi Biotec
LAG-3	17B4	FITC	Miltenyi Biotec
CTLA-4	BN13	PE	Miltenyi Biotec
Isotype control (CD49b)	Mouse IgG2a (S43.10)	FITC	Miltenyi Biotec
Isotype control (PD-1)	Mouse IgG2 b (IS6-11E5.11)	PE Vio-770	Miltenyi Biotec
Isotype control (LAP)	Mouse IgG1 k (IS5-21F5)	VioBright™FITC	Miltenyi Biotec
Isotype control (CD226)	Mouse IgG2a (S43.10)	PE	Miltenyi Biotec
Isotype control (CTLA-4)	Mouse IgG1 (IS5-21F5)	PE	Miltenyi Biotec
Isotype control (LAG-3)	Mouse IgG1 k (IS5-21F5)	FITC	Miltenyi Biotec

2.8.2: Intracellular staining

Intracellular staining was performed with fluorophore-conjugated anti-human antibodies matched against corresponding anti-mouse isotype control antibodies (Table 2.3).

Intracellular staining for IL-4 or transcription factors (TFs), was performed following CSA for IL-10 or IFN-ɣ expression using an intracellular staining kit (FoxP3 Staining Buffer Set, P130-093-142, Miltenyi Biotec). Cell pellet from the final CSA wash step was disrupted and resuspended in ~ 100µL of residual MACS buffer. 1mL of Fixation/Permeabilization buffer was added to tube and mixed using the pulse vortex (Stuart SA8 Vortex Mixer, Variable Speed, Product no.: LAB086) and cells incubated for 60 minutes at 4°C in the fridge and away from light. Following the incubation period, 2mL of 1X Permeabilization buffer was added to the setup and centrifugation performed at 500g (speed optimised for this step to minimise cell loss as there is usually a substantial cell density loss following permeabilization) for 10 minutes at room temperature. Supernatant was discarded, cell pellet disrupted, and cells resuspended in residual MACS buffer and stained for various TFs with fluorochrome-conjugated antibodies as listed in Table 2.3 below. Cells were then incubated for 45 minutes at room temperature in the dark. After incubation, 2mL of 1X Permeabilization buffer was added to tube and sample centrifuged at 500g for 10 minutes. After the final wash, cells were resuspended in

200µl of FACS buffer for immediate acquisition or fixed with 200µl of 4% paraformaldehyde in PBS, wrapped in foil and stored at 4^0C for acquisition on the flow cytometer later following the antibody panel in Table 2.3.

Table 2.3: Panel for intracellular staining (Two colour flow cytometry with IL-10 APC/marker)

Marker	Clone	Fluorochrome	Manufacturer
c-MAF	Sym0F1	PE	eBiosciences
IL-4	7A3-3	PE	Miltenyi Biotec
T-bet	REA102	PE	Miltenyi Biotec
RoRɣ(t)	REA278	PE	Miltenyi Biotec
FoxP3	REA1253	PE	Miltenyi Biotec
Helios	REA829	PE	Miltenyi Biotec
Granzyme B	REA226	FITC	Miltenyi Biotec
Perforin	REA1061	PE	Miltenyi Biotec
GATA-3	REA174	FITC	Miltenyi Biotec
Isotype control (c-MAF)	Mouse IgG2b k (eBMG2b)	PE	eBiosciences
Isotype control (IL-4)	Mouse IgG1 k (IS5-21F5)	PE	Miltenyi Biotec
Isotype control (REA PE Abs)	REA human IgG1 (REA293)	PE	Miltenyi Biotec
Isotype control (REA FITC Abs)	REA human IgG1 (REA293)	FITC	Miltenyi Biotec

2.9: Evaluation of cytokine secretion

2.9.1: Enzyme-linked Immunosorbent Assay (ELISA)

The presence of IL-10 and IFN-γ was assessed in cell culture supernatants harvested 24 hours after cell stimulation (unless otherwise stated). This was performed with Human IL-10 DuoSet ELISA (Product code: DY217B, R&D Systems) and Human IFN-γ ELISA Set (Product code: 555142, BD Biosciences) following the manufacturer's recommended protocol. High-binding ELISA plates (3590, Costar®) were coated with 100µl per well of the IL-10 or IFN-γ capture antibody as appropriate, diluted to the recommended concentration with PBS, and incubated overnight at 4°C.

The following day, plates were washed three times using 300µl/well washing buffer (0.05% Tween®20 in PBS, pH 7.2-7.4) and blocked with 300µl per well of reagent diluent (1% BSA in PBS) for 1 hour at room temperature. Plates were then washed three times using 300µl/well of washing buffer and diluted samples were added. Samples and provided standard were diluted in reagent diluent. Every plate had a seven point 1:2 serial dilution of the provided standard in reagent diluent, with a top concentration of 2000pg/mL. After adding samples and standard, plates were incubated for two hours, and afterwards unbound components were removed by washing (three times) using 300µl per well of washing buffer. The plates were then incubated for 2 hours at room temperature with 100µl per well of the biotinylated goat anti-human IL-10 detection

antibody, diluted to the recommended concentration with reagent diluent. The plates were washed three times using 300µl per well of washing buffer for each wash. The plates were incubated for 20 minutes at room temperature in the dark with 100µl per well of streptavidin-HRP, diluted to the recommended concentration with reagent diluent. The plates were washed three times using 300µl per well of washing buffer for each wash. The plates were developed with 100µl per well of TMB substrate (T0440-1L, Sigma Aldrich) solution, at room temperature in the dark for about 20 minutes. The reaction was stopped by adding 50µl per well of 2N sulphuric acid. The optical density (OD) for each well was read at 450nm using a microplate reader (Tecan). The ODs of the serially diluted standards were used to plot a standard curve and the equation of the standard curve was used to calculate the concentration of IL-10 secreted by cells in culture. Samples from the same experiments were analysed at the same time and the percentage Coefficient of Variabilities (CVs) for inter and intra assays were within acceptable range of < 15 and < 10, respectively. The manufacturer's protocol was also followed for IFN-γ ELISA using cell culture supernatant.

2.9.2: Cytokine Secretion Assay (CSA)

Evaluation of cytokine expression by stimulated cells was done using Cytokine Secretion Assay (CSA) and data acquired by Flow cytometry. IL-10 Secretion Assay Detection Kit -APC (Human, Product code: 130-090-761, Miltenyi Biotec) was used for IL-10 capture while IFN-γ Secretion Assay Detection Kit-FITC (Human, Product code: 130-090-433, Miltenyi Biotec) was used for IFN-γ capture. For single cytokine capture, the manufacturer's recommended protocol was followed. However, for dual (IL-10/IFN-γ) CSA, an optimised protocol was followed. CSA was performed 24 hours after cell stimulation (unless otherwise stated).

Cells were harvested into a 15mL tube and washed with 5mL cold MACS buffer (4°C) at 380g for 7 minutes. The supernatant was discarded, cell pellet disrupted, and cells resuspended in ~80μL of buffer. 40μL of cold TCM (containing 5% FBS) was added to the cells. The "Catch Reagent" (primary antibody) for IL-10 (10μL/test) and IFN-γ (7μL/test) were pre-mixed and total 17μL of catch reagent mix was added to each sample, mixed gently and incubated on ice (to prevent cytokine secretion) for 10 minutes. Cells were made to secrete cytokines by adding 5mL of warm TCM to each sample and placing tubes on a rotating platform (25rpm) at 37°C for 45 minutes. Then 10mL of cold MACS buffer was added to each tube and cells were washed at 300g for 10 minutes. After wash, the supernatant was discarded, cell pellet disrupted and fluorochrome-

conjugated Detection antibody (secondary ab) was added (7μL of IL-10 detection ab and 6μL of IFN-γ detection ab were pre-mixed for each sample), mixed gently and incubated at 4°C for 15 minutes. Unbound detection antibody was removed by adding 7mL of MACS buffer/ sample and washing cells at 300g for 10 minutes. After the wash, supernatant was discarded, cell pellet disrupted and cells resuspended in 200µl of FACS buffer for immediate acquisition or fixed with 200µl of 4% paraformaldehyde in PBS, wrapped in foil and stored at 4^0C for acquisition on the flow cytometer later. There was no significant difference in marker expression if samples were run immediately through flow cytometer or fixed, although some background was observed in some cases with fixed samples, and this was excluded on the forward (FSC) and side (SSC) scatter during sample acquisition.

Matched cells from the same donor were maintained as negative control, in which case they were stained with only cytokine detection antibody (instead of using isotype control) to determine the background level of non-specific binding. Cells were not assessed for viability during acquisition as assays were mostly performed following a short period of costimulation (mostly 24 hours). However, viability staining could have demonstrated that only fluorescence from live cells were analysed.

2.9.3 Intracellular cytokine staining

IL-10 production in response to CD55 costimulation was attempted by intracellular staining but this method was not reliable for assessing IL-10^+ cells. $CD4^+$ T cells were stimulated with plate bound CD3/CD55 for 24 hours. Brefeldin A (1:1000) was added to cell culture in the final 4-6 hours of stimulation after which cells were permeabilised and stained following the intracellular staining protocol (2.8.2). Staining for IL-10 was weak and unconvincing (data not shown), the reason the cytokine secretion assay was used for measuring cytokine expression in this study.

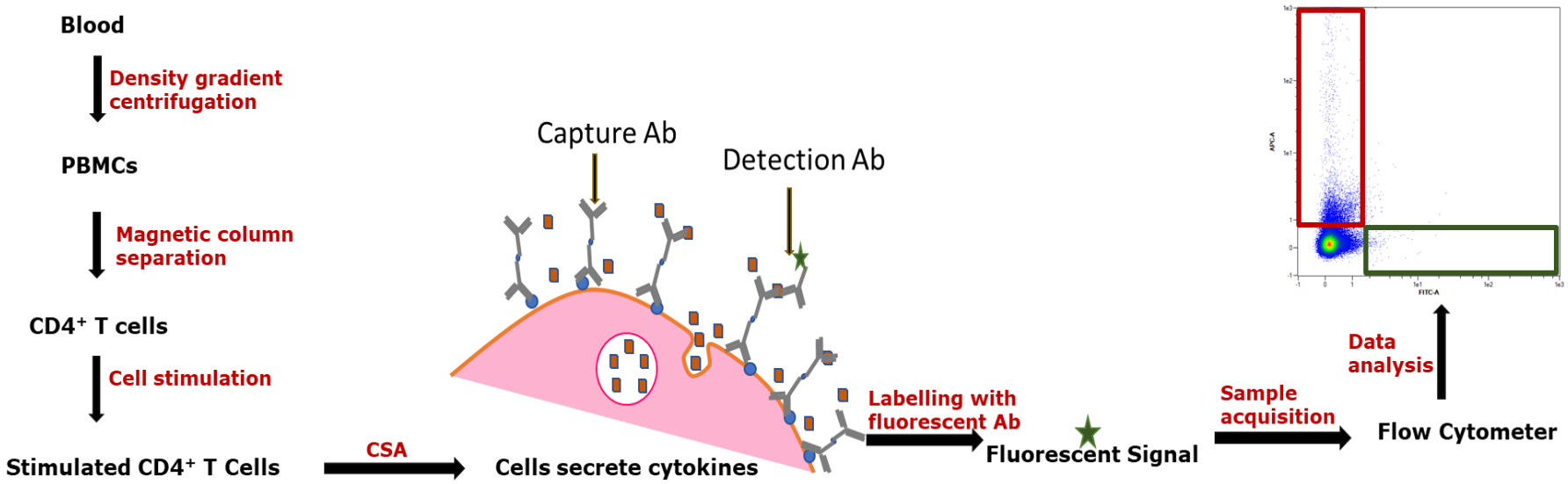


Figure 2.1: Experimental design for cytokine secretion assays

Fresh blood was obtained from healthy human donors. Diluted in PBS, layered in conical tubes followed by density gradient centrifugation. Cloudy PBMC layer was harvested, and appropriate isolation reagents added followed by enrichment of $CD4^+$ T cells. $CD4^+$ T cells were stimulated (with plate-bound antibodies or PMA/I) and CSA performed to capture secreted cytokines using IL-10/IFN-γ kits (Miltenyi Biotec). Detection antibody labelled cells were then acquired using the Flow Cytometer and data analysed using MACSQuantify or FlowJo software.

2.10: Data Analysis

2.10.1: Analysis of ELISA data

Data from ELISA experiments were analysed using Microsoft Excel. All statistical analyses for the data collected from various experiments were analysed using GraphPad Prism software (version 8).

2.10.2: Flow cytometry data analysis

All the Flow cytometry data was acquired with MACSQuant® Analyzer 10 (Miltenyi Biotec) flow cytometer and the collected data was analysed using MACSQuantify® Software (Version 2.11.5). Analyses were carried out on a Forward Scatter – Area (FSC-A), Side Scatter – Area (SSC-A) and doublet discrimination was done on a Forward Scatter – Area (FSC-A), Forward Scatter – Height (FSC-H). To compensate for multicolour flow cytometry experiments, a tube was labelled for each fluorochrome, and compensation beads (MACs COMP beads, anti-human IgK) was pulse-vortexed to mix after which one drop was added to each tube followed by antibody conjugate. Tubes were pulse-vortexed and compensation beads run through MACSQuant® Analyzer 10. Compensation matrix was saved and applied during subsequent sample acquisition. CTV proliferation data were also analysed with FlowJo™ (Version 10).

2.10.3: Statistical analysis

GraphPad Prism 8 software was used for all statistical analyses. Two-tailed, Paired Student's *t* test was used for analysing data from multiple donors with normal distribution. Repeated Measures One-way ANOVA was used for comparing more than two groups followed by Dunnett's Post hoc test to determine the point of significant difference. Data was statistically significant at $p \leq 0.05$, ns = not significant. Many thanks to Dr Andrea Venn (Statistician with the School of Medicine, University of Nottingham) for her invaluable input and kind review of statistical analyses.

2.11: Gating strategies for analysis of flow cytometry data

2.11.1: Setting IL-10 and IFN-γ gates

Gates were set at the junction of cytokine-positive and cytokine-negative cell populations. The gates were verified against detection antibody (only) control conditions. Figures 4.1,5.2, 6.1, and 7.5 show detailed gating strategies for the cytokine capture assay. Gates for T cell memory subsets (Figure 5.2E) were set at the junction of respective T memory subsets with unstimulated cells or at timepoint 0 (for time course), then applied to the test samples while Gates for Tr1 markers and Transcription factors (Figure 6.1) were set against detection antibody (IL-10) and isotype controls (markers/Transcription factors) then applied to test samples.

2.11.2: All other gates

In all other experiments, control conditions that contained positive and negative cell populations for the respective antibodies were included. Gates were set at the junction of the positive and negative cells populations of the controls and were applied to the samples. All gates were verified to be correct against isotype control conditions set against 0.22% to 0.26% positive events (Figure 3.1 and Figure 3.4). Appropriate control data are shown along with main data sets in all result chapters.

Chapter 3

CD55-97 interactions drive the production of IL-10 in a population of peripheral blood $CD4^+$ T cells

3.1: Introduction

CD55, Decay Accelerating Factor (DAF) belongs to the family of regulators of complement activation (RCA), protecting host cells from complement system attack by binding to C3b and C4b, thereby preventing formation of the membrane attack complex. CD55 is highly expressed on different cell types including T cells and possesses an N-terminal extracellular portion of four SCR domains linked through O-glycosylated spacer to a C terminal glycosylphosphatidylinositol anchor(H. H. Lin et al., 2001). Domains 2–4 of the SCR complex are involved in complement regulation and in binding of viral and bacterial pathogens. However, it is the protein-protein interactions between the EGF domains of CD97 and SRC domains of CD55 that demonstrated a novel natural function for CD55:CD97 interaction(Hamann et al., 1998; Hamann et al., 2000).

CD97 is a member of the epidermal growth factor (EGF)-TM7 subfamily of G protein coupled receptors (ADGRE5), that mediate cell-cell interactions. It was first characterized by Eichler W et al., as an early activation marker detectable on lymphocytes between 2-4 hours of activation, distinct from other activation-dependent cells surface antigens, CD69, CD25 and 4F2(Eichler, Aust, & Hamann, 1994).

CD97 is (~80KDa glycoprotein) detectable on resting lymphocytes at low levels but highly expressed by monocytes, granulocytes and activated B and T cells(Kop et al., 2009). It exists as 3 isoforms, containing different numbers of EGF like domains (EGF 1,2,5), (EGF 1,2,3,5) and (EGF 1,2,3,4,5), which changes their ligand interactions. The 1,2,5 isoform of CD97 mediates its specific interaction with CD55 [dissociation constant (K_D) of 86μM] in a calcium dependent interaction (Kwakkenbos et al., 2006; H. H. Lin et al., 2001).

T Cell receptor (TCR) engagement coupled with CD55-CD97 costimulation initiate signaling events within cells leading to cell proliferation and cytokine production. Our lab previously reported that CD55-CD97 costimulation resulted in activation, proliferation, and differentiation of naïve $CD4^+$ T cells, leading to a small population of IL-10 producing cells (Sutavani et al., 2013). Therefore, we hypothesized that CD55-CD97 costimulation was able to restimulate a population of IL-10 positive cells within human peripheral blood $CD4^+$ T cells.

3.2: Results

3.2.1: Activation of human peripheral blood $CD4^+$ T cells

We have previously shown that CD55 costimulation results in the differentiation of naïve $CD4^+$ T cells, giving a small IL-10 producing population. The effect of CD55 costimulation on peripheral blood $CD4^+$ T cells is yet to be examined. TCR engagement ('signal 1') and costimulation ('signal 2') can both be mimicked using monoclonal antibodies against the receptors CD3 and CD55, respectively. We therefore assessed the phenotype of purified $CD4^+$ T cells stimulated with anti-CD3 and anti-CD55 (monoclonal antibody) or CD97Fc (cellular ligand to CD55).

In an earlier study, our group reported that CD3/ CD55 costimulation of naïve $CD4^+$ T cells for 72 hours induced a population of $IL\text{-}10^+$ cells while CD3/ CD28 costimulation failed to induce this rare population. To reproduce these results, naïve $CD4^+$ T cells were purified and stimulated with CD3/CD55 antibodies. To determine the concentration at which the anti-CD55 antibody optimally stimulates naïve cells, dose titration of CD55 antibody was performed at a fixed concentration of CD3 antibody (1µg/ml) for 72 hours and cell proliferation measured by thymidine uptake. It was observed that dose titration of CD55 antibody at a fixed concentration of CD3 antibody (1µg/ml) resulted in proliferation of naïve $CD4^+$ T cells that reached a maximum response at 5µg/ml (Figure 3.2). Further analysis revealed high amount of secreted IL-10 in culture supernatant in multiple donors, up to about 1.2ng/ml in some donors (Figure 3.3).

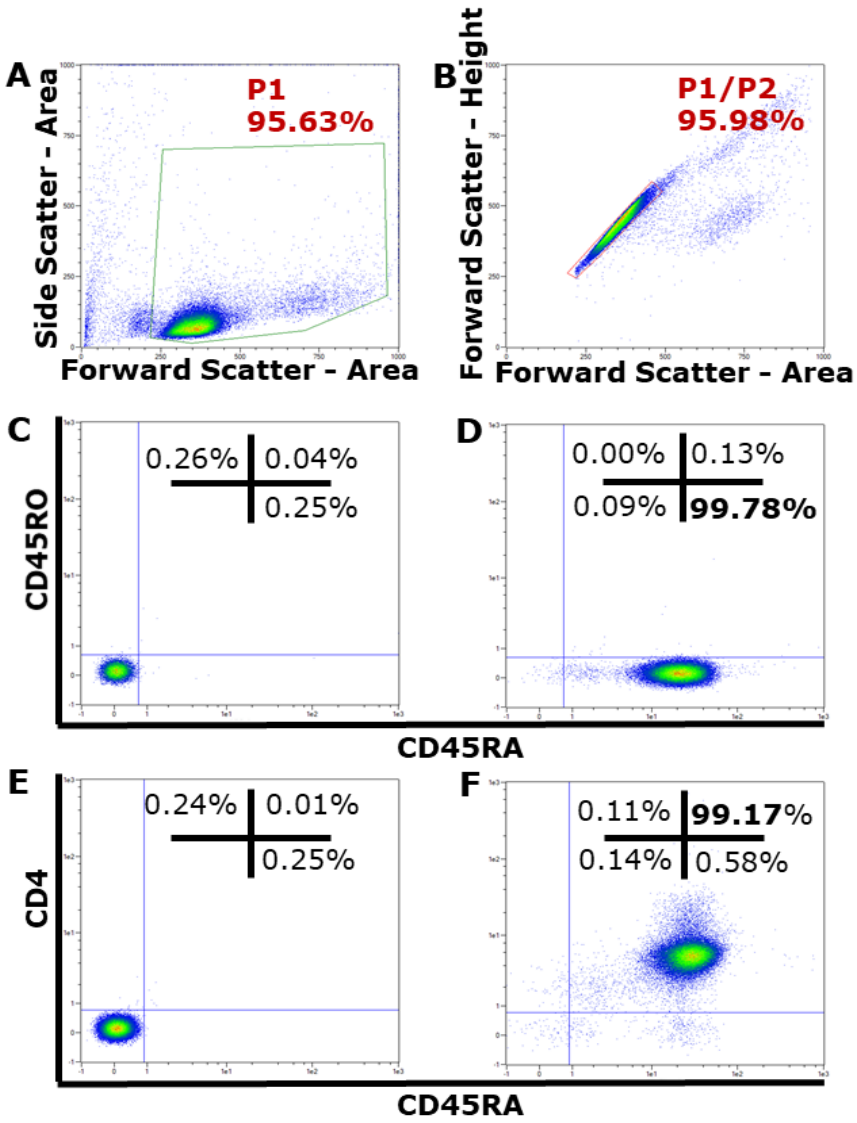


Figure 3.1: Purity of naïve CD4⁺ cells were >99% CD45RA⁺ CD45RO⁻

Following enrichment from PBMCs by MACS naïve CD4 isolation kit, naïve $CD4^+$ cells were assessed for purity using flow cytometer. Cells were co-stained for CD4, CD45RA and CD45RO. **A** Analysis was carried out on a forward scatter – area, side scatter – area. **B** doublet discrimination on a forward scatter – area, forward scatter – height plot. **C & E** Isotype controls. **D** Cells were >99% $CD45RA^+$ $CD45RO^-$**.** **F** cells were >99% $CD4^+$ $CD45RA^+$ in all experiments.

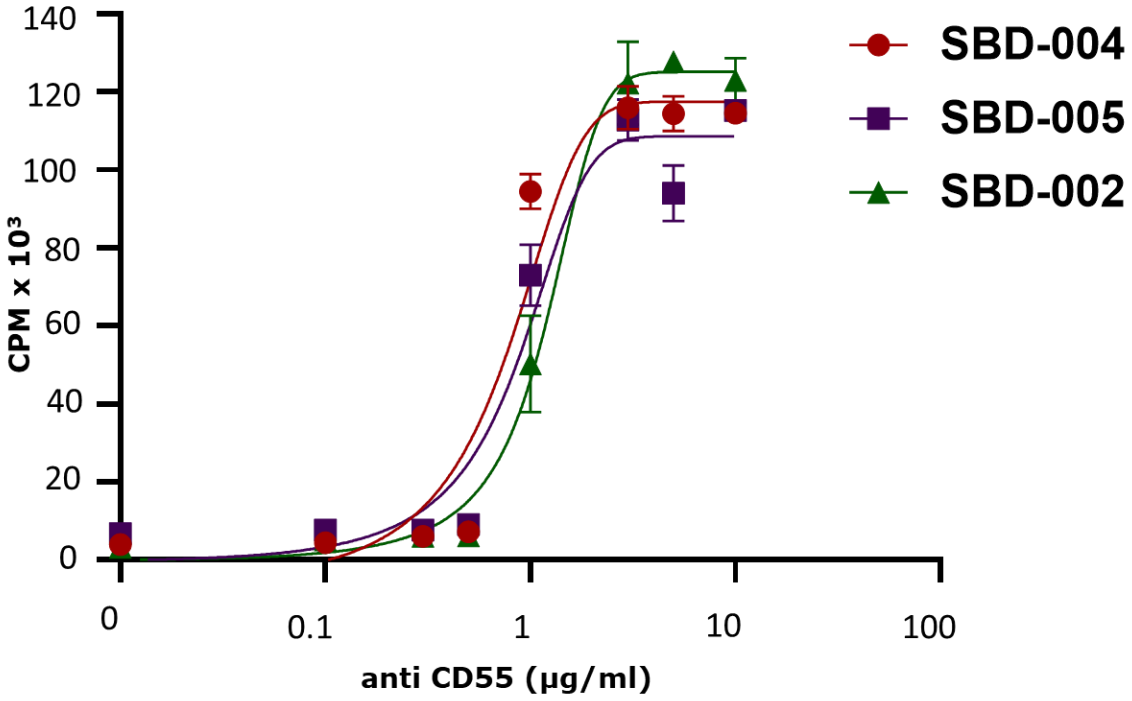


Figure 3.2: Naïve CD4$^+$ T cells proliferation by anti CD3 is enhanced by increasing doses of CD55 antibody

Titration of anti-CD55 antibody with a constant concentration of plate bound anti CD3 antibody (1µg/ml) was used to stimulate naïve CD4$^+$ T cells (1.0 x 10^5 cells/well) in 96 well plate for 72 hours. Cell proliferation was assessed by tritiated thymidine incorporation in the last 15 hours of culture measured in Counts Per Minute (CPM). Results show mean ± SD from three donors.

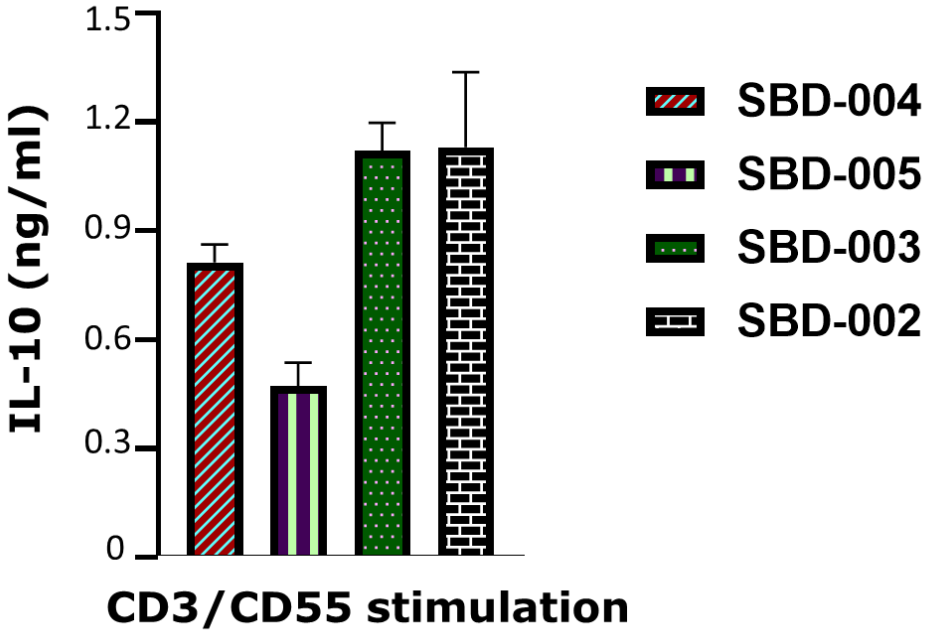
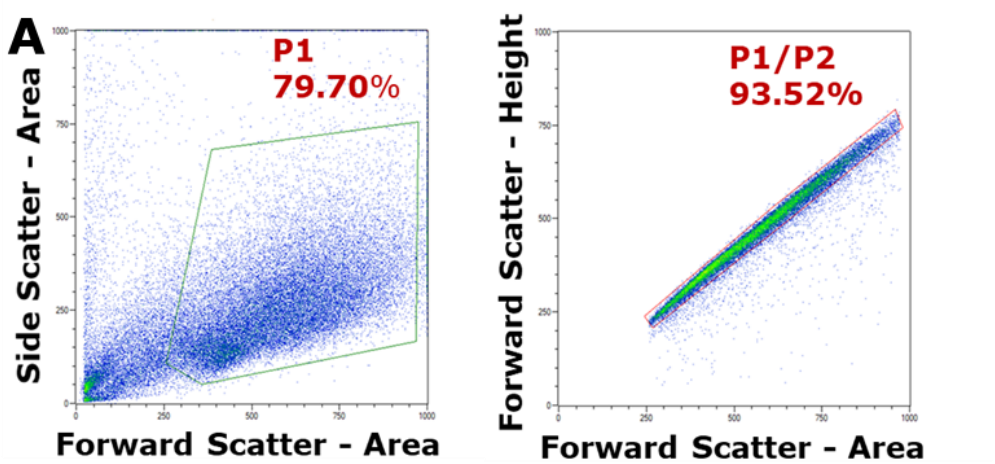


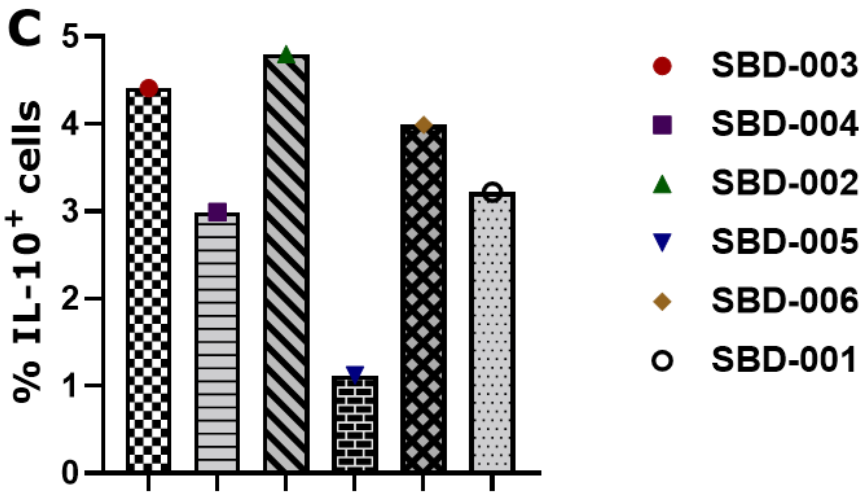
Figure 3.3: CD3/CD55 costimulation of naïve CD4$^+$ T cells results in IL-10 production in culture

Purified naïve CD4$^+$ T cells (1.0 x 10^5 cells/well) were costimulated with plate bound CD3/CD55 (1µg/mL and 5µg/mL, respectively) antibody in a 96 well plate for 72 hours. ELISA for IL-10 was performed from harvested culture supernatant. Results shown are Mean ± S.D from four donors.

Naïve T cells remain naïve or antigen inexperienced until they encounter MHC peptide complex. When presented on a professional APC, such as a DC, this provides costimulation to assist in the differentiation of naïve cells to effector cells. The kinetics of a naïve response is 10-14 days (Berard & Tough, 2002; Croft, Bradley, & Swain, 1994; Dubey, Croft, & Swain, 1995). However, once stimulated, antigen experienced T cells respond with much shorter kinetics, usually 2-4 days and are more sensitive to lower doses of antigenic stimulations (Constant et al., 1994).

Previous observations showed that naïve $CD4^+$ T cells proliferate and secrete cytokines after stimulation. Figure 3.4 shows that these cells produce IL-10, which can be measured after 72 hours of stimulation with CD3/CD55 antibody. Flow cytometry plots show IL-10 expression from three donors (Figure 3.4 B), with the percentage of IL-10 expression ranging between 1–5% across six donors (Figure 3.4C).





CD3/CD55 costimulation of naive CD4+ T cells

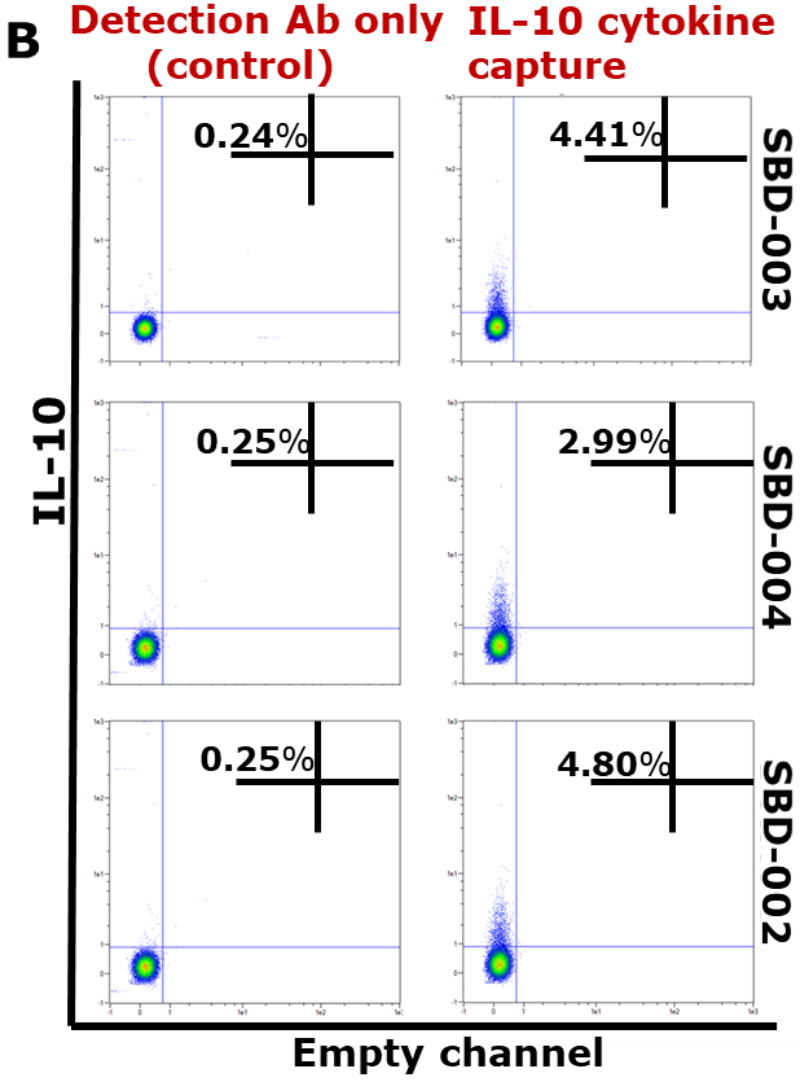


Figure 3.4: CD3/CD55 costimulation of naïve $CD4^+$ T cells reveal a small population of IL-10 producing cells

Purified naïve $CD4^+$ T cells, isolated from peripheral blood of healthy donors were stimulated with plate bound CD3/CD55 for 72 hours. **A** Data analysis was carried out on a forward scatter – area, side scatter – area and cell clumps excluded by doublet discrimination on a forward scatter – area, forward scatter – height plot. **B** IL-10 cytokine capture performed after 72 hours costimulation. Results shown indicate positive control (detection Ab only) to the left and IL-10 surface capture to right for three donors. **C** percentage IL-10 positive cells for six donors.

To validate these observations on total $CD4^+$ T cells, highly purified $CD3^+CD4^+$ T cells (>98%, Figure 3.5) were used in all experiments. Anti-CD3 antibody (clone OKT-3) was titrated on purified $CD4^+$ T cells (Figure 3.6) and a dose-dependent effect on cell proliferation resulted in a logistic growth curve. The concentration of anti-CD3 antibody at which cell proliferation was halfway to the top (suboptimal dose -1µg/ml) was chosen for use with anti-CD55 in order to measure any potential positive or negative effects of the costimulatory molecule CD55.

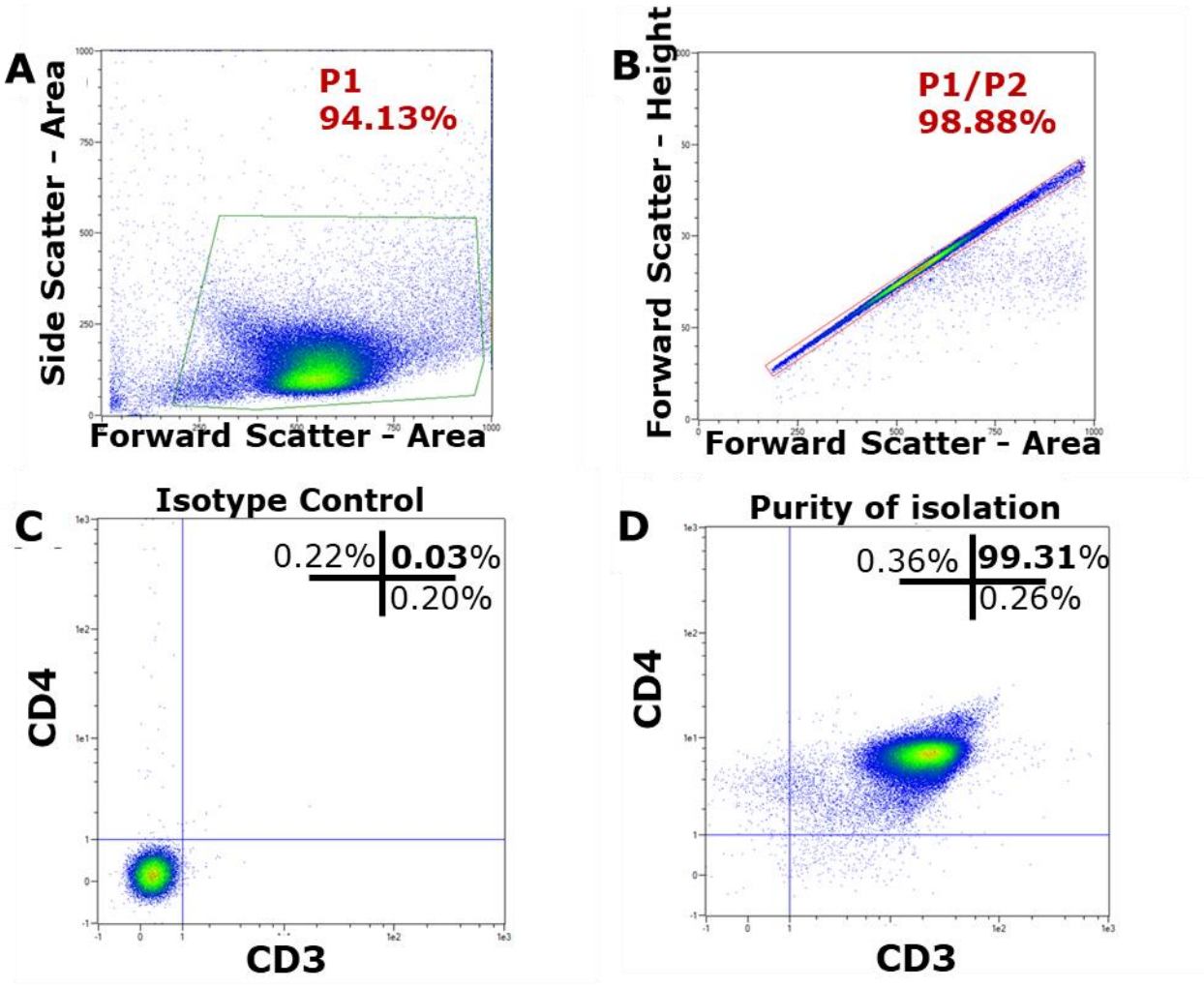


Figure 3.5: Purity of isolated $CD4^+$ T cells were >98% $CD4^+CD3^+$

Isolated $CD4^+$ T cells were assessed for purity by dual staining for CD4 and CD3, using flow cytometer. **(A)** Flow cytometry plot showing whole population on forward scatter-area, side scatter – area. **(B)** Flow plot showing doublet discrimination by forward scatter- Area, forward scatter – Height. **(C)** Isotype control gate and **(D)** Highly pure (>98% $CD4^+CD3^+$) were used in all subsequent experiments.

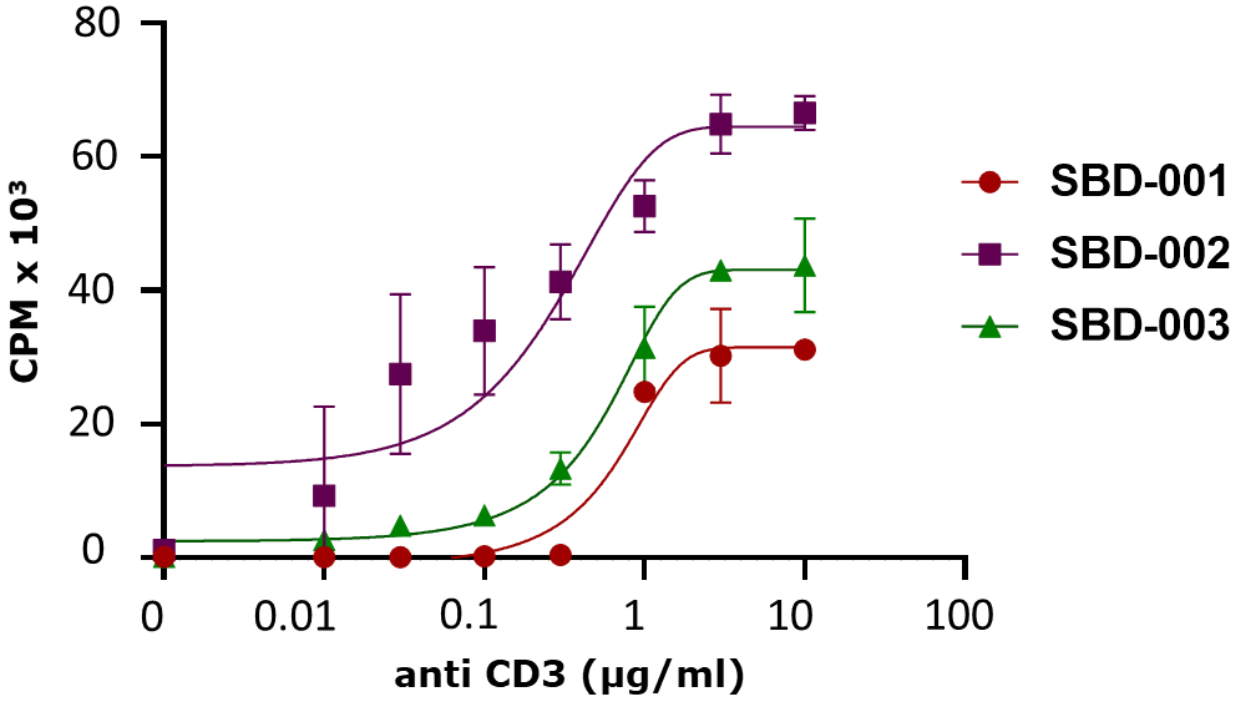


Figure 3.6: Dose titration of anti CD3 on proliferation of $CD4^+$ T cells

Plate-bound anti-CD3 was used to stimulate $CD4^+$ T cells at density 1.0 x 10^5 cells/well in a 96 well plate for 72 hours. Cell proliferation was assessed by incorporation of tritiated thymidine in the last 15 hours of culture measured in Counts Per Minute (CPM). Results show best fit curves for mean ± SD from three donors.

Having determined the sub-optimal concentration of antibody (anti-CD3) that provides 'signal 1' the next task was to determine the optimal concentration of anti-CD55 antibody that gave 'signal 2'. Optimal concentration of costimulatory molecule is needed to fully activate the cells. Therefore, a dose titration of anti-CD55 antibody at sub-optimal CD3 signal (1µg/ml) revealed (Figure 3.7) cellular proliferation increased with increase in dose of anti-CD55 to levels higher than observed with CD3 stimulation alone. A concentration of 5µg/ml anti-CD55 antibody was selected for use with 1µg/ml anti-CD3 antibody in all future experiments.

Previous studies have shown that even at a range of concentrations of anti CD3, anti CD55 was able to enhance T cell proliferation and even at optimal CD3 (3-4ug/ml), a titration of anti CD55 can increase the activation and proliferation of T cells. This is one of the assays used to define costimulatory activity.

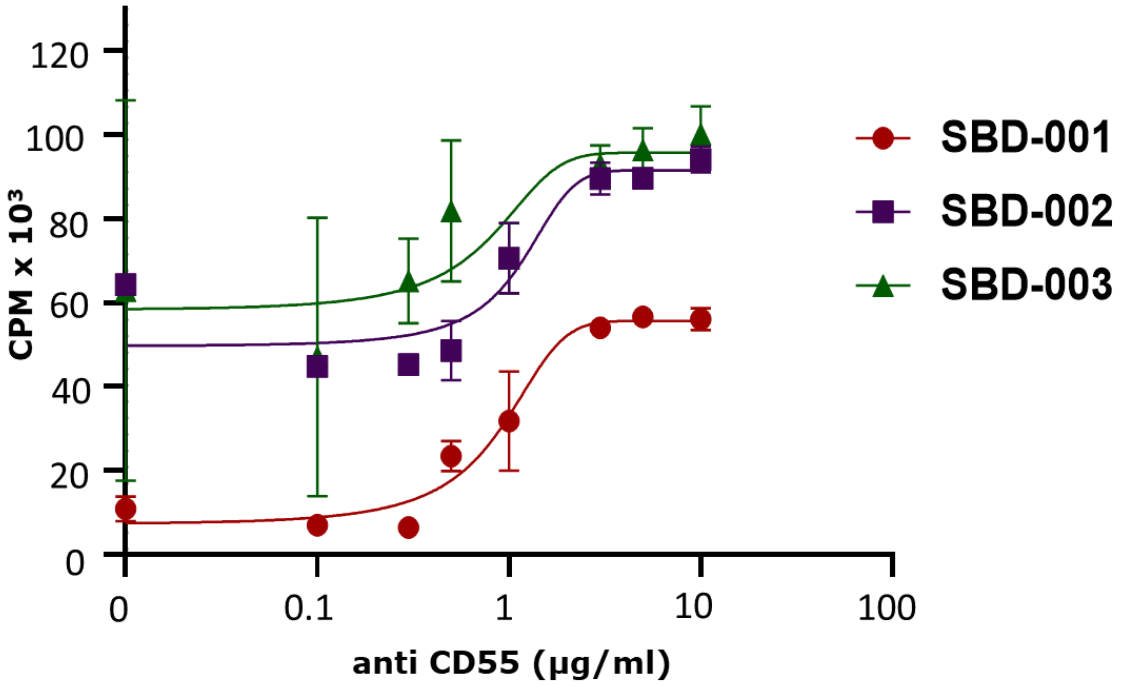


Figure 3.7: T cell proliferation by anti-CD3 is enhanced by increasing doses of anti-CD55 antibody

Plate-bound anti-CD3 antibody at a constant concentration of 1µg/ml with titration of anti-CD55 antibody was used to stimulate $CD4^+$ T cells at a density of 1.0 x 10^5 cells/well in a 96 well plate for 72 hours. Cell proliferation was assessed by tritiated thymidine incorporation in the last 15 hours of culture measured in Counts Per Minute (CPM). Results show mean ± SD from three donors.

3.2.2: Generation of CD97 fusion protein

To determine whether stimulation with the natural ligand for CD55 (CD97) produced similar results as stimulation with anti-CD55 antibodies, recombinant CD97 fusion protein was generated. CD97mFc previously cloned into pcDNA3.1 by Abbott, et al., (Figure 3.8 A) was used (Abbott et al., 2007; Stacey et al., 2003).

To validate the sequence, it was first aligned and confirmed from both ends (forward from T7 site and reverse from SP6 site) to be CD97 isoform 2 (CD97 1, 2, 5) as shown in Figure 3.8 C.

The sequence translation data is shown in Figure 3.9. The CD97Fc vector was then amplified in E. coli and purified by Qiagen maxiprep, quantitated and transfected into expi 293 cells. The CD97 fusion protein generated in a 7-day transient transfection was purified from culture supernatant by Fast Protein Liquid Chromatography (FPLC) on a Protein-G affinity column.

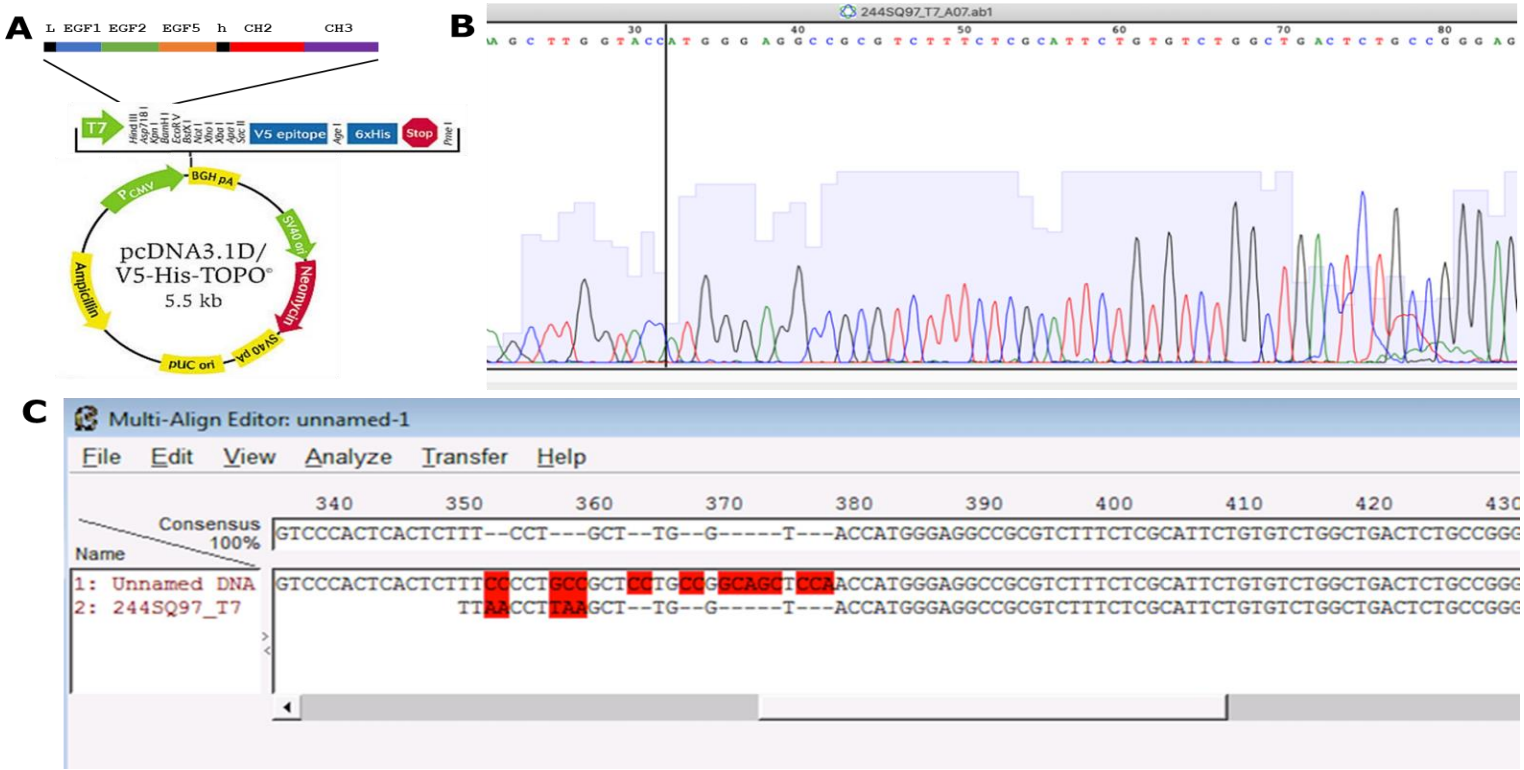


Figure 3.8: CD97-mFc was cloned into pcDNA3.1

A Sequence was verified by automated sequencing from the T7 site. **B** and aligned. **C** confirming the sequence to be CD97 isoform 2 (CD97 1,2, 5).(Stacey et al., 2003)

CD97-> Leader / Signal sequence -->|<-- EGF 1

```
     M  G  G  R  V  F  L  A  F  C  V  W  L  T  L  P  G  A  E  T  Q  D  S  R
   1 ATGGGAGGCCGCGTCTTTCTCGCATTCTGTGTCTGGCTGACTCTGCCGGGAGCTGAAACCCAGGACTCCA  70

     G  C  A  R  W  C  P  Q  N  S  S  C  V  N  A  T  A  C  R  C  N  P  G
  71 GGGGCTGTGCCCGGTGGTGCCCTCAGAACTCCTCGTGTGTCAATGCCACCGCCTGTCGCTGCAATCCAGG 140

                                EGF 2
     F  S  S  F  S  E  I  I  T  T  P  T  E  T  C  D  D  I  N  E  C  A  T
 141 GTTCAGCTCTTTTTCTGAGATCATCACCACCCCGACGGAGACTTGTGACGACATCAACGAGTGTGCAACA 210

     P  S  K  V  S  C  G  K  F  S  D  C  W  N  T  E  G  S  Y  D  C  V  C  S
 211 CCGTCGAAAGTGTCATGCGGAAAATTCTCGGACTGCTGGAACACAGAGGGGAGCTACGACTGCGTGTGCA 280

     P  G  Y  E  P  V  S  G  A  K  T  F  K  N  E  S  E  N  T  C  Q  D  V
 281 GCCCGGGATATGAGCCTGTTTCTGGGGCAAAAACATTCAAGAATGAGAGCGAGAACACCTGTCAAGATGT 350

                                EGF 5
     D  E  C  S  S  G  Q  H  Q  C  D  S  S  T  V  C  F  N  T  V  G  S  Y
 351 GGACGAGTGCAGCTCCGGGCAGCATCAGTGTGACAGCTCCACCGTCTGCTTCAACACCGTGGGTTCATAC 420

     S  C  R  C  R  P  G  W  K  P  R  H  G  I  P  N  N  Q  K  D  T  V  C  E
 421 AGCTGCCGCTGCCGCCCAGGCTGGAAGCCCAGACACGGAATCCCGAATAACCAAAAGGACACTGTCTGTG 490

                                                          |<--mFc Hinge
     D  M  T  F  S  T  W  T  P  P  P  G  V  H  S  Q  E  P  S  G  P  I  S
 491 AAGATATGACTTTCTCCACCTGGACCCCGCCCCCTGGAGTCCACAGCCAGGAGCCCAGCGGGCCCATTTC 560

                                mFc Hinge   -->|<--  Ch2 of mFc
     T  I  N  P  C  P  P  C  K  E  C  H  K  C  P  A  P  N  L  E  G  G  P
 561 AACAATCAACCCCTGTCCTCCATGCAAGGAGTGTCACAAATGCCCAGCTCCTAACCTCGAGGGTGGACCA 630

     S  V  F  I  F  P  P  N  I  K  D  V  L  M  I  S  L  T  P  K  V  T  C  V
 631 TCCGTCTTCATCTTCCCTCCAAATATCAAGGATGTACTCATGATCTCCCTGACACCCAAGGTCACGTGTG 700

     V  V  D  V  S  E  D  D  P  D  V  Q  I  S  W  F  V  N  N  V  E  V  H
 701 TGGTGGTGGATGTGAGCGAGGATGACCCAGACGTCCAGATCAGCTGGTTTGTGAACAACGTGGAAGTACA 770

     T  A  Q  T  Q  T  H  R  E  D  Y  N  S  T  I  R  V  V  S  T  L  P  I
 771 CACAGCTCAGACACAAACCCATAGAGAGGATTACAACAGTACTATCCGGGTGGTCAGCACCCTCCCCATC 840

     Q  H  Q  D  W  M  S  G  K  E  F  K  C  K  V  N  N  K  D  L  P  S  P  I
 841 CAGCACCAGGACTGGATGAGTGGCAAGGAGTTCAAATGCAAGGTCAACAACAAAGACCTCCCATCACCCA 910

  Ch2 of mFc  -->|          |<--          Ch3 of mFc
     E  R  T  I  S  K  I  K  G  L  V  R  A  P  Q  V  Y  I  L  P  P  P  A
 911 TCGAGAGAACCATCTCAAAAATTAAAGGGCTAGTCAGAGCTCCACAAGTATACATCTTGCCGCCACCAGC 980

     E  Q  L  S  R  K  D  V  S  L  T  C  L  V  V  G  F  N  P  G  D  I  S
 981 AGAGCAGTTGTCCAGGAAAGATGTCAGTCTCACTTGCCTGGTCGTGGGCTTCAACCCTGGAGACATCAGT 1050

     V  E  W  T  S  N  G  H  T  E  E  N  Y  K  D  T  A  P  V  L  D  S  D  G
1051 GTGGAGTGGACCAGCAATGGGCATACAGAGGAGAACTACAAGGACACCGCACCAGTCTTGGACTCTGACG 1120

     S  Y  F  I  Y  S  K  L  N  M  K  T  S  K  W  E  K  T  D  S  F  S  C
1121 GTTCTTACTTCATATATAGCAAGCTCAATATGAAAACAAGCAAGTGGGAGAAAACAGATTCCTTCTCATG 1190

     N  V  R  H  E  G  L  K  N  Y  Y  L  K  K  T  I  S  R  S  P  G  K
1191 CAACGTGAGACACGAGGGTCTGAAAAATTACTACCTGAAGAAGACCATCTCCCGGTCTCCGGGTAAA 1257
```

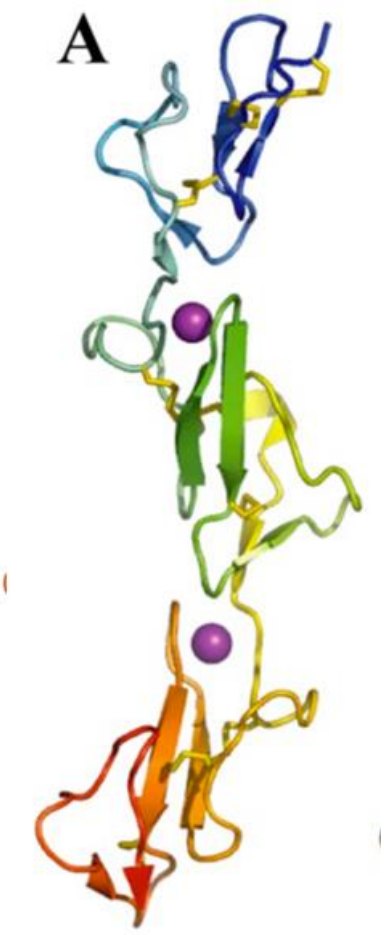


Figure 3.9: Translation of the sequence data from CD97mFc vector

CD97 sequence obtained from T7 and SP6 forward and reverse primers. The translation shows the CD97 leader sequence **(black),** the three EGF like domains, 1,2 and 5 **(Blue, Green and Orange)** with a 5 amino acid portion or the stalk region **(black)** separating CD97 from the hinge region **(*Italic*)** of the mouse IgG2b constant heavy domains two **(red)** and three **(purple). A** ribbon diagram of the three EGF like domains in CD97 (1, 2, 5) coloured from ***blue*** at the N terminus to ***red*** at the C terminus with disulphide bonds in ***yellow***, and calcium ions displayed as ***purple*** spheres (Abbott et al., 2007).

N/B: Rachel J. M. Abbott and Ian Spendlove did the cloning, validation and translation of CD97 plasmid DNA.

Figure 3.10 shows the elution spectrogram for CD97Fc fusion protein at absorbance (A_{280}). The peak fractions were collected and dialysed in 2 litres buffer containing 150mM NaCl, 10mM CaCl2 and 10mM Tris HCl. Purified and dialysed samples were assessed for approximate protein concentration by nanodrop.

The Fc fusion protein was validated in SDS-PAGE under reducing and non-reducing conditions and Coomassie stained. The native Mr of CD97Fc is 46.3KDa. There are 3 putative glycosylation sites in the EGF domains that contribute to the activity of CD97. The predominant band of 80-90KDa may represent glycosylated CD97Fc. This runs as a dimeric form on non-reducing gels due to the Fc region and appears at approximately 180KDa (Figure 3.11) confirming previous observations (Eichler et al., 1994; Kop et al., 2009; Labrijn et al., 2017).

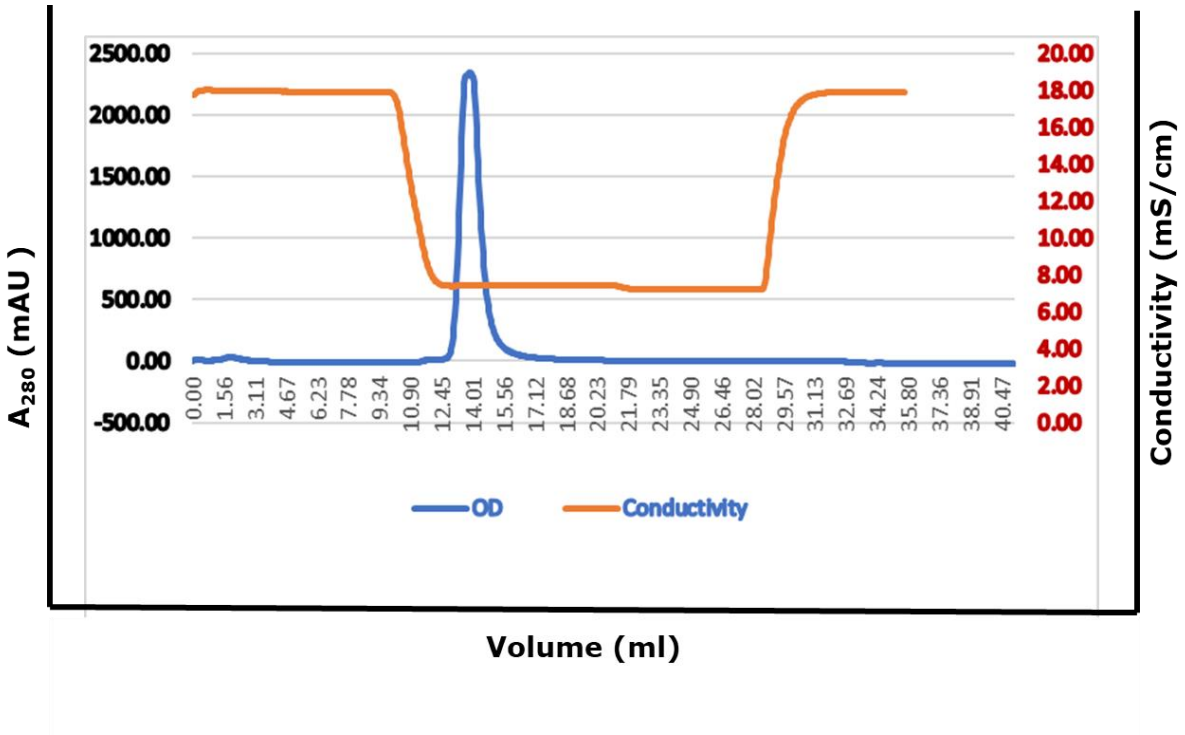


Figure 3.10: FPLC spectrogram for purification of CD97Fc fusion protein

CD97 fusion protein was purified from transfection supernatant by Fast protein liquid chromatography (FPLC) on a protein G column. Fractions 11-16 were eluted at absorbance A_{280}, pooled, neutralized and dialysed against 10mM Tris-HCl (pH 8.0) buffer overnight at room temperature.

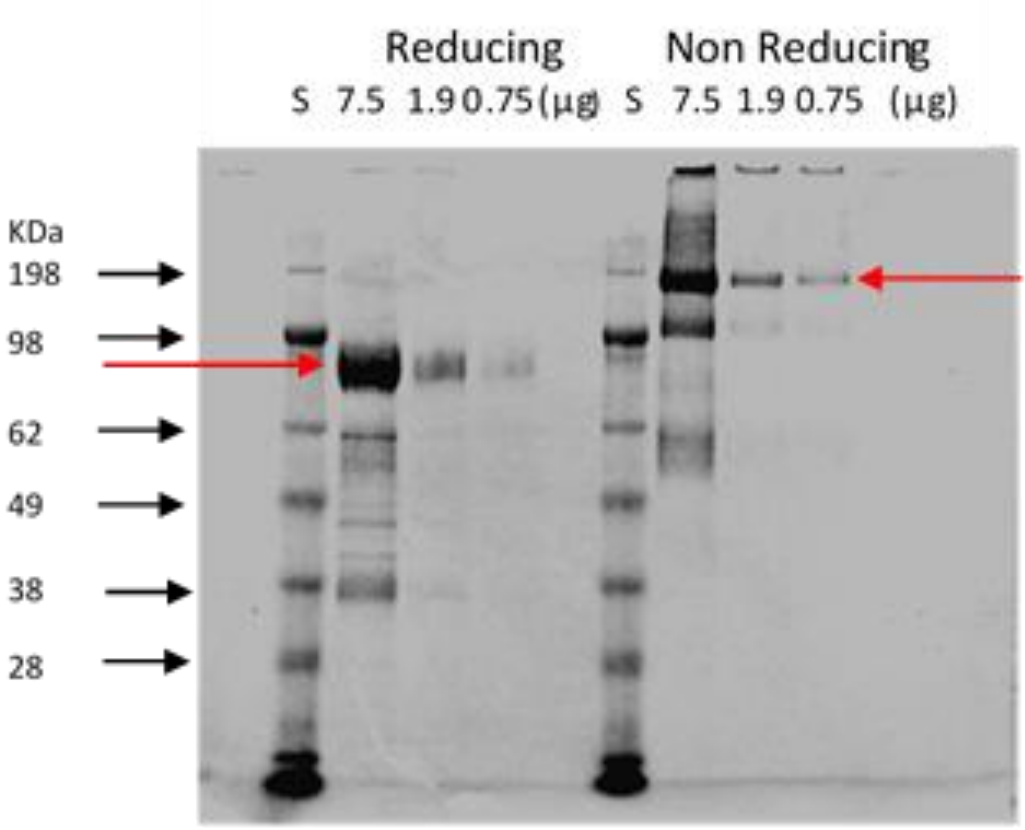


Figure 3.11: SDS-PAGE and Coomassie staining of reducing and non-reducing conditions for purified CD97Fc fusion protein

Three concentrations (7.5. 1.9 and 0.75 µg/ml) of CD97Fc were run on a standard 10% SDS-PAGE gel under either reducing or non-reducing conditions and stained with Coomassie blue dye to determine approximate size and purity of the recovered protein. **S** represents the protein standard marker. Red arrows indicate the position of CD97Fc fusion protein.

3.2.3: Costimulation with CD97Fc potently drives cell proliferation

Purified CD97Fc was used to costimulate $CD4^+$ T cells. Proliferation assays were carried out to determine the optimal dose at which CD97Fc costimulated $CD4^+$ T cells (Figure 3.12). Dose titration of CD97Fc together with a fixed concentration of anti-CD3 antibody was used to stimulate purified $CD4^+$ cells. Optimal cell proliferation occurred at 2-5µg/ml and this concentration was selected for use in all future experiments.

Figure 3.13 shows the effects of CD3 and CD97Fc alone or in combination to demonstrate a costimulatory effect. Cell proliferation due to costimulation with CD97Fc, was significantly higher ($p \leq 0.05$) than that observed with either CD3 or CD97Fc alone.

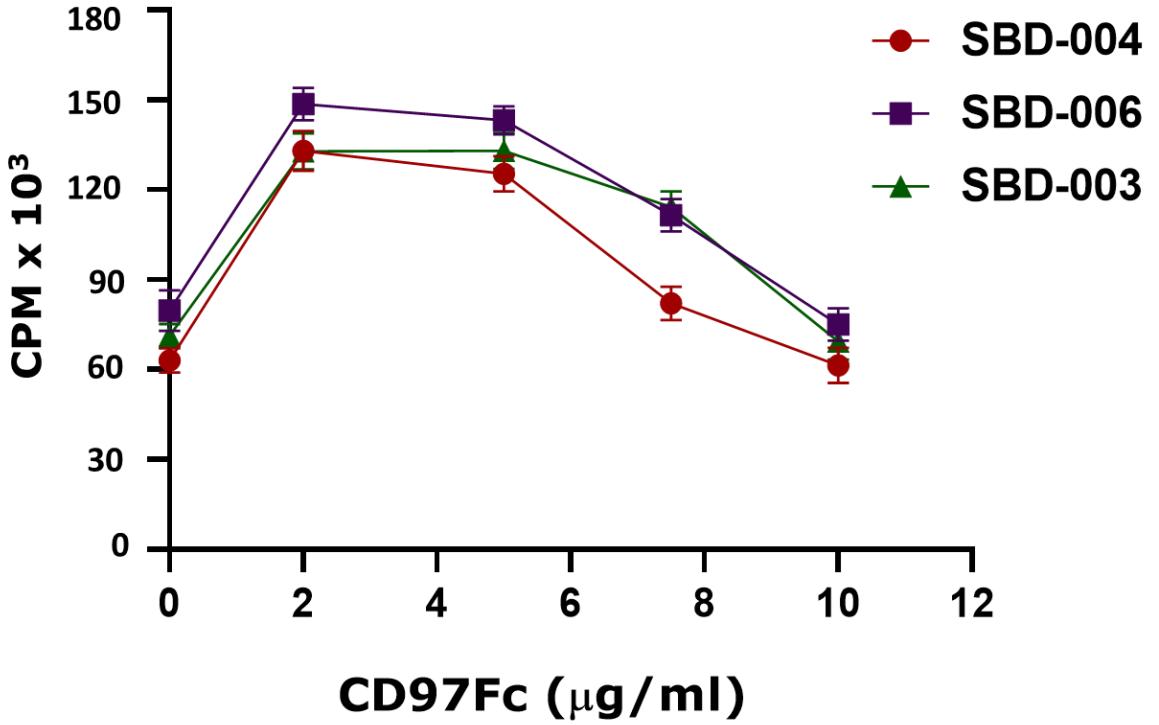


Figure 3.12: CD97Fc costimulation enhanced the proliferation of $CD4^+$ T cells in a dose dependent manner up to 2μg/mL at fixed concentration (1μg/ml) of anti-CD3.

Plate-bound anti-CD3 antibody (1μg/ml) with titration of CD97Fc was used to stimulate $CD4^+$ T cells at density of 1.0×10^5 cells/well in a 96 well plate for 72 hours. Cell proliferation was assessed by tritiated thymidine incorporation in the last 15 hours of culture measured in Counts Per Minute (CPM). Results show mean ± SD from three donors.

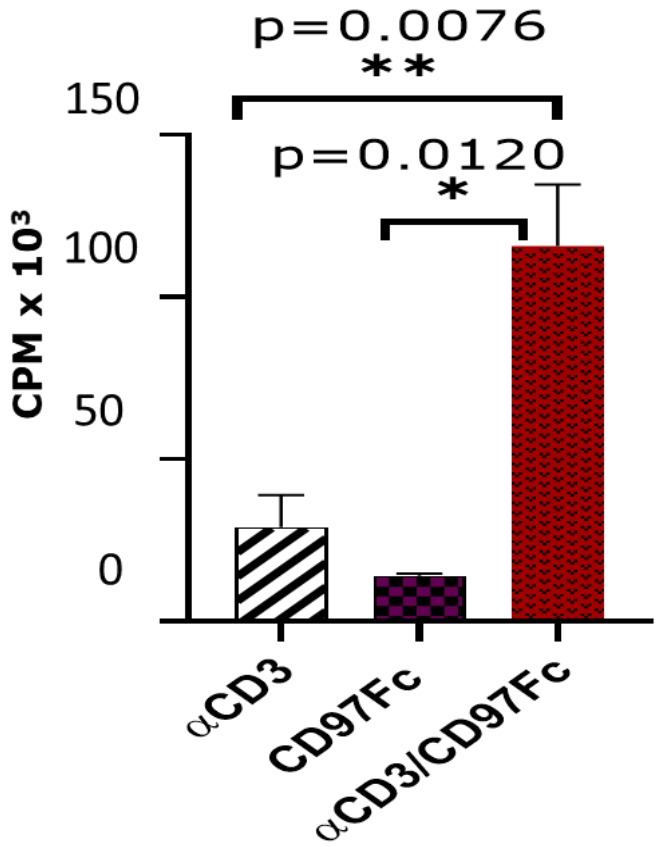


Figure 3.13: Anti-CD3/CD97Fc potently drives cell proliferation, greater than anti-CD3 or CD97Fc alone

Purified $CD4^+$ T cells at 1.0 x 10^5 cells/well were stimulated with plate bound anti CD3 (1µg/ml) and/or CD97Fc (2µg/ml) in a 96 well plate for 72 hours. Proliferation was assessed by ^{3}H-thymidine incorporation in the last 15 hours of culture. CD97Fc with sub-optimal CD3 stimulation induced significantly higher cell proliferation than stimulation with CD3 or CD97Fc alone. Statistical significance was determined by two-tailed, paired Student's *t* test (n = 6). Data shown are mean ± SD from six donors in six independent experiments, statistically significant $p \leq 0.05$.

Following activation, $CD4^+$ T cells increase in size and proliferating cells formed characteristic blasts (Figure 3.13) where $CD4^+$ T cells were stimulated with anti-CD3, anti-CD3/ anti-CD55, anti-CD3/ anti-CD28 or anti-CD3/CD97Fc for 72 hours.

Tiny blasts started to form when cells were stimulated with anti-CD3 antibody alone (Figure 3.14A), but larger cell blasts were observed in cultures where cells were stimulated with anti-CD3/ anti-CD28, anti-CD3/CD97Fc or, anti-CD3/ anti-CD55. The size of cell blasts seen with anti-CD28 and anti-CD55 costimulation were larger than those observed with CD97Fc (Figure 3.14 B-D).

Cell proliferation dye (Cell Trace Violet – CTV) was used to determine the proliferation cycles under costimulation. After 72 hours of CD3/CD97Fc costimulation, cells were observed to have gone through multiple rounds of proliferation cycles for the three donors assessed (Figure 3.15).

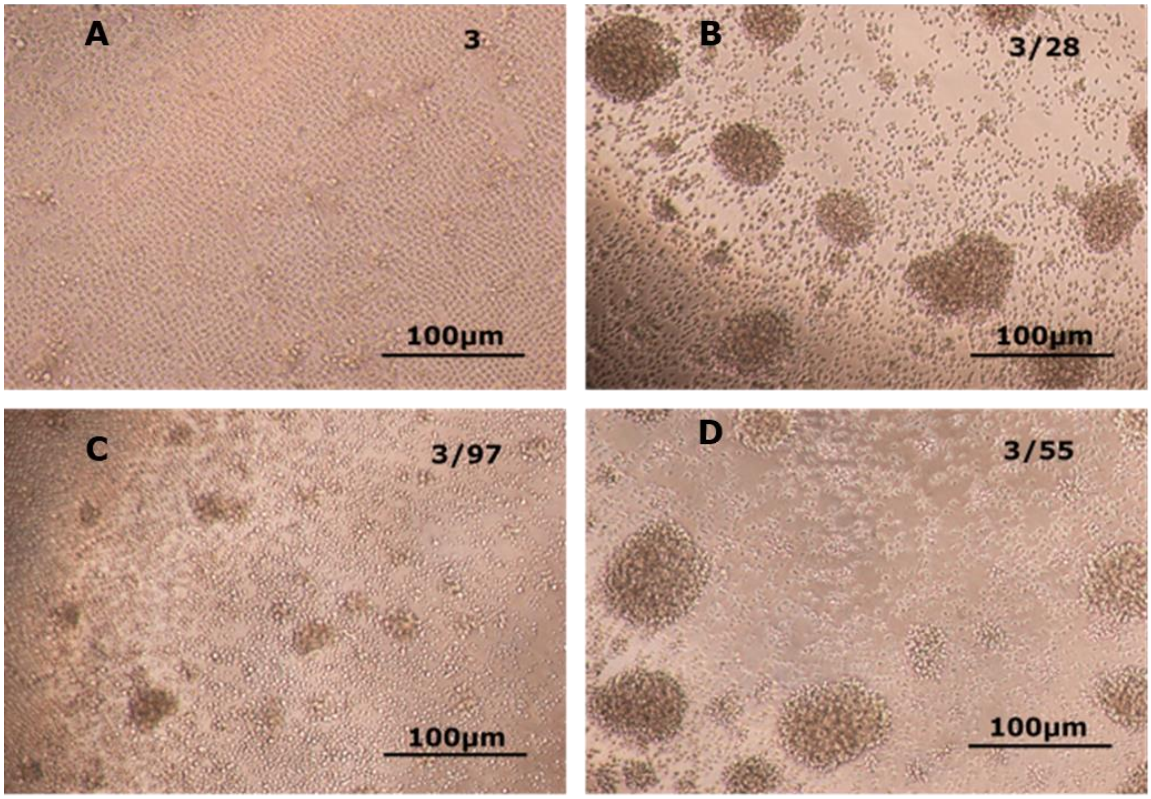


Figure 3.14: Costimulation-driven blast formation of CD4$^+$T cells

Stimulation of purified CD4$^+$ T cells results in proliferation and blast formation. Cell culture images were taken after 72 hours of stimulation with **A** anti-CD3 alone; **B** anti-CD3 /anti-CD28; C anti-CD3 /CD97Fc; **D** anti-CD3/anti-CD55.

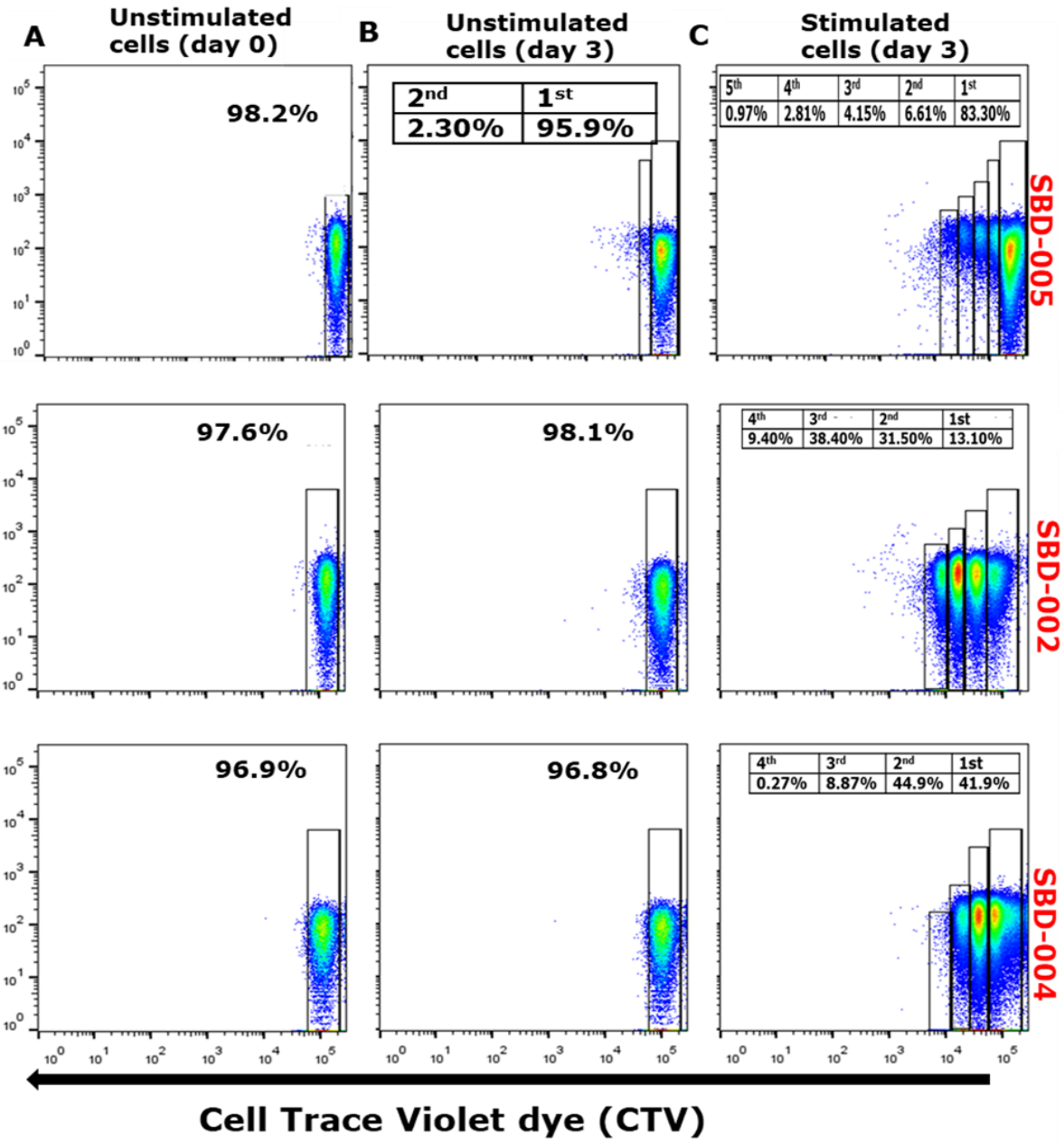


Figure 3.15: CD4$^+$ T cells undergo multiple rounds of proliferation cycles after costimulation with anti-CD3/CD97Fc

CD4$^+$ T cells were labelled with CellTrace Violet (CTV) dye and costimulated with anti CD3/CD97Fc for 72 hours. Proliferation was assessed by CTV dye dilution. **(A)** CTV labelled cells before stimulation. **(B)** CTV labelled cells in culture (without stimulation) **(C)** CTV labelled cells following CD3/CD97Fc costimulation. Data shown are for three donors in three independent experiments.

3.2.4: CD55-CD97 costimulation of $CD4^+$ T cells led to IL-10 secretion

Having established that CD55-CD97 interaction resulted in the proliferation of $CD4^+$ T cells, the next question was to assess whether costimulation via CD55 resulted in IL-10 expression. To achieve this, purified cells were stimulated with anti-CD3 and with different concentrations of CD97Fc.

Costimulation of $CD4^+$ T cells with anti-CD3/ titration of CD97Fc and anti-CD3/anti-CD55 (comparator) for 24 hours resulted in IL-10 secretion in cell culture supernatant (Figure 3.16) measured by Elisa. Elisa data were obtained from culture supernatant during dose titration of CD97Fc around the optimal concentration of CD97Fc (2µg/ml) based on data from previous experiments. The peak point (optimal concentration) for IL-10 secretion with CD97Fc was compared to Ab (CD55) stimulated cells- the reason for the short range. IL-10 secretion observed by CD3/CD97Fc costimulation at optimum concentration (2µg/ml) was comparable to levels obtained with CD3/CD55 costimulation.

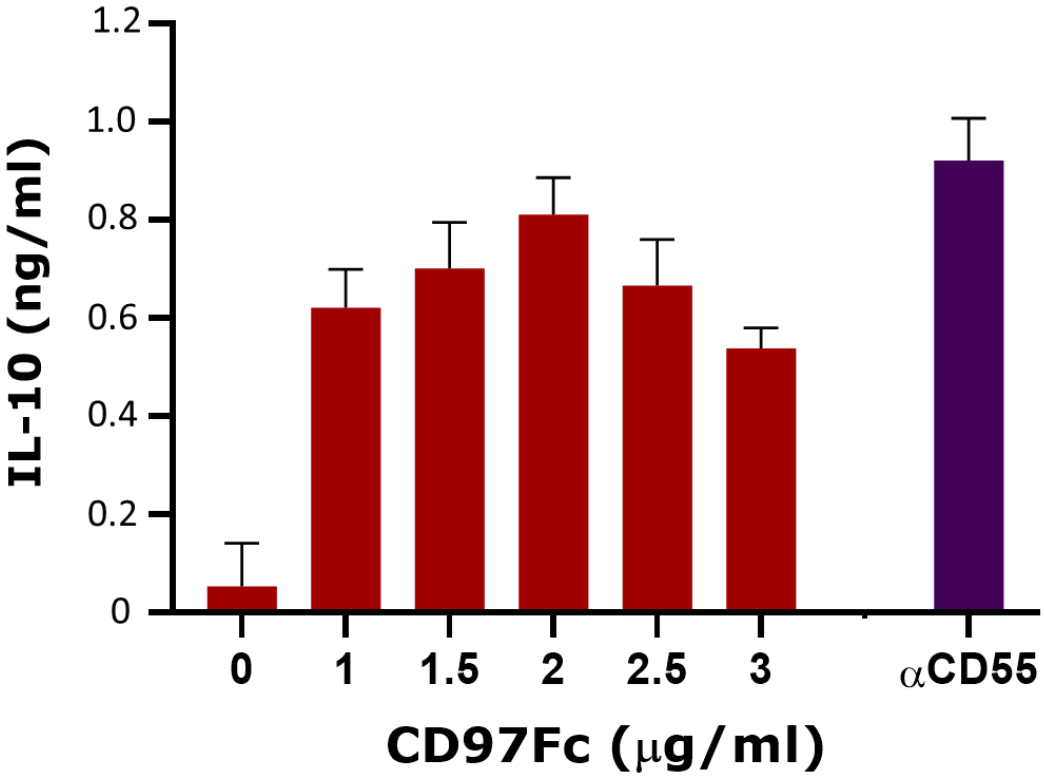


Figure 3.16: Costimulation of CD4⁺ T cells by titration of CD97Fc at a fixed concentration of anti-CD3 (1μg/ml) resulted in IL-10 secretion in culture to similar levels achieved by anti-CD55 costimulation.

$CD4^+$ T cells isolated from peripheral blood of healthy donors, were stimulated with plate bound CD3 and/or titration of CD97Fc. Culture supernatant was harvested after 24 hours of stimulation and assayed for IL-10 using ELISA technique. Results are mean ± S. D from three donors in three independent experiments.

High amount of IL-10 was secreted following CD3/CD97Fc costimulation of $CD4^+$ T cells, but the cells responsible for the IL-10 production are not known. To measure IL-10 expression by cells, the cytokine secretion assay technique (2.9.2) was utilised.

The expression of IL-10 by stimulated cells increased in a dose dependent manner from 1 µg/ml of CD97Fc (3.79%) and peaked at 2 µg/ml (4.15%) and decreased to 2.87% at 3 µg/ml of CD97Fc (Figure 3.17B). The dose titration curve of CD97Fc showed a peak IL-10 expression by $CD4^+$ T cells at 2.0 µg/ml and this observation was consistent across all three donors assayed (Figure 3.17C).

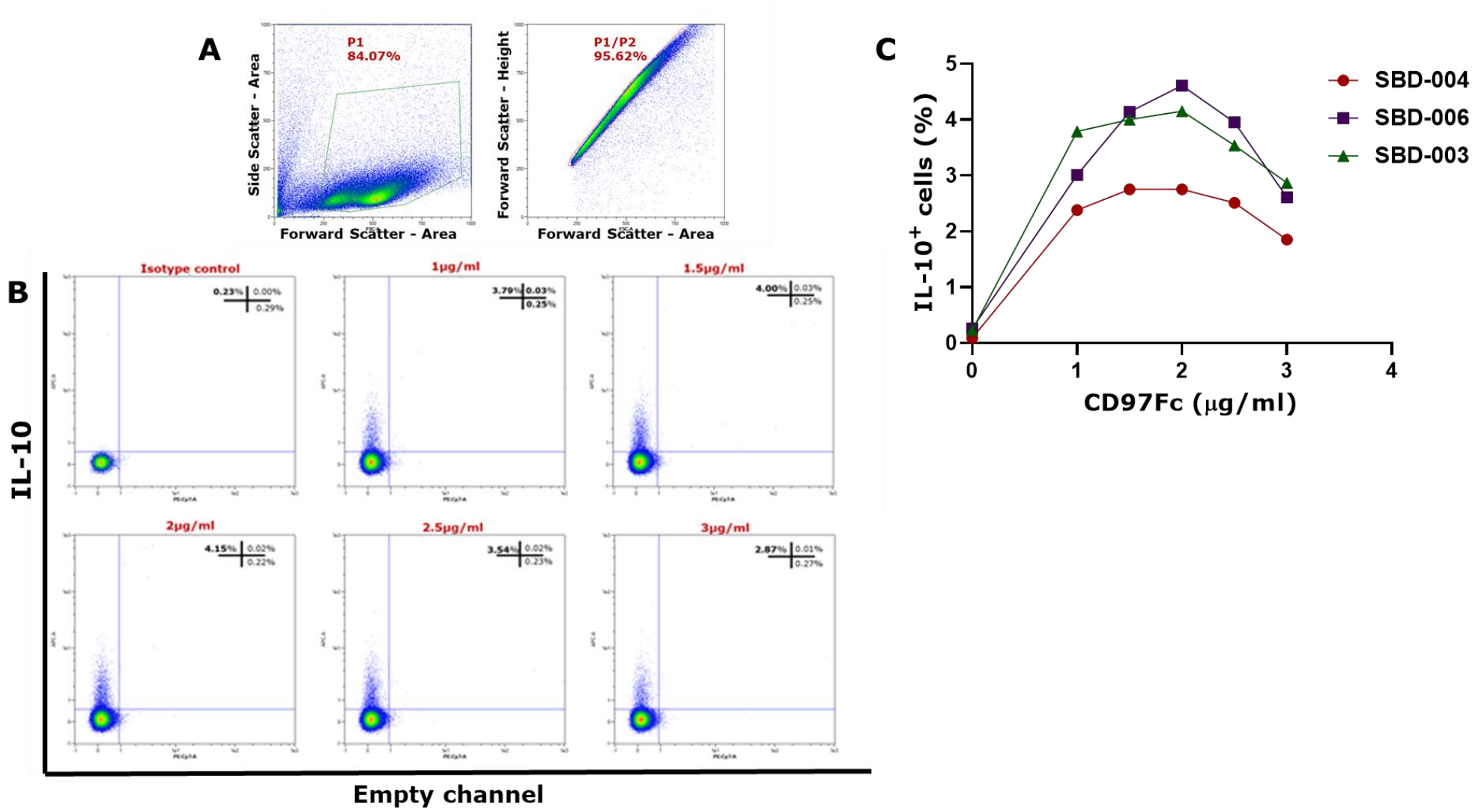

A
P1
84.07%
Side Scatter - Area
Forward Scatter - Area
P1/P2
95.62%
Forward Scatter - Height
Forward Scatter - Area
B
Isotype control
0.23%
0.00%
0.29%
1μg/ml
3.79%
0.03%
0.25%
1.5μg/ml
4.00%
0.03%
0.25%
2μg/ml
4.15%
0.02%
0.22%
2.5μg/ml
3.54%
0.02%
0.23%
3μg/ml
2.87%
0.01%
0.27%
IL-10
Empty channel
C
IL-10+ cells (%)
CD97Fc (μg/ml)
SBD-004
SBD-006
SBD-003

Figure 3.17: Dose titration of CD97Fc at fixed concentration of anti-CD3 (1µg/ml) resulted in IL-10 secretion by $CD4^+$ T cells in a dose dependent manner up to 2µg/mL.

$CD4^+$ T cells, isolated from peripheral blood of healthy donors, were stimulated with plate bound CD3 and titration (1, 1.5, 2, 2.5 and 3 µg/ml) of CD97Fc. **A** Analysis was carried out on a forward scatter – area, side scatter – area and doublet discrimination on a forward scatter – area, forward scatter – height plot. **B** percentage of cells producing IL-10 in the cytokine secretion assay at 24 hours costimulation in one donor. **C** Percentage of IL-10 positive cells increased with CD3/CD97Fc costimulation in all three donors.

Costimulation of naïve $CD4^+$ T cells with antibodies against CD3/CD55 revealed a small percentage of IL-10 positive cells. Our lab previously demonstrated that it takes up to three days for a naïve $CD4^+$ T cells to secrete effector cytokines while effector cells typically respond quicker. Therefore, this study sought to investigate the existence of these IL-10 positive cells within the $CD4^+$ T cell pool in human peripheral blood.

$CD4^+$ T cells purified from human peripheral blood were stimulated with anti-CD3/anti-CD55 or anti-CD3/CD97Fc for 24 hours. Results showed that stimulation of cells with anti-CD55 antibody or CD97Fc (natural ligand for CD55) resulted in IL-10 expression by a small percentage of cells (Figure 3.18 A). This observation was consistent across five donors assayed (Figure 3.18 B).

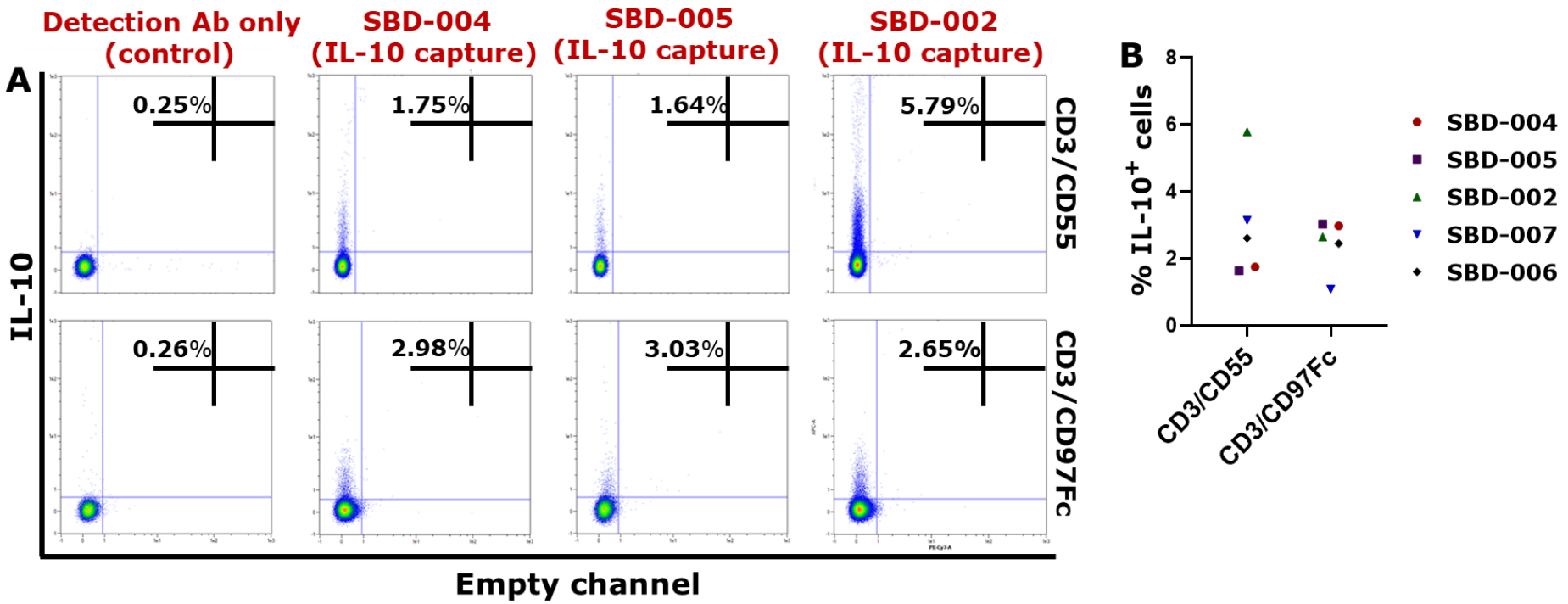


Figure 3.18: Percentage IL-10 expression following CD3/CD55 or CD3/CD97Fc costimulation of $CD4^+$ T cells for 24 hours

$CD4^+$ T cells isolated from PBMCs of healthy donors and costimulated with plate bound anti-CD3/CD55 or CD97Fc for 24 hours. **A** IL-10 expression measured by cytokine secretion assay against detection Ab (only) control for three donors. **B** percentage IL-10 positive cells for five donors.

3.2.5: IL-10$^+$ cells went through several rounds of proliferation cycles upon costimulation with CD3/CD97Fc

Tr1 cells have previously been reported from several *in vitro* studies to be anergic, non-proliferating IL-10 producing cells (Levings & Roncarolo, 2000). However, since TCR engagement via CD3 stimulation together with CD97Fc costimulation resulted in proliferation of cells and blasts formation (Figure 3.13) leading to IL-10 expression by a small percentage of cells (Figure 3. 18). The aim of this assay was to demonstrate that the IL-10$^+$ cells in our model proliferate. It's likely that IL-10$^-$ cells would go through more proliferation cycles than IL-10$^+$ cells as the kinetics of IL-10$^+$ cells in our model peaks earlier (24 to 36 hours) while the IL-10$^-$ cells peaks later (30 to 48 hours). We thought to assess the proliferative capacity of these IL-10$^+$ cells following CD3/CD97Fc costimulation.

CD4$^+$ T cells were stained with Cell Trace Violet proliferation dye (described in 2.6.2) and costimulated with CD3/CD97Fc for 72 hours followed by IL-10 CSA to assess IL-10 expression and cell proliferation (Figure 3.19). Results showed that IL-10$^+$ cells (1.97% to 3.95%) went through multiple rounds of proliferation cycles together with the IL-10$^-$ cells for the three donors assessed. (Figure 3.19C).

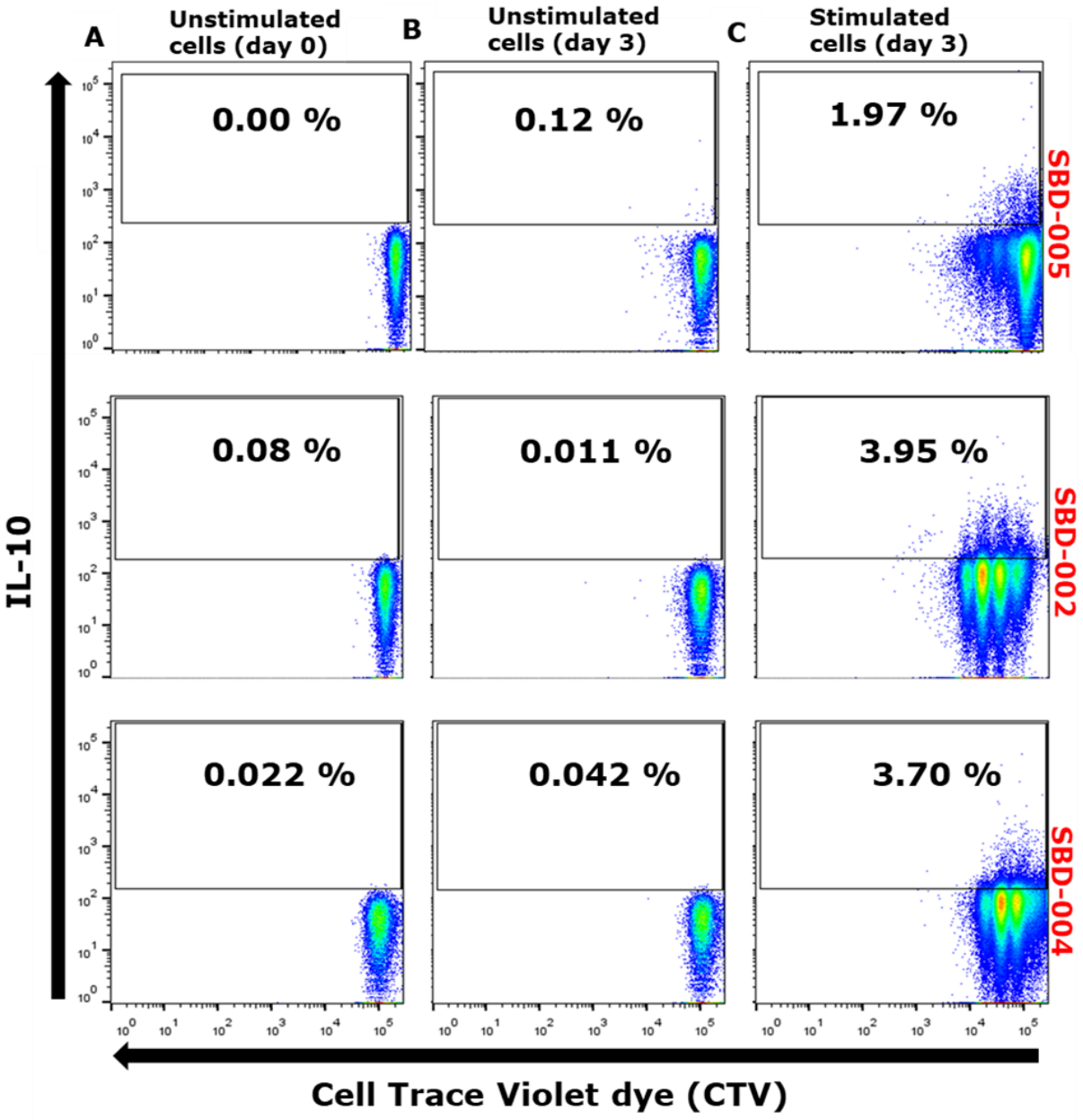


Figure 3.19: IL-10 producing cells proliferate after costimulation of CD4$^+$ T cells with anti-CD3/CD97Fc for 72 hours

CD4$^+$ T cells were labelled with CellTrace Violet (CTV) dye and costimulated with anti-CD3/CD97Fc for 72 hours. Proliferation was assessed by CTV dye dilution and IL-10 expression measured using the cytokine secretion assay. **A** CTV labelled cells before stimulation. **B** CTV labelled cells in culture (unstimulated). **C** IL-10 expression by CTV labelled cells. Data shown are for three donors in three independent experiments.

3.3: Discussion

The interactions between CD97 and its cellular ligand CD55 was assessed based on their ability to activate, drive proliferation and cytokine production in a population of peripheral blood $CD4^+$ T cells. Data showed that CD55-CD97 costimulation together with TCR engagement resulted in cell proliferation and cytokine production on both naïve $CD4^+$ T and total $CD4^+$ T cells. However, anti-CD55 antibodies or CD97Fc alone failed to stimulate $CD4^+$ T cells, indicating the need for TCR engagement in T cell activation.

TCR engagement together with CD55-CD97 interactions initiate signalling events within T cells that enhance proliferation and cytokine secretion. This bares great similarities with CD28 costimulation, which initiates a complex series of signalling events leading to T cell growth, cytoskeleton reorganisation, proliferation, and secretion of cytokines. Activation of these signalling pathways result in increased intracellular calcium levels, activation of MAPKs (mitogen activated protein kinases), activation of PI3K (phosphoinositol-3-kinase) and translocation to the nucleus of transcription factor NF-κB (Acuto & Michel, 2003; Takeda et al., 2008). However, crosslinking of CD55 with monoclonal antibody (mAb) results in phosphorylation of Fyn and Lck, an effect also seen with CD97 engagement of CD55(Shenoy-Scaria et al., 1992b). Fyn and Lck are strongly associated with the TCR complex and the activation Fyn and/or Lck phosphorylate ITAM (immunoreceptor tyrosine-based

activation motifs) provides a mechanism by which CD55 and other costimulatory molecules can enhance TCR stimulation of T cells (J. R. Hwang et al., 2020). However, while it is thought that CD28 costimulation promotes the translocation of nuclear factor kappa B (NF-κB) to the nucleus where it initiates transcription of the IL-2 gene and other genes essential for T cell proliferation(L. P. Kane, Lin, & Weiss, 2002), the mechanisms that CD97 engagement of CD55 promotes TCR signalling resulting in T cell activation is still not fully understood. Additionally, there must be a simultaneous engagement of TCR-MHC for CD55-CD97 to be able to stimulate T cells. When CD55 costimulation of naïve $CD4^+$ T cells was studied, it was observed that naïve cells had the capacity to differentiate into a small population of IL-10 producing cells. Further analyses by IL-10 capture assay showed that this small population of cells was responsible for the high IL-10 production in culture supernatant (Sutavani et al., 2013) demonstrated the capacity of CD55 engagement to preferentially induce IL-10 production from naïve $CD4^+$ T cells when compared with CD28 suggesting a role for CD55 in the induction of Tr1-like cells. However, the role of CD97 in relation to IL-10 production by peripheral blood $CD4^+$ T cells has not been fully described. Here, we showed that CD97, through engagement with its cellular ligand CD55, in combination with CD3 stimulation induced the activation of $CD4^+$ T cells from human peripheral blood leading to IL-10 production.

Stimulation of $CD4^+$ T cells with mAb against CD3 showed activation of the cells in a dose dependent manner as measured by thymidine uptake. There was a gradual increase in cell proliferation with increase in TCR engagement to levels where no further increase was observed by increasing the stimulus. TCR engagement alone was able to stimulate $CD4^+$ T cells because of the possibility that they are an already primed population of cells with lower activation threshold which makes them able to respond to very low concentrations of stimuli (Berard & Tough, 2002). Furthermore, effector cells which have been reported to have a greater frequency of antigen specific responses are stimulated by all APC types including resting B cells and to some extent by macrophages (Croft et al., 1994). CD55 costimulation led to greater activation and proliferation of cells to levels higher than observed with anti-CD3 alone. This demonstrated that CD55 costimulation enhanced the proliferation of $CD4^+$ T cells leading to secretion of effector cytokines including IL-2 necessary for cell proliferation (Capasso et al., 2006a; Sutavani et al., 2013).This is not the case for naïve $CD4^+$ T cells as they are antigen inexperienced and the conditions for activating them must be exactly right. Therefore, TCR engagement alone has been reported to induce proliferation of only a percentage of naïve $CD4^+$ T cells and in the absence of costimulation, these cells become anergic or unresponsive (Appleman & Boussiotis, 2003; Haynes, Eaton, & Swain, 2002). Our data showed that suboptimal TCR engagement with optimal CD55 costimulation resulted in cellular activation, proliferation, and the

production of high amount of IL-10 by naïve $CD4^+$ T cells, again demonstrating an absolute need for costimulation in the differentiation of naïve cells.

In this study, human CD97 isoform (EGF 1,2,5) was generated and validated. This isoform is reported to bind to CD55 with highest affinity and the construct was specifically generated to study the CD97-CD55 interactions as its necessary for the binding of CD55 (Abbott et al., 2007). Data showed that plate bound CD97Fc together with anti-CD3 showed strong costimulation of $CD4^+$ T cells leading to cellular activation and proliferation. However, it was observed that proliferation and IL-10 secretion decreased as the strength of CD97Fc increased beyond 2.5ug/ml. The reason for this observation was not immediately clear but it might be that IL-10 positive (regulatory) cells are more sensitive to costimulation and therefore at lower signal strength are triggered in preference to proinflammatory cells and increasing the signal strength dampens the response and may represent another mechanism of regulating plasticity in T cells. It might also be as a result of The Hook (prozone) effect in which the ability of CD97Fc to form immune complex reduces with increase in concentration.

Of note is the fact that CD97-CD55 engagement alone did not have any significant effect on the T cells, but costimulation via CD3 and CD97 demonstrated a great capacity to stimulate $CD4^+$ T cells to levels significantly higher than observed for CD3 stimulation alone. Notably,

our data showed CD97Fc costimulation of $CD4^+$ T cells led to the secretion of IL-10, typically ≤ 5% of cells. The small population of IL-10^+ cells observed in his study was consistent with data previously generated by other researchers in our laboratory and were consistent among multiple donors in this study.

The cytokine milieu is crucial in determining the quality of T cell responses to exogenous antigens and based on the cytokine profile, $CD4^+$ T cells can be divided into subsets. T_H1 cells promote cell-mediated immune responses by producing IFN-γ, while the T_H2 cells enhance antibody-mediated immune responses by producing high levels of IL-4, IL-13, and IL-5. However, novel $CD4^+$ T cell subset often termed Tr1 cells are thought to promote immune resolution and immunosuppression by producing high levels of immunoregulatory cytokines IL-10 and /or transforming growth factor (TGF)-β (Z. Li, Liu, Guo, & Wang, 2016; O'Garra, 1998). The anti-inflammatory role of Tr1 cells is mediated in part by production of high levels of IL-10 which dampen the function of both antigen-presenting cells and antigen-specific effector T cells (N. Chihara, A. Madi, K. Karwacz, A. Awasthi, & V. K. Kuchroo, 2016). Levings et al., demonstrated that treatment of T cells with exogenous IL-10 resulted in a long-lasting state of antigen-specific T-cell unresponsiveness, irreversible by CD3/CD28 costimulation or the presence of IL-2 (Levings & Roncarolo, 2000). Furthermore, IL-10 has been reported to strongly reduce the antigen specific proliferation of T cells by both indirect and direct mechanisms:

indirectly by downregulating the expression of MHC class II, CD80 and CD86; and directly by inhibiting IL-2 production, thereby promoting cell cycle arrest (Sieg, King, Huang, & Kaplan, 1996; Taga & Tosato, 1992).

However, our data showed that $CD4^+$ T cells went through several proliferation cycles upon costimulation with CD97Fc which may suggest that the IL-10 produced by the cells did not interfere with cellular proliferation. Interestingly, the data also showed that IL-10 positive cells went through several rounds of proliferation cycles following CD3/CD97Fc costimulation. These results suggest that IL-10 positive cells in the peripheral $CD4^+$ T cell pool are responsive to CD55 costimulation. Several mechanisms of peripheral tolerance have been proposed including the death or development of a state of non-responsiveness by T cells (T cell anergy) or by active suppression of T cell responses and Tr1 cells have been shown to play crucial role in peripheral tolerance (Fathman & Lineberry, 2007; Kalekar et al., 2016; Levings & Roncarolo, 2000; Macián, Im, García-Cózar, & Rao, 2004). Several groups have used exogenous IL-10 in inducing anergic conditions in $CD4^+$ T cells and have described the resulting cells as Tr1 cells. Here we showed that the high amount of IL-10 observed are a consequence of CD55 costimulation and are produced by a small population of actively dividing cells.

Chapter 4
Identifying Tr1 cells in human peripheral blood

4.1: Introduction

Tr1 cells have been differentiated and expanded *in vitro,* but the presence of Tr1 cells in human peripheral blood (*ex vivo),* is yet to be fully defined. The role of Tr1 cells in maintaining peripheral tolerance has been well established(Battaglia, Gregori, Bacchetta, & Roncarolo, 2006; Groux, 2003), which makes characterizing these cells in human peripheral blood pertinent. However, the molecular mechanisms and phenotype of Tr1 cells have not been fully understood since discovery by Groux group in 1997 (Groux et al., 1997).

Most studies, both *in vitro* and *in vivo*, have demonstrated the induction and expansion of Tr1 cells through: stimulating human peripheral blood mononuclear cells (PBMCs) with allogeneic monocytes in the presence of high dose IL-10 (Groux et al., 1997); the use of immature dendritic cells (Roncarolo, Levings, & Traversari, 2001); the use of vitamin D3 in the presence of dexamethasone through autocrine IL-1 production (Barrat et al., 2002) and rapamycin (Battaglia, Stabilini, et al., 2006; Bergmann, Strauss, Zeidler, Lang, & Whiteside, 2007); the use of IL-10 with IFN-α (Levings et al., 2001); influence of DC-10 (S. Gregori et al., 2010) and tolerogenic DCs (Weiner, 2001a); IL-6 (Jin, Han, & Yu, 2013) or in the presence of IL-27 (Apetoh et al., 2010). Other groups have

used alternate co-stimulation with antibodies against molecules like CD46, CD2 (CD58 ligand) and CD55 to differentiate naïve $CD4^+$ T cells into Tr1 cells (Kemper et al., 2003; Sutavani et al., 2013; Wakkach, Cottrez, & Groux, 2001) while *in vivo* data from Burton et al., showed the induction of $CD4^+IL\text{-}10^+$ T cells by dose escalation protocol by administration of soluble acetylated N-terminal peptide (Ac1-9) of Myelin Basic Protein [MBP Ac1-9(4Y)] containing the immunodominant $CD4^+$ T cell epitope (Burton et al., 2014). It has also been proposed that fully differentiated Th1 and Th2 cells can be converted into Tr1 cells through chronic stimulation (Hawrylowicz & O'Garra, 2005).

Unlike natural regulator T (nTreg) cells that emerge from the thymus after thymic selection with a defined phenotype ($CD3^+CD4^+CD25^{hi}CD127^{lo}FoxP3^+$) (Asano, Toda, Sakaguchi, & Sakaguchi, 1996; Fontenot, Dooley, Farr, & Rudensky, 2005), Tr1 cells have a phenotype that results from *in vitro* / *in vivo* induction, and differs depending on their mode of induction. However, the hallmark of their activity has been the production of high levels of IL-10 with little or no other cytokine production (Groux et al., 1997; Kunicki, Amaya Hernandez, Davis, Bacchetta, & Roncarolo, 2018; Moore, de Waal Malefyt, Coffman, & O'Garra, 2001). Whether these cells are a differentiated population of Tr1 cells or an induced phenotype of IL-10 producing cells converted from Th1/Th2, for example, is yet to be confirmed.

Upon T cell receptor (TCR) mediated activation, T cells are primed to differentiate, proliferate, and secrete an array of relevant cytokines following co-stimulation. Sutavani et al*.,* in their work demonstrated how TCR engagement (with anti CD3) and CD55-CD97 interaction drive the differentiation of Tr1 cells from naïve precursors (Sutavani et al., 2013). The study showed that CD55 costimulation (signal 2) induced *in vitro* differentiation of naïve $CD4^+$ T cells to Tr1 cells. However, proper phenotyping of Tr1 cells in human peripheral pool would have far reaching applications in both cancer and autoimmune conditions.

Effector cells do not have the absolute need for costimulation upon antigen re-challenge but would rapidly expand and secrete cytokines in response to antigen specific stimulation (Farber et al., 2016). The costimulatory ability of CD55 via CD97 interactions is well established (Capasso et al., 2006a) and this interaction has been shown to drive Tr1 induction *in vitro*. This supports the idea that once differentiated from naïve precursors, Tr1 cells respond to CD55 costimulation to maintain a committed phenotype.

We therefore hypothesised that Tr1-like cells exist as a differentiated population in human peripheral blood and responded to CD55 costimulation, maintaining the phenotype identified *in vitro*.

4.2: Results

4.2.1 Kinetics of IL-10 production are faster than for IFN-ɣ

Immune cells respond to antigen specific stimulation, proliferate, and secrete of cytokines which modulate immune responses. These cytokines possess either pro-inflammatory or anti-inflammatory functions and their peak secretion times differ depending on immune cell type (Kum, Cameron, Hung, Kalyan, & Chow, 2001; Romagnani, 1994). As shown in the previous chapter (Figure 3.17), naïve $CD4^+$ T cell required longer stimulation time (72 hours) for cytokine expression compared to the effector counterpart which have shorter kinetics. However, the kinetics of pro inflammatory cytokine (IFN-ɣ) and anti-inflammatory cytokine (IL-10) by $CD4^+$ T cells following specific stimulation by CD3/CD55 has not been previously reported. Immunologically, proinflammatory and anti-inflammatory cells are capable of cross regulating the functions of each other by their signature cytokine profiles. Therefore, the hallmark of Tr1 cells is the production of high amounts of the anti-inflammatory cytokine, IL-10 (Roncarolo et al., 2018) while Th1 cells produce mainly IFN-ɣ (pro-inflammatory cytokine) (J. Chen & X. Liu, 2009). Some studies also report Th1 cells as being able to produce IL-10 (Cope, Le Friec, Cardone, & Kemper, 2011).To distinguish these two cell phenotypes, we used dual surface capture assay to measure the presence of single and double positive cells (IL-10 & IFN-ɣ).

IL-10 mediated suppression of Th1 cells is widely reported (Cope et al., 2011), therefore the kinetics of IL-10 expression was ascertained following time course costimulation of $CD4^+$ T cells with CD3/CD55.

$CD4^+$ T cells were purified from peripheral blood of healthy donors, rested overnight, and stimulated with plate bound anti-CD3/anti-CD55 antibodies. Optimisation and gating strategy for dual cytokine (IL-10 & IFN-ɣ) capture assay was established as shown in Figure 4.1. Optimum cytokine production was assessed using a nine- point, 54-hour time course (Figure 4.2). Cytokine production was observed to have started as early as 6 hours after stimulation (Figure 4.2). Peak cytokine production occurred at 24 hours for IL-10 and 30 hours for IFN-ɣ, indicating a faster IL-10 kinetics with CD3/CD55 stimulation.

The observation that IL-10 secretion peaked around 24 hours was consistent across multiple donors (Figure 4.3 A). Figure 4.3B shows that IFN-ɣ secretion by $CD4^+$ T cells reached peak amounts between 30 to 36 hours and was consistent across multiple donors while a small percentage of double positive (IL-10 and IFN-ɣ) cells peaked between 24 and 36 hours (Figure 4.3C).

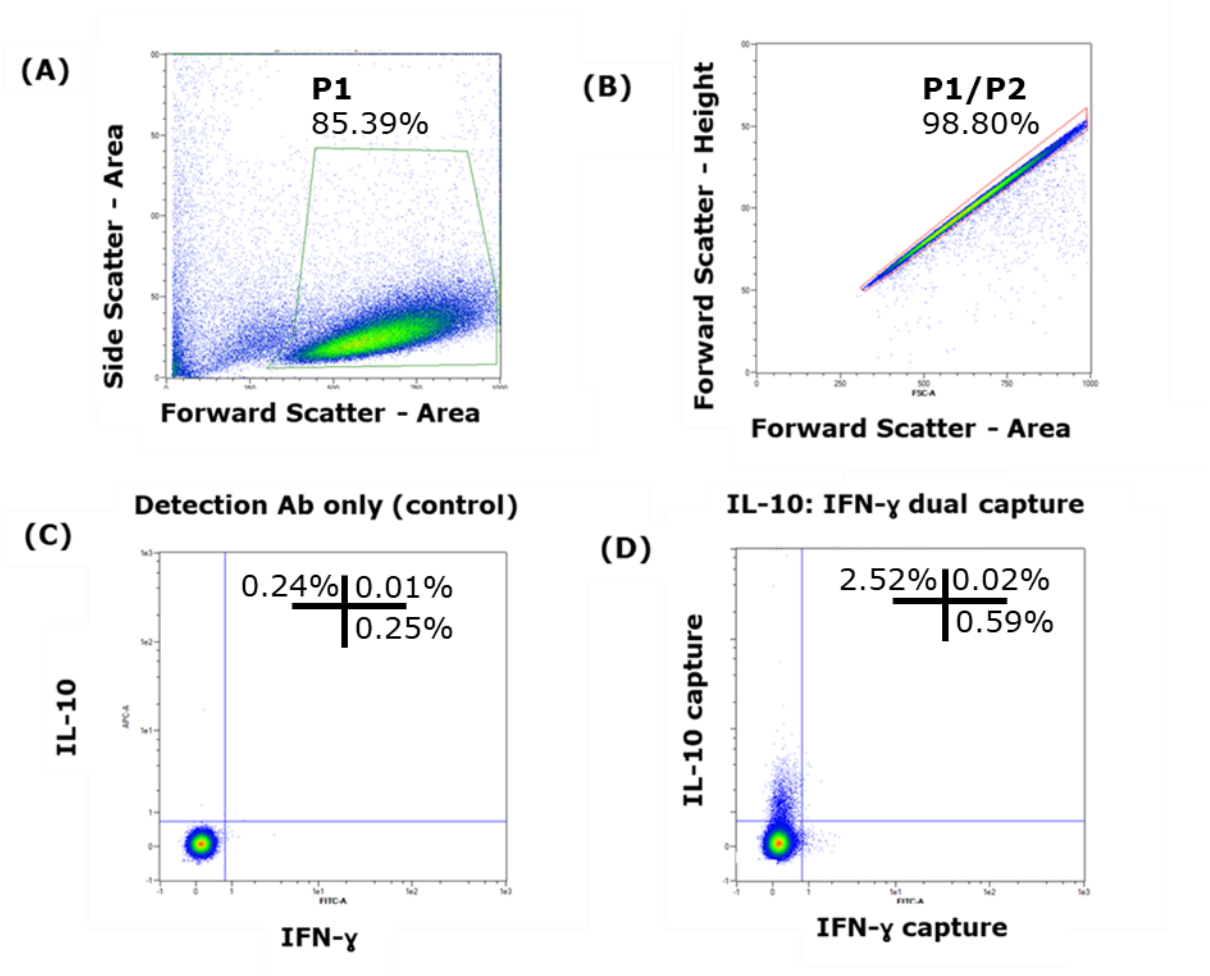


Figure 4.1: Determining the IL-10 and IFN-ɣ populations in purified CD4⁺ T cells

Flow cytometer plots showing IL-10 and IFN-ɣ gates set at the junction of cytokine-negative and cytokine-positive cell populations checked against control conditions. **(A)** dead cell exclusion on forward scatter-area, side scatter – area. **(B)** doublet discrimination and cell clumps exclusion by forward scatter- area, forward scatter – height. **(C)** IL-10/IFN-ɣ dual capture with detection antibody only – control **(D)** Assessment of cytokine expression (IL-10/IFN-ɣ) following cytokine secretion assay of T cells stimulated for 24 hours with CD3/CD55. Same gating approach was used for experiments with CD3/CD28 or Phorbol-Myristate-Acetate and Ionomycin (PMA/I).

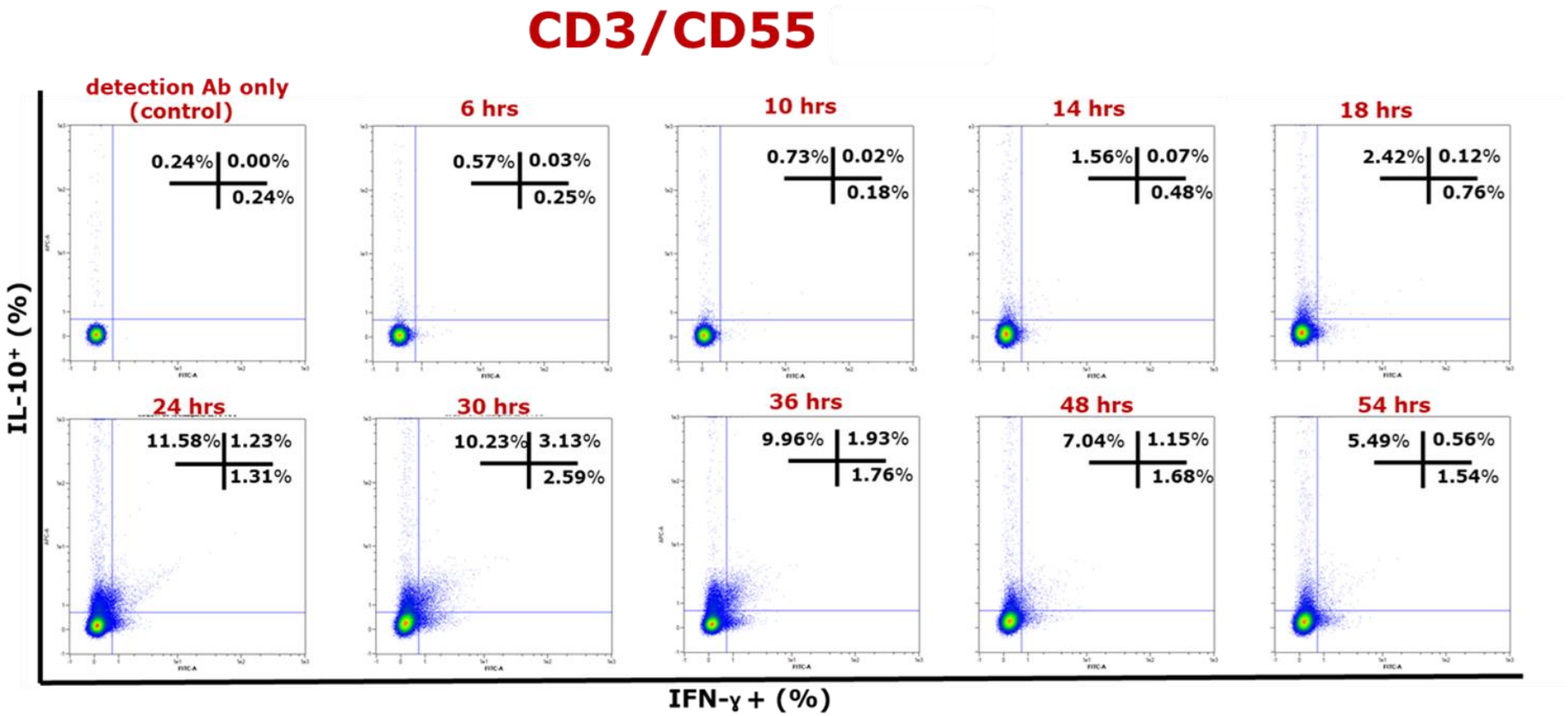


Figure 4.2: Kinetics of IL-10 and IFN-ɣ expression over 54 hours following CD3/CD55 costimulation

$CD4^+$ T cells, isolated from peripheral blood of healthy donors were stimulated with plate bound CD3/CD55. Time course (6, 10, 14, 18, 24, 30, 36, 48, 54 hours) assessment of IL-10 and IFN-ɣ expression was carried out using the cytokine capture assay. Flow cytometry plots showing cytokine expression (IL-10, IFN-ɣ and double positive cells) for single donor. Data shown is from a single donor and representative of three independent experiments.

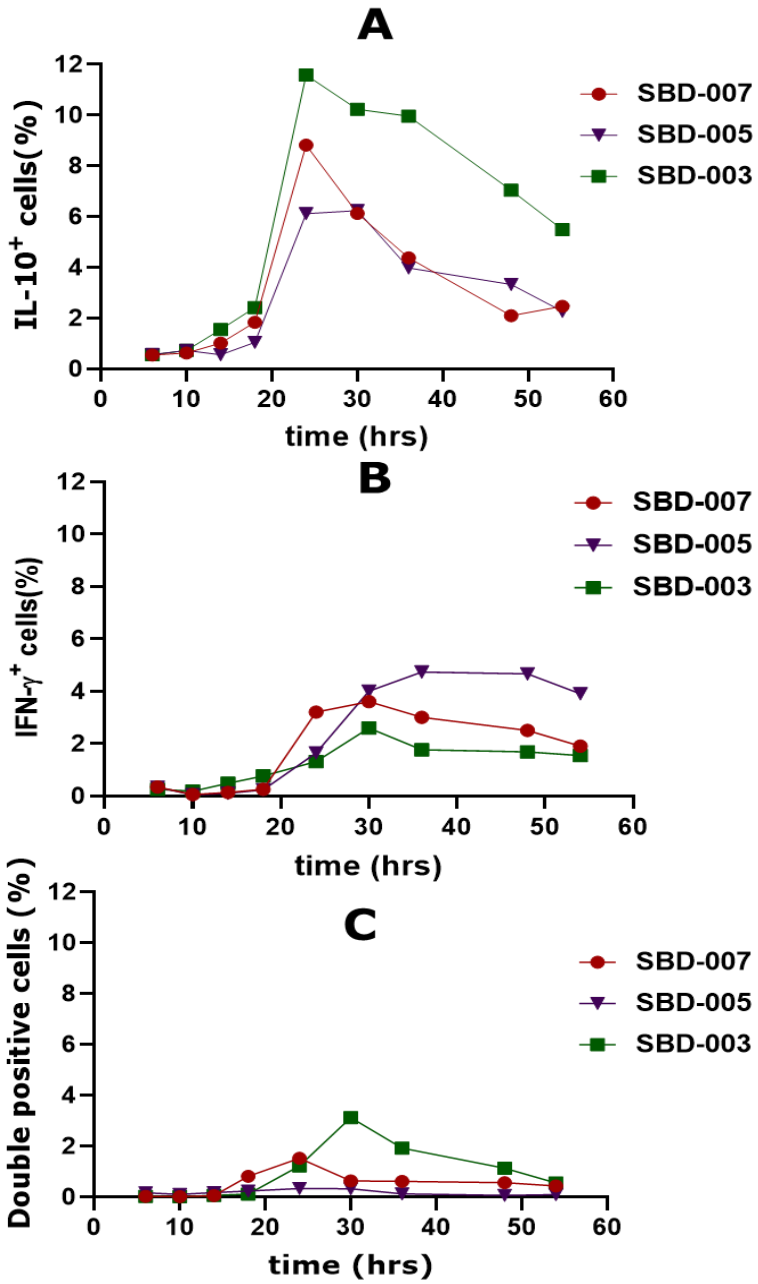


Figure 4.3: Kinetics of IL-10 positive, IFN-ɣ positive and IL-10/IFN-ɣ double positive cells

$CD4^+$ T cells, isolated from peripheral blood of healthy donors and stimulated with plate bound CD3/CD55 over 54hours time course was assessed for IL-10 and IFN-ɣ. IL-10 and IFN-ɣ expression was measured by cytokine secretion assay. **A** percentage of IL-10 positive cells, **B** percentage of IFN-ɣ positive cells and **C** percentage of cells expressing both IL-10 & IFN-ɣ (double positive) at different time points after stimulation. Plots show data from three donors.

4.2.2: IL-10 accumulates in culture supernatant following CD3/CD55 costimulation of $CD4^+$ T cells

Kinetic analysis showed that the number of IL-10 positive cells peaked at 24 hours and before the IFN-ɣ positive cells at 30 hours. However, the fate of the secreted IL-10 remained undetermined. We tested the hypothesis that $IL\text{-}10^+$ cells do not utilise the IL-10 produced in cultures, by harvesting culture supernatant at each timepoint for Enzyme Linked Immunosorbent Assay (ELISA).

In Figure 4.4, we show that IL-10 secreted in culture supernatant was observed to have been induced as early as six hours after stimulation and maintained a steady increase through the 54-hour time course. The donors accumulated 10, 20 and 30ng/ml of IL-10 over 54 hours. This is a very high amount of IL-10 from a relatively small number of cells (6, 8 and 10%) at their peaks, showing that IL-10 produced over 54 hours appeared to accumulate in all three donors.

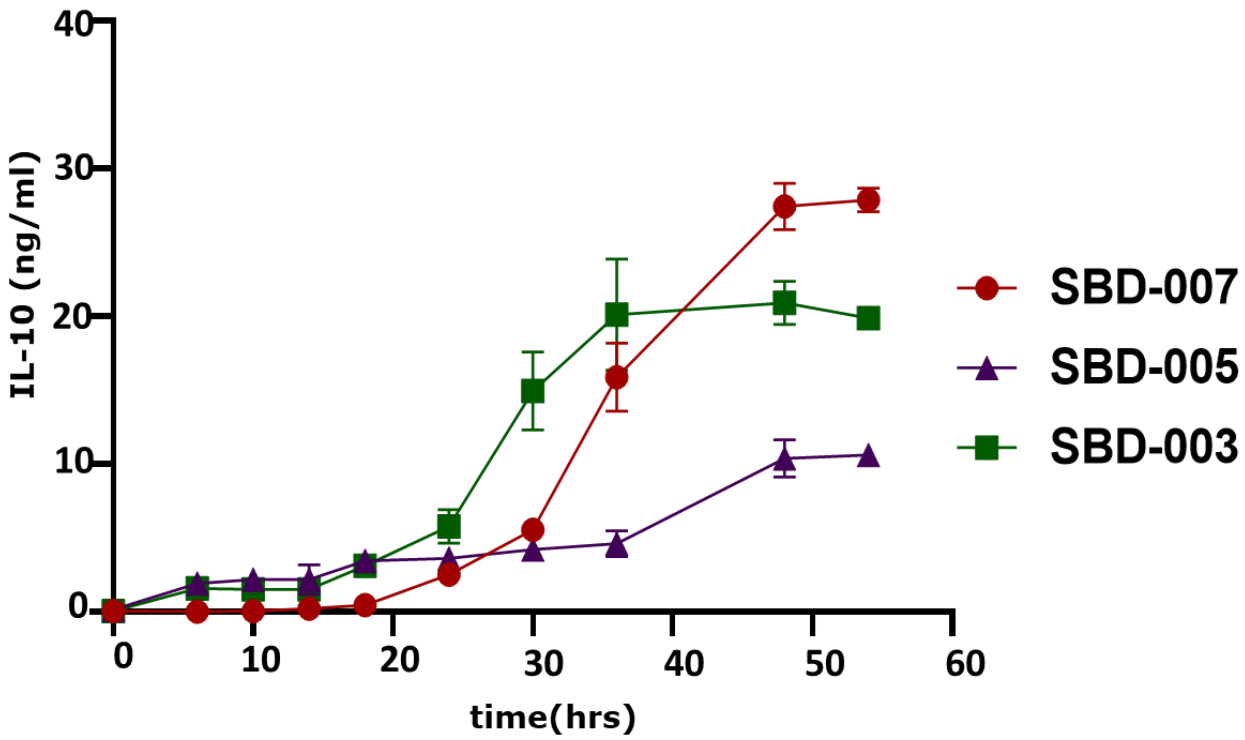


Figure 4.4: Cumulative IL-10 in culture supernatant over 54 hours, following CD3/CD55 costimulation of $CD4^+$ T cells

$CD4^+$ T cells were stimulated as indicated and ELISA performed from culture supernatants for the respective timepoints. The experiment was set up to allow culture supernatant to be sampled from different wells at each time point. Data shown are mean ±SD of triplicates from three donors.

We have shown that costimulation of $CD4^+$ T cells with CD3/CD55 induced cytokine expression over 54 hours. Kinetics of IL-10 expression peaked around 24 hours of stimulation and this time point was selected for subsequent assays involving IL-10. The kinetics of this, 24 hours, would suggest that this is the stimulation of an effector or memory phenotype, rather than the three-day response observed for naïve T cells(Sutavani et al., 2013). As effector cells should be responsive to TCR stimulation only, we used CD3 stimulation alone to determine if similar number of cells responded and cytokine production (IL-10 & IFN-ɣ) was observed.

To achieve this, $CD4^+$ T cells purified from peripheral blood of healthy human donors were stimulated with different concentrations of anti-CD3 for 72 hours. A titration of anti-CD3 antibody was used to confirm its ability to stimulate cells. $CD4^+$ T cells were stimulated with anti-CD3 for 72 hours and cell proliferation measured by tritiated thymidine incorporation during the last 15 hours of culture and even with the lowest concentration of anti-CD3, cell proliferation was observed to be considerably higher than the unstimulated control as seen in Figure 3.6.

Cytokine expression, measured by dual capture cytokine secretion assay showed that IL-10 and IFN-ɣ expression increased with increase in concentration of anti-CD3 antibody, from 0.01 µg/ml to 3 µg/ml in a dose dependent manner (Figure 4.5). Figure 4.6 shows effect of the titration of CD3 on cytokine production in three donors. Data showed that CD3 stimulation alone was enough to induce a substantial expression (1 to 3.5%) of IFN-ɣ, minimal IL-10 (0.6 to 1.16 %) and less than 1% double positive expression at the highest dose of anti-CD3.

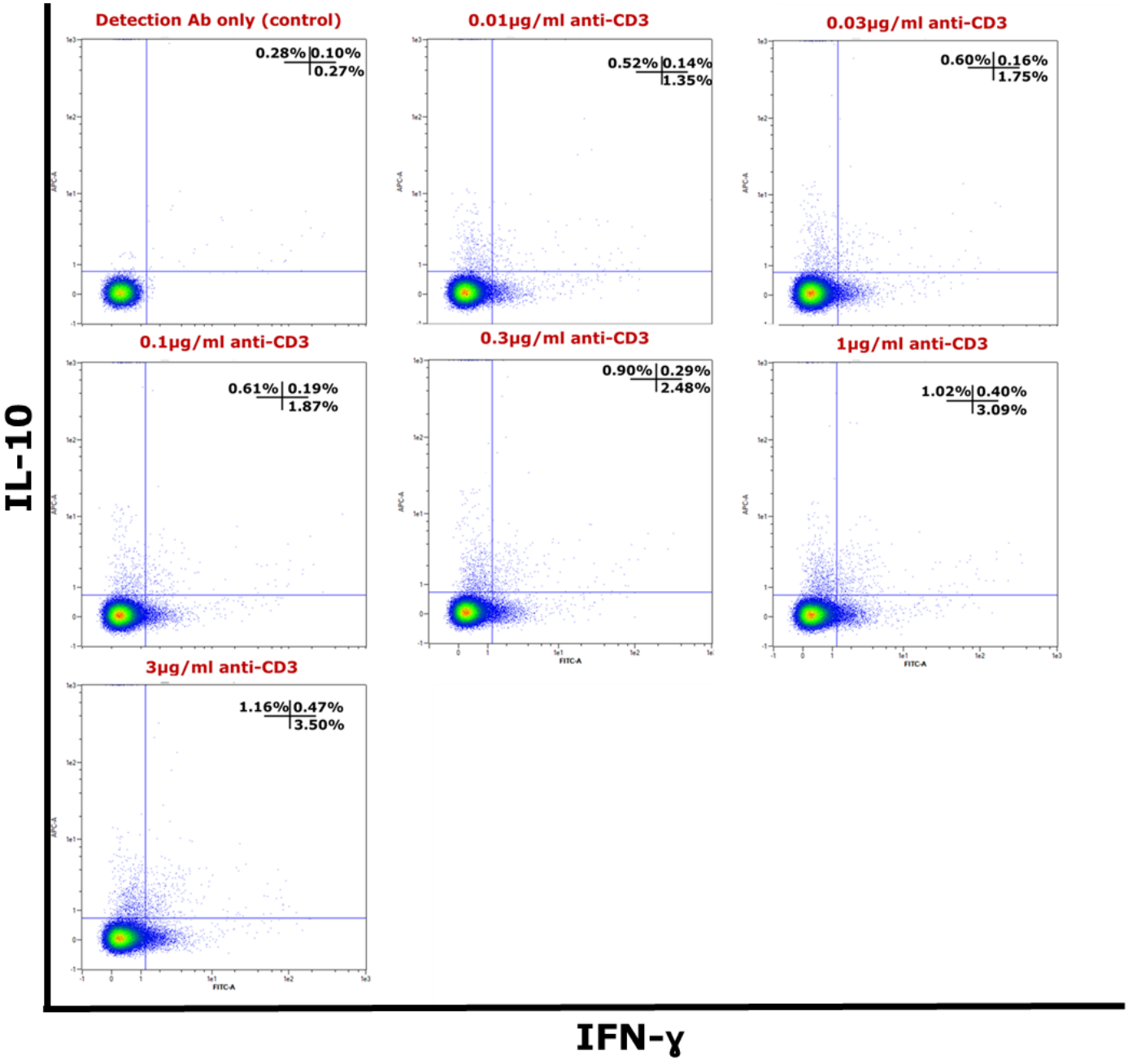


Figure 4.5: Stimulation of CD4⁺ T cells with anti-CD3 alone revealed dose dependent cytokine induction

$CD4^+$ T cells, isolated from peripheral blood of healthy donors, were stimulated with plate bound anti CD3 (0.01, 0.03, 0.1, 0.3, 1, and 3 µg/ml) at 1.5 x 10^5 cells/well for 24 hours. Flow cytometry plots show IL-10, IFN-γ and double positive cells as assessed by dual cytokine secretion assay. Data shown is for a single donor, representative of three donors.

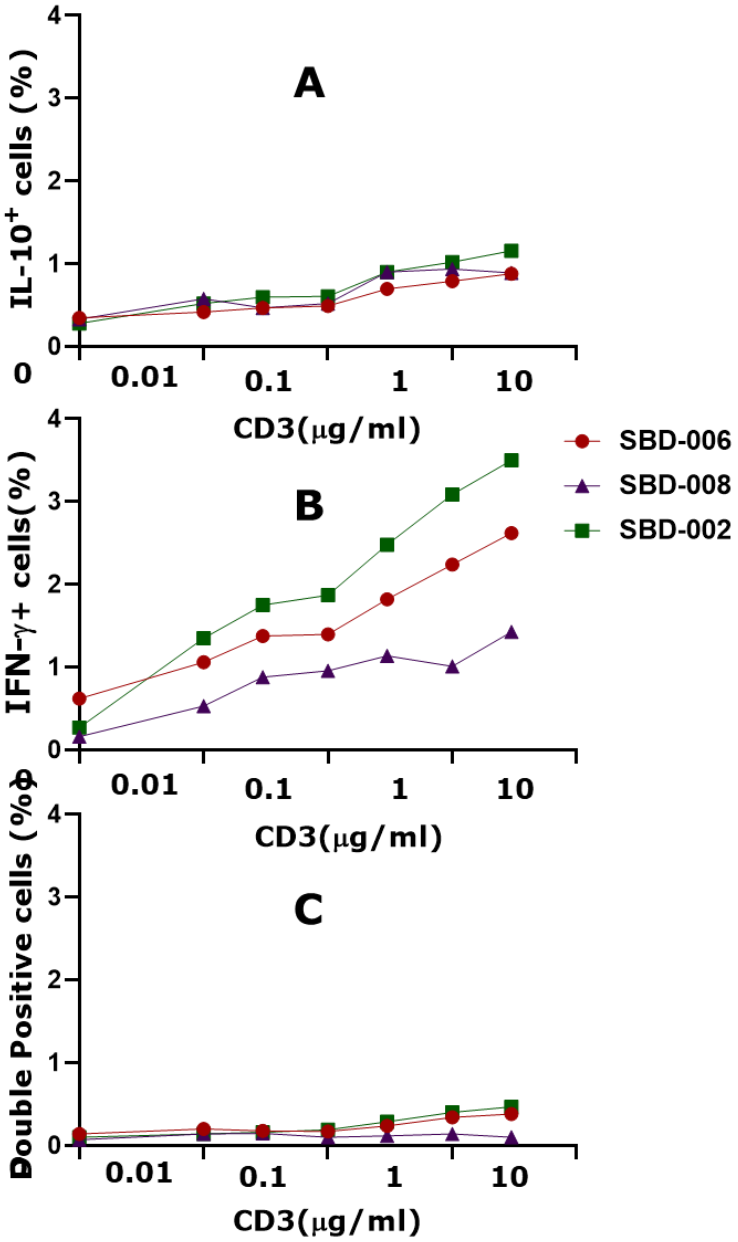


Figure 4.6: Expression of IL-10, IFN-ɣ and double positive (IL-10/IFN-ɣ) cells from titration of anti CD3

$CD4^+$ T cells, isolated from peripheral blood of healthy donors and stimulated with a titration of plate bound anti-CD3 for 24 hours. IL-10 and IFN-ɣ expression was measured by cytokine secretion assay. **A** percentage of IL-10 positive cells, **B** percentage of IFN-ɣ positive cells and percentage of double positive cells. Plots show data from three donors.

4.2.3: Proliferation of $CD4^+$ T cells via costimulation with CD3/CD55 or CD3/CD28 are equipotent

Stimulation via CD3 alone is sufficient to drive proliferation and cytokine production in effector cells. However, this restimulation might usually be mediated by antigen presenting cells for $CD4^+$ T cells. Under these circumstances, it is feasible that one of the many costimulatory molecules is engaged to enhance and sometimes modify T cell activation.

Stimulation of $CD4^+$ T cells either through CD3/CD55 or CD3/CD28 have been shown to result in cell activation, proliferation, and acquisition of effector functions at levels higher than those obtained with CD3 alone. Comparison of the two stimuli showed that CD55 costimulation is as potent as CD28 in inducing cell proliferation which were significantly higher than stimulation with CD3, CD55 or CD28 alone.

In Figure 4.7, stimulation of $CD4^+$ T cells with antibody against CD3 alone resulted in cell proliferation to a level higher than that observed for cell cultures stimulated with CD55 and CD28 alone. Cell proliferation induced by CD55 and CD28 costimulation in the presence of sub-optimal CD3 stimulation were significantly higher than proliferation seen with CD55 or CD28 alone, proliferation was comparable in magnitude between CD55 and CD28 costimulation.

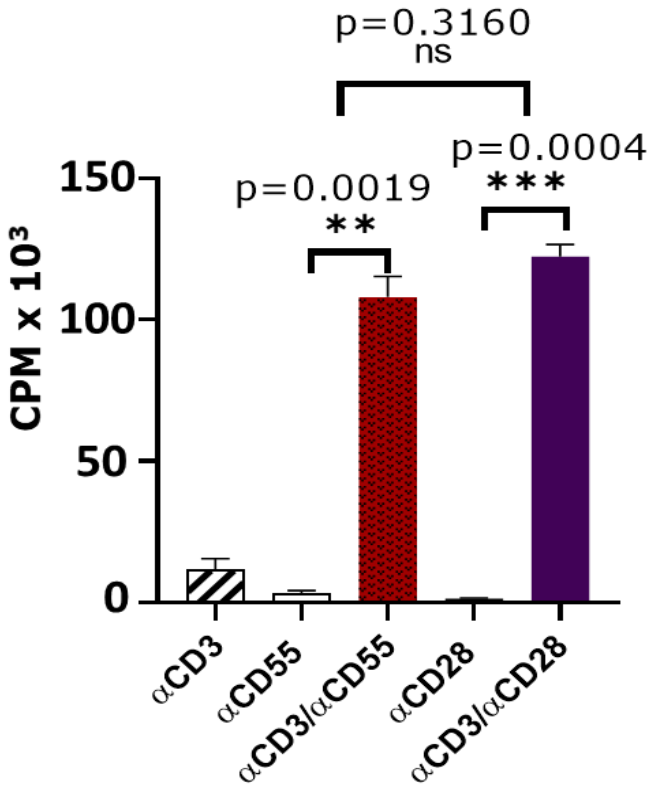


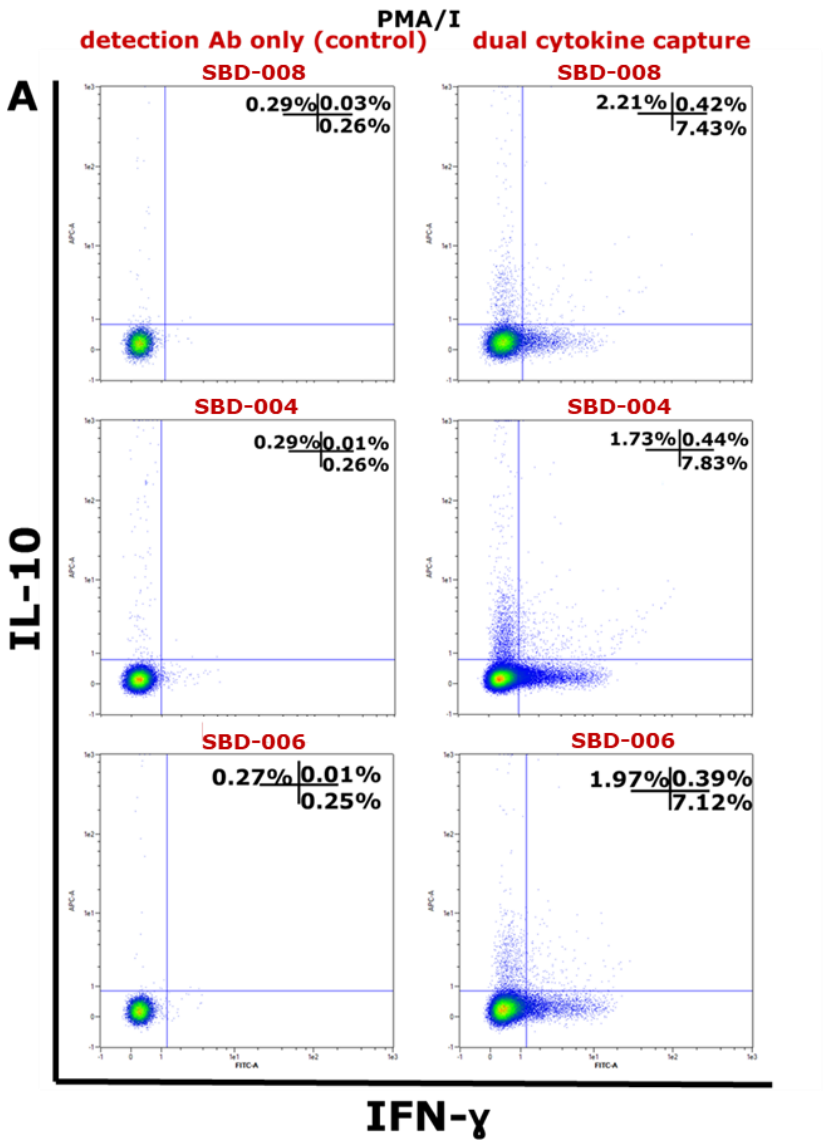
Figure 4.7: Cell proliferation in response to different stimuli, assessed by tritiated thymidine uptake

Purified $CD4^+$ T cells at 1.0 x 10^5 cells/well were stimulated with plate bound antibodies [CD3 (1µg/ml); CD55 & CD28 (5µg/ml)] as indicated, in a 96 well plate for 72 hours. Proliferation was assessed by ^{3}H-thymidine incorporation in the last 15 hours of culture. Statistical significance was determined by two-tailed, paired Student's *t* test (n = 6). Data shown (mean ± SD) from six independent experiments.
** $p \leq 0.05$, *** $p \leq 0.005$, ns = not significant.

4.2.4: Stimulation of CD4$^+$ T cells with PMA/Ionomycin, CD3/CD28 or CD3/CD55 results in a small population of IL-10 single positive cells and a population of positive for IFN-ɣ single cells

CD4$^+$ T cells effector function can be initiated using mitogens such as PMA and Ionomycin (I) that activate the protein kinase C (PKC) and calcium mobilisation pathways. Phytohemagglutinin (PHA), a mitogenic lectin, which also utilises calcium dependent – receptor independent pathway has also been used to stimulate T cells (M. K. Yong et al., 2017). This generally provides strong stimuli that activate all T cell populations leading to cytokine production, representing non-specific stimulation of CD4$^+$ T cells. During reactivation of effector and memory cells by antigen presenting cells, TCR recognition of MHC peptide provides the primary impetus for T cells function. This is supported by several other potential costimulatory molecules, including the prominent CD28 which results in the stimulation of Th1 cells

Stimulation of total CD4$^+$ T cells with PMA/I (Figure 4.8) revealed populations of IL-10$^+$ and IFN-ɣ$^+$ (single positive) cells and a small population of cells double positive for IL-10 and IFN-ɣ. In Figure 4.8A, flow cytometry plots showed about 2% of cells were single positive for IL-10 while approximately 7.46% (mean) of stimulated cells were positive for IFN-ɣ. This observation was consistent across seven donors (Figure 4.8B).


PMA/I
detection Ab only (control)
dual cytokine capture
A
SBD-008
0.29%
0.03%
0.26%
SBD-008
2.21%
0.42%
7.43%
SBD-004
0.29%
0.01%
0.26%
SBD-004
1.73%
0.44%
7.83%
SBD-006
0.27%
0.01%
0.25%
SBD-006
1.97%
0.39%
7.12%
IL-10
IFN-γ

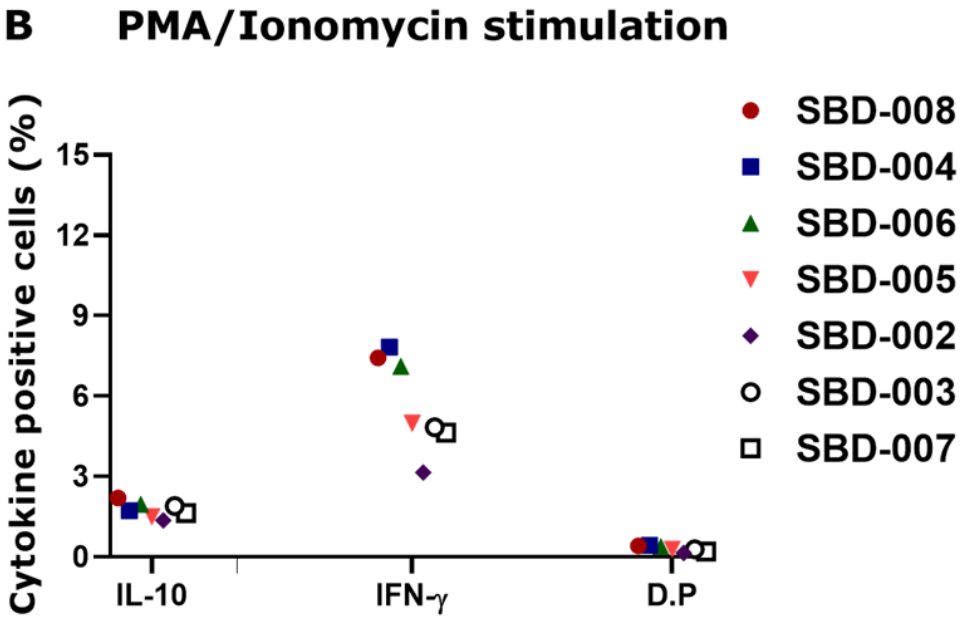
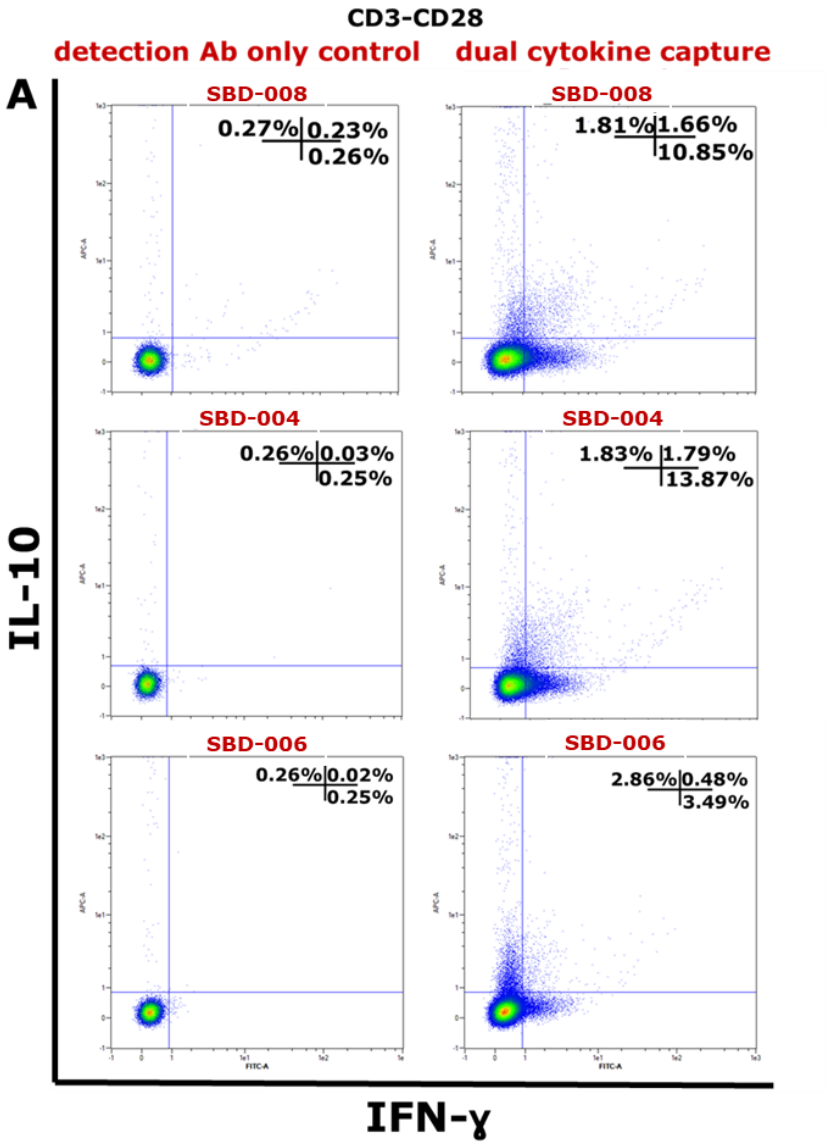

B
PMA/Ionomycin stimulation
Cytokine positive cells (%)
15
12
9
6
3
0
IL-10
IFN-γ
D.P
SBD-008
SBD-004
SBD-006
SBD-005
SBD-002
SBD-003
SBD-007

Figure 4.8: PMA/I stimulate IL-10$^+$ and IFN-γ$^+$ populations of cells in peripheral blood CD4$^+$ T cells

$CD4^+$ T cells, isolated from peripheral blood of healthy donors, were stimulated with PMA/Ionomycin (100ng/2µg /ml, respectively) for 24 hours. IL-10 and IFN-ɣ positive cells were assessed by dual cytokine capture assay. **(A)** IL-10 and IFN-ɣ surface capture with controls from three donors **(B)** percentage IL-10, IFN-ɣ and double positive (D.P) cells from cytokine secretion assay on seven donors

Having shown that PMA/I predominantly stimulated IFN-ɣ positive cells, we compared this to the classical costimulatory pair, CD3/CD28. $CD4^+$ T cells stimulated with CD3/CD28 for 24 hours (Figure 4.9), showed a robust response to CD28 costimulation leading to high expression of pro inflammatory cytokine (IFN-ɣ) and anti-inflammatory cytokine (IL-10) from separate populations of cells. Data showed that IFN-ɣ expression ranged between 3.49% and 13.87%; IL-10 between 1.79% and 4.36% across seven donors assessed (Figure 4.9B).


CD3-CD28
detection Ab only control
dual cytokine capture
A
SBD-008
0.27%
0.23%
0.26%
SBD-008
1.81%
1.66%
10.85%
SBD-004
0.26%
0.03%
0.25%
SBD-004
1.83%
1.79%
13.87%
SBD-006
0.26%
0.02%
0.25%
SBD-006
2.86%
0.48%
3.49%
IL-10
IFN-γ

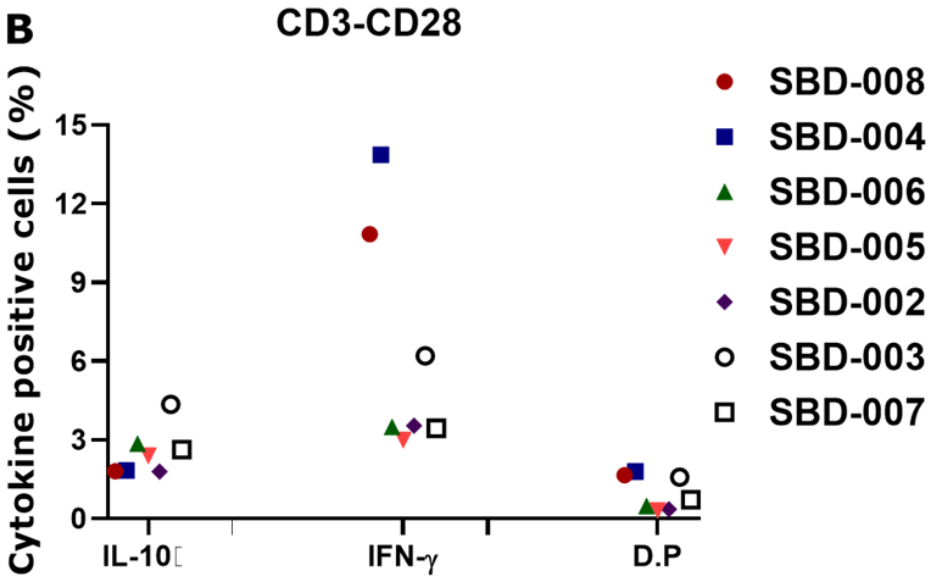
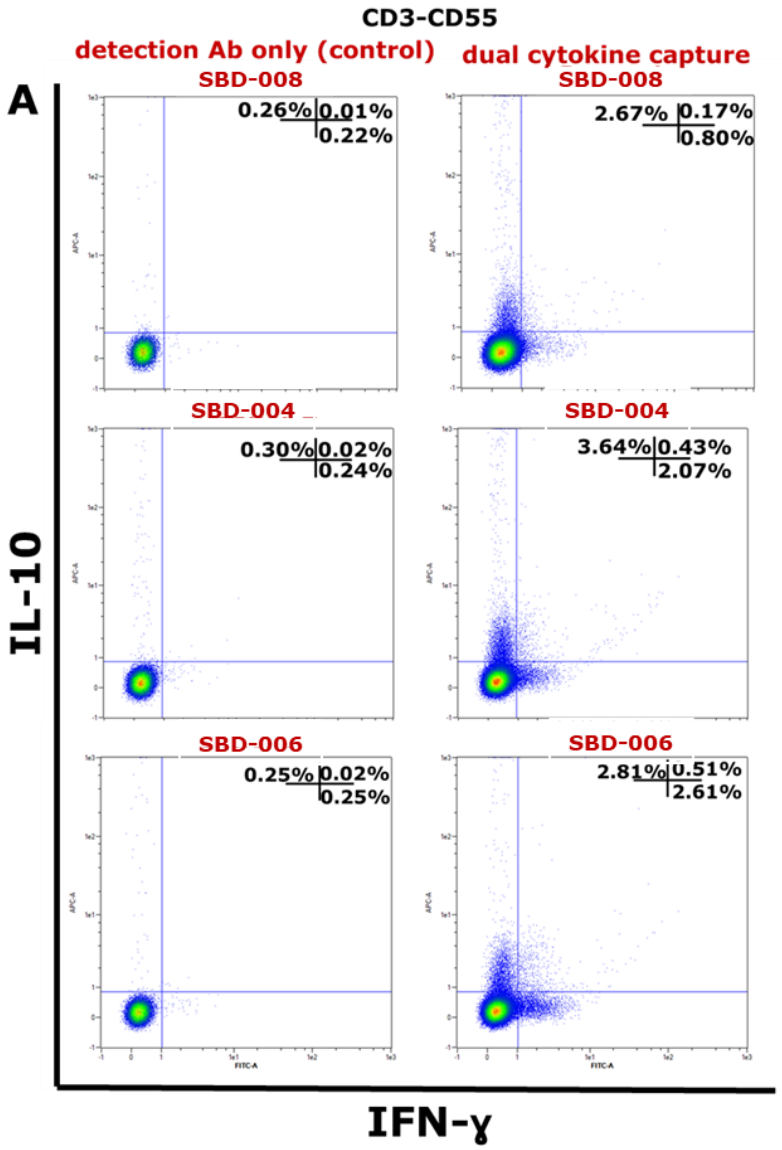

B
CD3-CD28
Cytokine positive cells (%)
15
12
9
6
3
0
IL-10
IFN-γ
D.P
SBD-008
SBD-004
SBD-006
SBD-005
SBD-002
SBD-003
SBD-007

Figure 4.9: CD3/CD28 costimulate IL-10^{+} and IFN-γ^{+} populations of cells in peripheral blood CD4^{+} T cells

CD4^{+} T cells, isolated from peripheral blood of healthy donors, were stimulated with plate bound anti CD3/ anti CD28 for 24 hours. IL-10 and IFN-ɣ positive cells were assessed by dual cytokine capture assay. **A** IL-10 and IFN-ɣ surface capture with controls from three donors. **B** percentage IL-10, IFN-ɣ and double positive (D.P) cells from cytokine secretion assay on seven donors

Like PMA/I, CD3/CD28 costimulation revealed a predominant population of IFN-ɣ positive cells with a small number of IL-10 positive cells. These were therefore compared to CD3/CD55 costimulation of total $CD4^+$ T cells which showed that both pro- and anti- inflammatory cells were stimulated to secrete their signature cytokines, IFN-ɣ & IL-10 respectively (Figure 4.10). Data showed that costimulation with CD3/CD55 resulted in cells expressing IL-10 (between 1.32% and 7.07%), IFN-ɣ (0.80% to 3.39%) with few cells expressing both cytokines (0.17% to 2.29%). Cytokine profile observed was similar across seven healthy donors assessed (Figure 4.10B).


A
CD3-CD55
detection Ab only (control)
dual cytokine capture
SBD-008
0.26%
0.01%
0.22%
SBD-008
2.67%
0.17%
0.80%
SBD-004
0.30%
0.02%
0.24%
SBD-004
3.64%
0.43%
2.07%
SBD-006
0.25%
0.02%
0.25%
SBD-006
2.81%
0.51%
2.61%
IL-10
IFN-γ

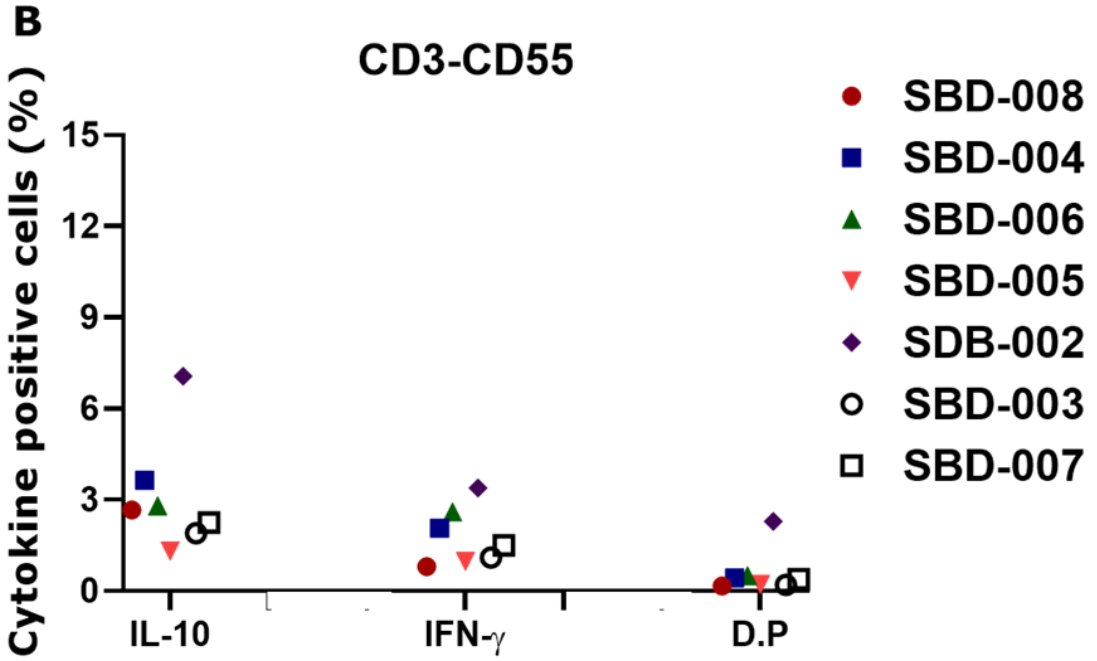

B
CD3-CD55
Cytokine positive cells (%)
15
12
9
6
3
0
IL-10
IFN-γ
D.P
SBD-008
SBD-004
SBD-006
SBD-005
SDB-002
SBD-003
SBD-007

Figure 4.10: CD3/CD55 costimulate IL-10$^+$ and IFN-γ$^+$ populations of cells in peripheral blood CD4$^+$ T cells

CD4$^+$ T cells, isolated from peripheral blood of healthy donors, were stimulated with plate bound anti CD3/ anti CD55 for 24 hours. IL-10 and IFN-ɣ positive cells were assessed by dual cytokine capture assay. **A** IL-10 and IFN-ɣ surface capture with controls from three independent experiments. **B** percentage IL-10, IFN-ɣ and D.P cells from cytokine secretion assay carried out on seven donors

4.2.5: Stimulation of $CD4^+$ T cells with PMA/I, CD3/CD28 or CD3/CD55 does not result in the co-expression of IL-4

Having shown that CD3/CD55 costimulation predominantly drives a population of IL-10 positive cells within $CD4^+$ T cells, we looked for co-expression of IL-4, a marker of Th2 cells (Howard et al., 1982) and a marker excluded from Tr1 cells, but IL-4 has been reported to be expressed at low levels by *in vitro* differentiated Tr1 cells (Roncarolo et al., 2014). Whether these cells co-express IL-4 in human peripheral blood remains unknown.

$CD4^+$ T cells were assayed for IL-10 and IL-4 co-expression after 24 hours stimulation with PMA/I; CD3/CD28; orCD3/CD55 (Figure 4.11). It was observed that $IL\text{-}10^+$ cells did not co-express IL-4 to any appreciable level across three donors analysed. It was also observed that, regardless of the type of stimuli, IL-10 positive cells were negative for IL-4.

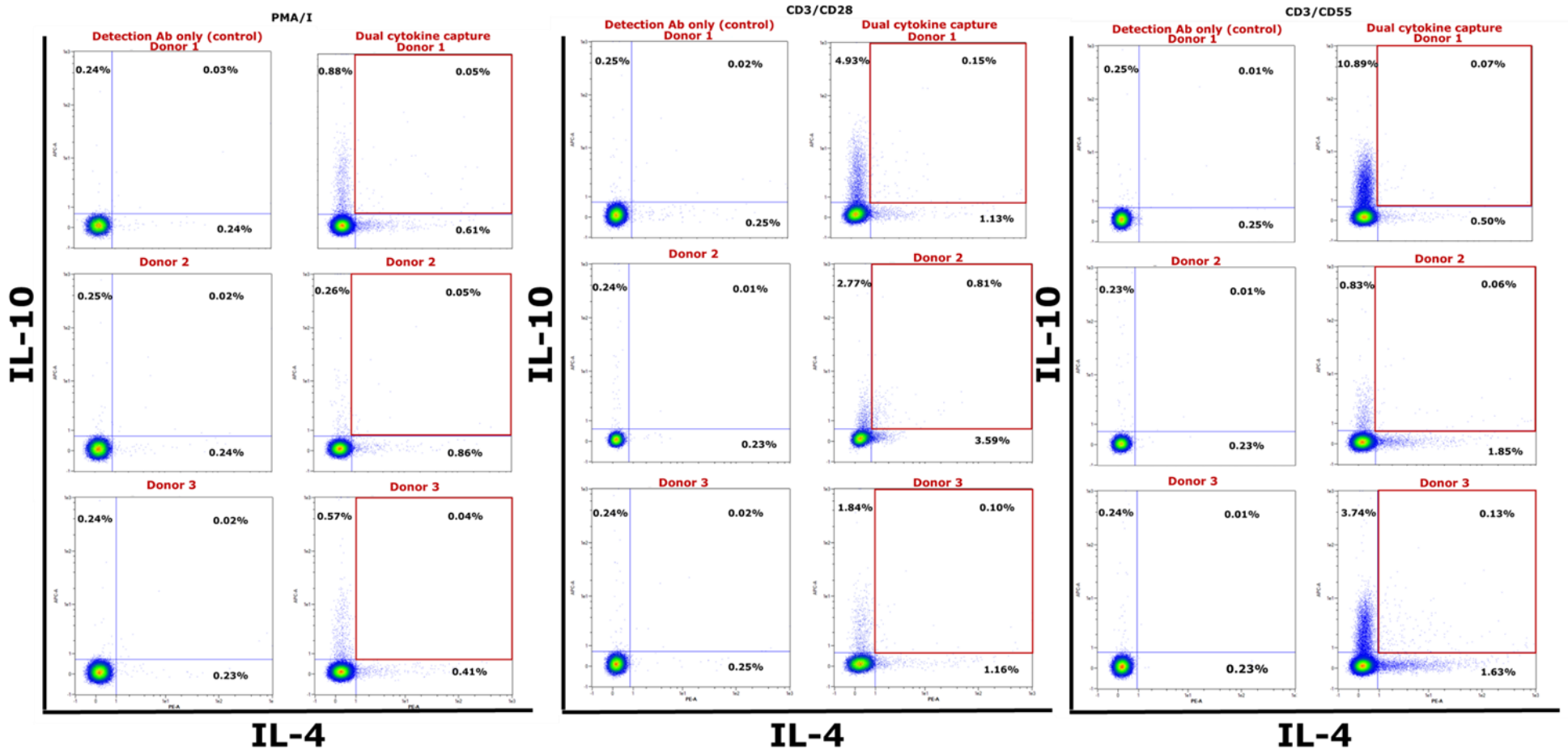

PMA/I
CD3/CD28
CD3/CD55
Detection Ab only (control)
Dual cytokine capture
Donor 1
Donor 2
Donor 3
0.24%
0.03%
0.24%
0.88%
0.05%
0.61%
0.25%
0.02%
0.24%
0.26%
0.05%
0.86%
0.24%
0.02%
0.23%
0.57%
0.04%
0.41%
0.25%
0.02%
0.25%
4.93%
0.15%
1.13%
0.24%
0.01%
0.23%
2.77%
0.81%
3.59%
0.24%
0.02%
0.25%
1.84%
0.10%
1.16%
0.25%
0.01%
0.25%
10.89%
0.07%
0.50%
0.23%
0.01%
0.23%
0.83%
0.06%
1.85%
0.24%
0.01%
0.23%
3.74%
0.13%
1.63%
IL-10
IL-4

Figure 4.11: IL-10$^+$ cells from PMA/I stimulation, CD3/CD28 or CD55 costimulation of CD4$^+$ T cells do not co-express IL-4

CD4$^+$ T cells isolated from peripheral blood of healthy donors and stimulated with plate-bound anti CD3/ anti CD55 for 24 hours, were assessed for IL-10 and IL-4. IL-10 positive cells were assessed by cytokine secretion assay while IL-4 expression was measured by intracellular cytokine assay. IL-10 and IL-4 positive cells with controls from three healthy donors. **Red box** (on plots to the right) indicates percentage of cells double positive IL-10 and IL-4

4.2.6: IL-10 positive cells undergo multiple rounds of proliferation cycles following CD3/CD55 costimulation

We have shown that both costimulation (CD3/CD55 or CD28) and non-specific (PMA/I) stimulation of human $CD4^+$ T cells reveal a small population of IL-10 single positive cells with CD55 stimulation showing a robust effect in activating this population of cells. High IL-10 expression is the hallmark of Tr1 cells and different models have been reported for Tr1 induction, most of which opine that Tr1 cells were anergic, non-proliferating cells. Our group had reported CD55 costimulation as an alternate costimulatory pathway for induction of Tr1 cells from human naïve T cells and available data also showed that CD55 co-stimulate the IL-10 population within effector $CD4^+$ T cells. However, whether this population of IL-10 expressing cells within the memory/effector cells are anergic and non-proliferating has not been reported.

To address this, we purified $CD4^+$ T cells from peripheral blood of healthy human donors and stained with CellTrace Violet (CTV) dye, then stimulated with CD3/CD55 antibodies for three days (72 hours). Prior to stimulation, cells were stained at day 0, and flow cytometry analysis demonstrated that cells were uniformly labelled by CTV dye (Figure 4.12A) and this served as the negative control.

In Figure 4.12B, stimulated cells went through several rounds of proliferation cycles after 72 hours of stimulation assessed by flow cytometry staining with IL-10 detection antibody only. This served as

the positive control for the experiment in Figure 4.12C. When cytokine expression was measured along with proliferation, it was observed that about 5% of proliferating cells were positive for IL-10. A closer look at this population showed IL-10^{+} cells had gone through several proliferation cycles with peak IL-10 expressions (36.12% and 32.12%) and the 94.49% IL-10^{-} cells were also observed to have proliferated.

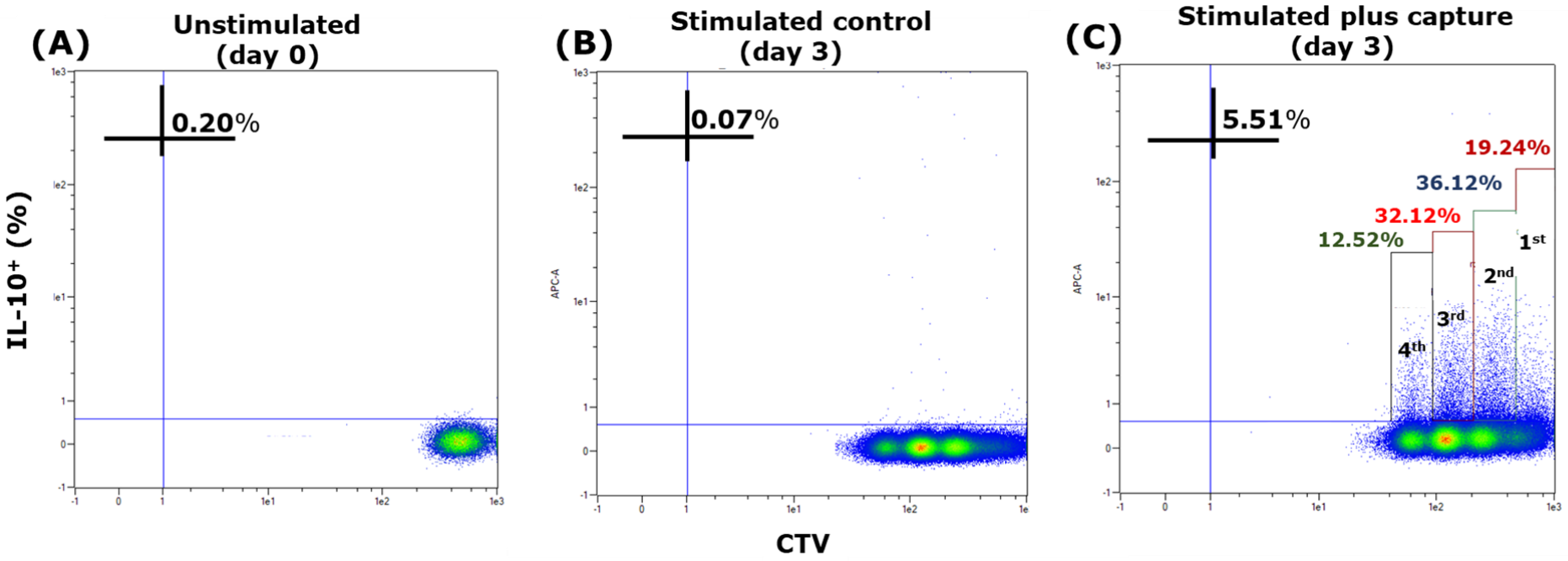


Figure 4.12: IL-10⁺ T cells undergo multiple rounds of proliferation cycles

CD4⁺ T cells were labelled with CellTrace Violet (CTV) dye and costimulated with CD3/CD55 for 72 hours. percentage (%) IL-10⁺ cells was assessed by IL-10 capture assay. Proliferation was assessed by CTV dye dilution in the context of IL-10 expression. **A** CTV labelled cells before stimulation. **B** CTV labelled cells after 72 hours costimulation and IL-10 capture (detection Ab only-control). **C** CTV labelled cells following CD3/CD55 costimulation for 72 hours and IL-10 capture. Data shown are representative of six independent experiments on six donors

4.2.7: Comparing CD55 and CD28 as costimulators of Tr1-like cells

CD55 costimulation has previously been reported to induce cell activation, differentiation and enhanced proliferation(Capasso et al., 2006a) and is also reported to preferentially induce Tr1-like cells from naïve $CD4^+$ T cells (Sutavani et al., 2013). Conversely, signalling through CD28 plays a vital role in many T cells processes ranging from cell differentiation, cytokine production to cell survival (J. H. Esensten, Y. A. Helou, G. Chopra, A. Weiss, & J. A. Bluestone, 2016).This study investigated the presence of a similar population in the peripheral blood and examined the role of CD55 and CD28 as costimulators of Tr1-like cells.

$CD4^+$ T cells isolated from human peripheral blood were costimulated with CD3/CD55 or CD28 for 24 hours. Figure 4.13 shows that cells expressed high levels of IL-10 across multiple (eight) donors following CD3/CD55 costimulation. Percentage expression ranged between 2% and 7% (against detection ab only controls). Cells were also able to express IFN-ɣ in response to CD55 costimulation (expression levels between 0.36% and 4.10%) but at lower levels when compared to IL-10 across multiple donors. Some cells were also positive for both cytokines (IL-10 & IFN-ɣ) and percentage expression ranged between 0.08% to 2.29%. Although variations in cytokine expression levels were observed to be donor dependent, expression pattern was consistent

across multiple donors, except for donor 5 where IL-10 was marginally lower than IFN-ɣ expression.

CD3/CD55

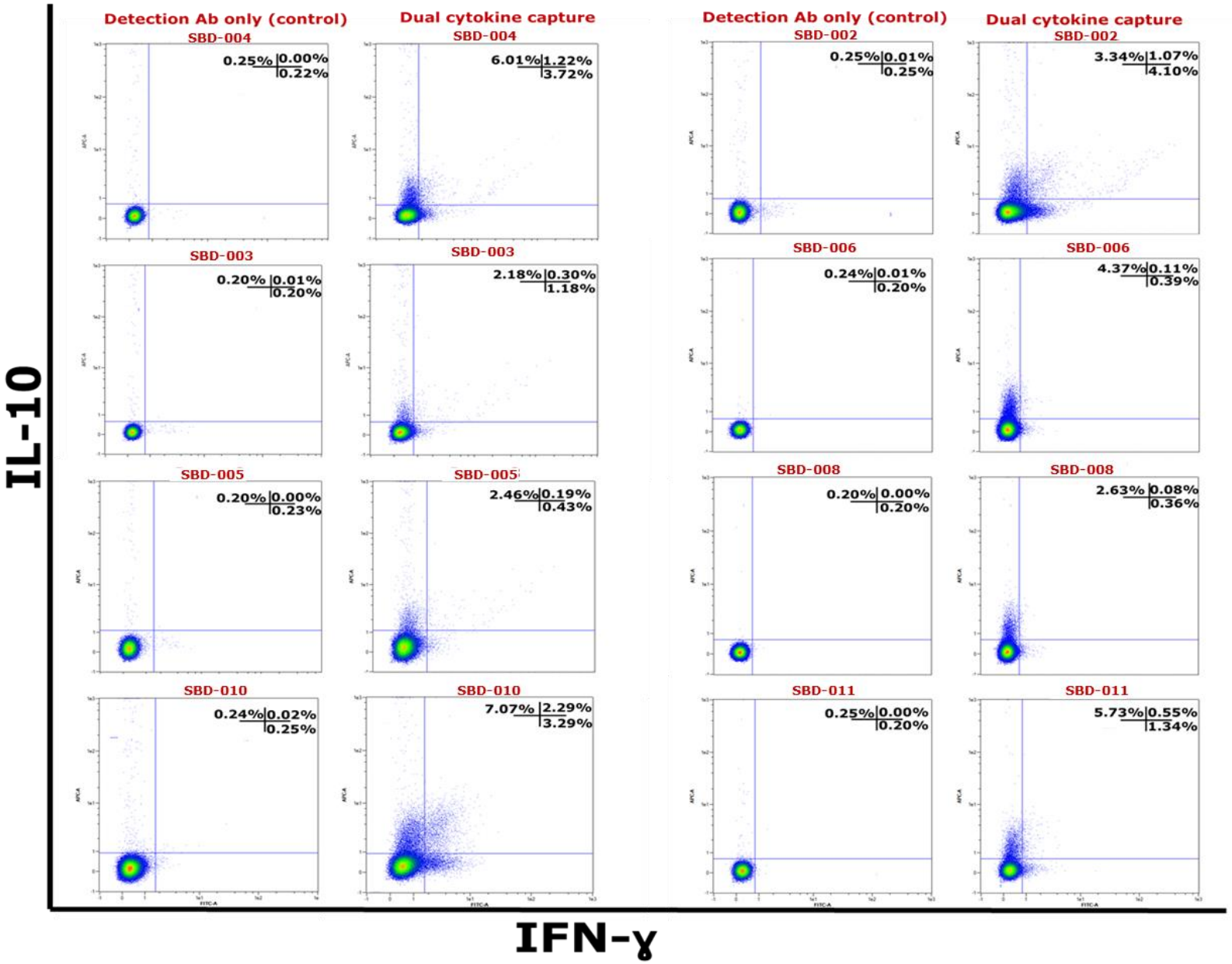

Detection Ab only (control)
Dual cytokine capture
Detection Ab only (control)
Dual cytokine capture
SBD-004
0.25% 0.00% 0.22%
SBD-004
6.01% 1.22% 3.72%
SBD-002
0.25% 0.01% 0.25%
SBD-002
3.34% 1.07% 4.10%
SBD-003
0.20% 0.01% 0.20%
SBD-003
2.18% 0.30% 1.18%
SBD-006
0.24% 0.01% 0.20%
SBD-006
4.37% 0.11% 0.39%
SBD-005
0.20% 0.00% 0.23%
SBD-005
2.46% 0.19% 0.43%
SBD-008
0.20% 0.00% 0.20%
SBD-008
2.63% 0.08% 0.36%
SBD-010
0.24% 0.02% 0.25%
SBD-010
7.07% 2.29% 3.29%
SBD-011
0.25% 0.00% 0.20%
SBD-011
5.73% 0.55% 1.34%
IL-10
IFN-γ

Figure 4.13: CD3/CD55 results in high IL-10 expression by IL-10 single positive cells

$CD4^+$ T cells, isolated from peripheral blood of healthy donors, were stimulated with plate bound CD3/ CD55 for 24 hours. IL-10 and IFN-γ positive cells were assessed using the IL-10 and IFN-γ dual capture assay. Cytokine positive cells were assessed against corresponding detection antibody (only) control for each donor. Flow cytometry plots shown are from eight donors in eight independent experiments.

CD3/CD28 costimulation on the other hand was observed to have driven a predominant IFN-ɣ positive phenotype (Figure 4.14). IFN-ɣ expression levels ranged from 2.04% to 4.74% across eight donors. It was further observed that CD28 costimulation was able to drive the expression of IL-10 but not to the high levels observed for IFN-ɣ. Percentage IL-10 positive cells ranged from 0.45% to 3.04% with double positive cells from 0.38% to 0.73% across multiple (eight) donors. Although donor variations were seen; the pattern of cytokine expression was similar in all donors.

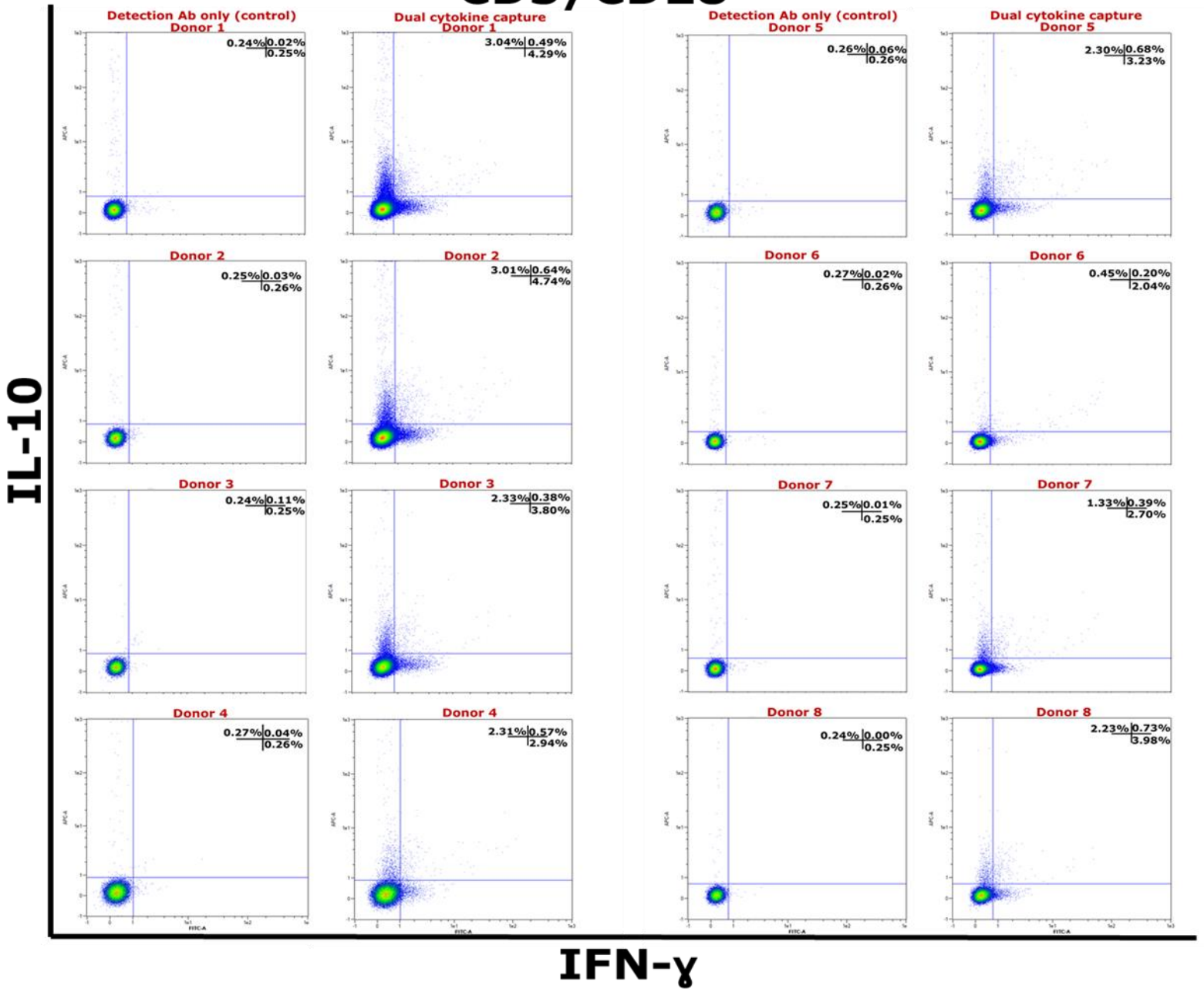

CD3/CD28
Detection Ab only (control)
Donor 1
0.24%
0.02%
0.25%
Dual cytokine capture
Donor 1
3.04%
0.49%
4.29%
Detection Ab only (control)
Donor 5
0.26%
0.06%
0.26%
Dual cytokine capture
Donor 5
2.30%
0.68%
3.23%
Donor 2
0.25%
0.03%
0.26%
Donor 2
3.01%
0.64%
4.74%
Donor 6
0.27%
0.02%
0.26%
Donor 6
0.45%
0.20%
2.04%
Donor 3
0.24%
0.11%
0.25%
Donor 3
2.33%
0.38%
3.80%
Donor 7
0.25%
0.01%
0.25%
Donor 7
1.33%
0.39%
2.70%
Donor 4
0.27%
0.04%
0.26%
Donor 4
2.31%
0.57%
2.94%
Donor 8
0.24%
0.00%
0.25%
Donor 8
2.23%
0.73%
3.98%
IL-10
IFN-γ

Figure 4.14: CD3/CD28 costimulation shows predominantly IFN-ɣ$^{+}$ phenotype

$CD4^{+}$ T cells, isolated from peripheral blood of healthy donors, were stimulated with plate-bound CD3/CD28 for 24 hours. IL-10 and IFN-ɣ positive cells were assessed by dual cytokine capture. Cytokine positive cells were assessed against corresponding detection antibody (only) control for each donor. Flow cytometry plots shown are from eight donors in eight independent experiments.

Figure 4.15 shows a summary of cytokine expression profile for both CD3/CD55 and CD3/CD28 costimulation. CD3/CD55 costimulation resulted in IL-10 expression levels significantly higher ($p \leq 0.05$) than IFN-ɣ and double positive (D.P) cells (Figure 4.15A). Results also showed that IFN-ɣ expression levels following CD3/CD28 costimulation, were significantly higher ($p \leq 0.05$) than IL-10 and D.P expressions (Figure 4.15B). Further analyses showed IL-10 expression levels from CD3/CD55 costimulation were significantly higher ($p \leq 0.05$) than levels seen with CD3/CD28 while IFN-ɣ expression levels were significantly higher for CD3/CD28 costimulation (Figure 4.15C).

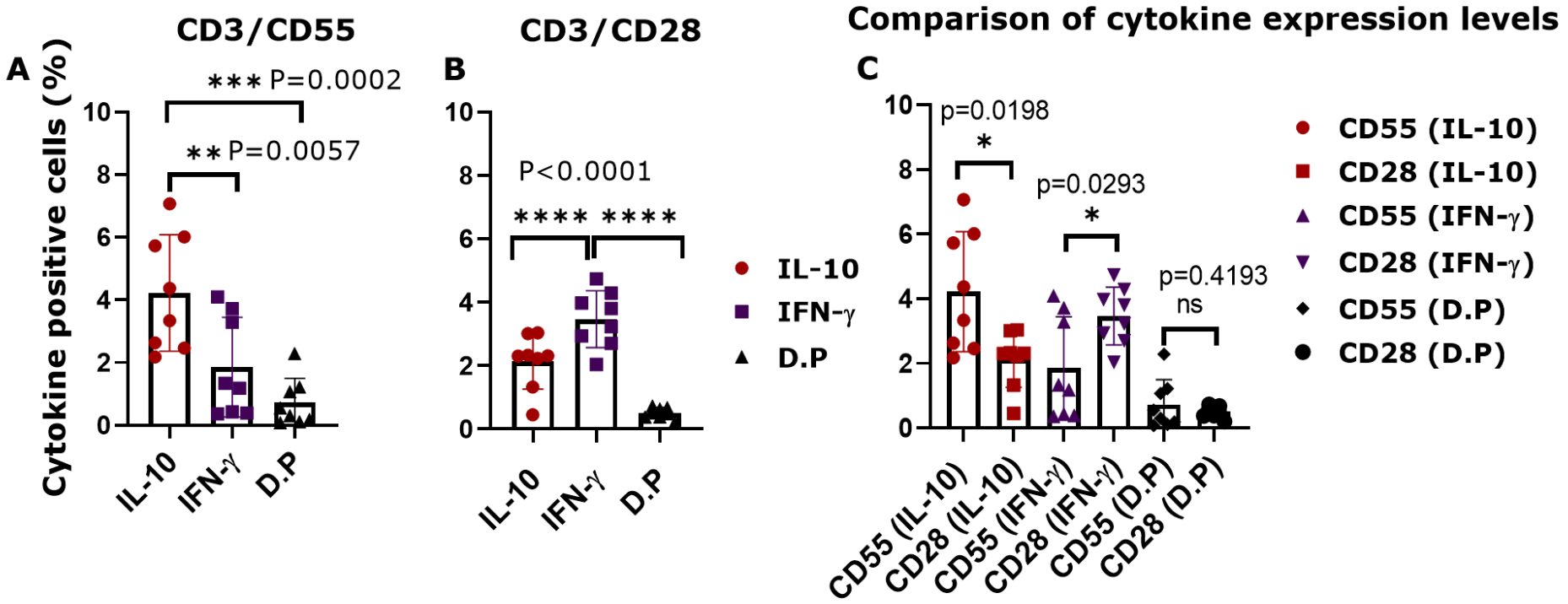


Figure 4.15: IL-10 and IFN-ɣ expression following CD3/CD55 or CD3/CD28 costimulation of $CD4^+$ T cells for 24 hours

Cytokine positive cells following CD3/CD55 or CD3/CD28 costimulation of $CD4^+$ T cells for 24 hrs. **A** cytokine profile following CD3/CD55 costimulation summarised from Figure 4.13. **B** cytokine profile following CD3/CD28 costimulation summarised from Figure 4.14. **C** comparison of cytokine expression profiles for CD3/CD55 and CD3/CD28 costimulation. Statistical significance was determined by two-tailed, paired Student's *t* test. Data shown are from eight donors. Data statistically significant at $P \leq 0.05$.

4.3: Discussion

This study showed that stimulation of $CD4^+$ T cells with nonspecific (PMA/I) and costimulation (CD3/CD28 or CD3/CD55) stimuli revealed a discrete population of cells in human peripheral blood, that were single positive for IL-10; negative for IFN-ɣ and IL-4 ($IL\text{-}10^+IFN\text{-}ɣ^-IL\text{-}4^-$).

Stimulation of $CD4^+$ T cells with PMA/I, a potent polyclonal nonspecific agent, activated cells to produce cytokines including IL-10 and IFN-ɣ. The use of costimulatory molecules (CD3/CD28 or CD3/CD55) against the respective receptors also potently activated $CD4^+$ T cells to secrete cytokines such as IL-10 and IFN-ɣ. As expected, CD28 costimulation resulted in a more Th1-like response by driving high IFN-ɣ expression (Jonathan H. Esensten et al., 2016), while CD55 costimulation favoured IL-10 single positive cell population, confirming previous reports of naïve $CD4^+$ T cells differentiation into Tr1 cells following CD55 costimulation (Capasso et al., 2006a; Sutavani et al., 2013). Furthermore, the $IL\text{-}10^+$ cells resulting from CD55 costimulation showed a small percentage of proliferating cells that produced high amounts of IL-10 (10-30ng/ml) in culture, but no IL-4.

The immune system fights off infections by producing effector cytokines and messenger molecules. These molecules then function in a balancing act between the pro- inflammatory and anti-inflammatory arms of the immune system. This complex homeostatic process is necessary to maintain tissue integrity and forge a delicate balance between effective immune responses for pathogen clearance during infection and the

appearance of tissue pathology or autoimmunity. Interferon gamma (IFN-ɣ), a pro-inflammatory cytokine predominantly produced by the Th1 cells, is required for the clearance of intracellular pathogens (J. Chen & X. Liu, 2009; Cope et al., 2011), whilst IL-10, a key member of the IL-10 family cytokines, represents a paradigm of anti-inflammatory mechanism exerting essential functions by restricting excessive inflammatory responses, upregulating innate immunity, and promoting tissue repairs(Ouyang & O'Garra, 2019; Saraiva, Vieira, & O'Garra, 2020). Other cytokines of the IL-10 super family including IL-19, IL-22, IL-24, IL-26, are produced by both innate and adaptive immune cells and function as effector and regulatory molecules of the immune system demonstrating profound and indispensable functional roles in infection, inflammation, tissue homeostasis, autoimmunity, and cancer (Ouyang, Rutz, Crellin, Valdez, & Hymowitz, 2011; Sabat, 2010; X. Wang, Wong, Ouyang, & Rutz, 2019; H. Wei, Li, Sun, & Guo, 2019).

The time course analyses of cytokines expression revealed an early peak in the kinetics of IL-10 expression around 24 to 30 hours after CD3/CD55 stimulation of $CD4^+$ T cells. Of note was the fact that IL-10 expression was noticed as early as 6 hours after stimulation and the cells in culture produced large amounts of IL-10 despite being relatively small in number. The overall kinetics of proliferation and cytokine secretion suggested that these cells responded in the same timeline as effector cells (Yssel et al., 1992), and much faster than reported for naïve cell stimulation. It also confirms that CD55 costimulation

preferentially activates the IL-10 pathway. Earlier studies on Naïve $CD4^+$ T cells differentiation with CD3/CD55 costimulation showed IL-10 peak time at around 72 hours (3 days). The early peak observed with total $CD4^+$ T cells indicated that the IL-10 producing cells might be effector phenotype within the peripheral blood. The underlying mechanisms for the induction of high IL-10 producing cells in human peripheral blood are not fully understood; but Capasso M., et al suggested that costimulation between CD55 (expressed by T cells) and CD97 (on APCs) were responsible for the induction of Tr1 cells in human peripheral blood (Capasso et al., 2006a). Report from other groups suggested that IL-10 producing tolerogenic dendritic cells (DC-10) induced antigen specific T regulatory Type 1 cells (M. Comi et al., 2018; Michela Comi, Giada Amodio, & Silvia Gregori, 2018) and Comi Michela et al., reported that co-expression of CD163 and CD141 together with CD14 and CD16 expression identified the tolerogenic DC population responsible for IL-10 secretion necessary for the induction of Tr1 cells *in vivo* in humans (Comi et al., 2020).

The kinetics of IFN-ɣ expression in response to CD3/CD55 stimulation was also analysed. It was observed that expression of IFN-ɣ peaked at a later point (30-36 hours) and data also showed that there was no meaningful expression of IFN-ɣ by cells until around 18 hours after stimulation. This observation may have been due to a couple of factors. There is a possibility that early secretion of IL-10 may have inhibited the activation, proliferation and expansion of existing Th1 cells. Similar

observation, first reported in mouse study by Fiorentino, D.F et al., showed that cytokine synthesis inhibitory factor (CSIF), subsequently named IL-10 secreted by Th2 cells inhibited the production of several cytokines including IFN-ɣ (Fiorentino, Bond, & Mosmann, 1989). These finding were corroborated by recent studies which showed that IL-10 is produced in a negative and positive feedback loop to forestall the uncontrolled production of pro-inflammatory cytokines during infection. Studies have also shown that IL-10 expression is tightly regulated by IFN-ɣ by blocking IL-10 production through phosphoinositide 3-kinase (PI3K) or by IL-10 itself by limiting its own expression or via a positive feedback to amplify production (Saraiva & O'Garra, 2010). In disease conditions or infections, increased IL-10 production has been shown to disrupt cellular responses at clearing pathogens by interfering with the IFN-ɣ production via reduction in macrophages activation (M. M. Kane & Mosser, 2001). However, the current data from $CD4^+$ T cells showed that there was no appreciable consumption of IL-10 by the mixed culture of IL-10, IFN-ɣ and IL-4 producing cells, although it doesn't exclude the possibility that IL-10 might be working in a self-limiting manner, by binding to the IL-10 producing cells and preventing the continued secretion of the cytokine.

Another angle is that CD3/CD55 costimulation, which has been demonstrated to drive the differentiation of Tr1 cells from naïve $CD4^+$ T cells, is not as potent in stimulating IFN-ɣ producing cells. Our data

showed that costimulation via CD55 was able to activate only about 3.39% of IFN-ɣ cells compared to about 14% of IFN-ɣ cells induced by CD3/CD28 costimulation and about 8% IFN-ɣ cells with PMA/I stimulation. Therefore, the early secretion of IL-10 supported the view that one role of IL-10 may be to cross-regulate Th1 development and function (Jankovic, Kugler, & Sher, 2010). The fact that our data showed that these $CD4^+$ T cells have the kinetics of effector/memory cells, produced large amount of IL-10, no IFN-ɣ or IL-4 supported our hypothesis that Tr1 cells were present in the human peripheral blood, mediating peripheral tolerance by secreting high levels of immune suppressive cytokines, IL-10 (Jankovic et al., 2010). Data also showed some cells were double positive for IL-10 and IFN-ɣ. These cells may be Th1 cells in immune resolution phase which are capable of intracellular switching from IFN-ɣ to IL-10 producing cells during the life cycle of Th1 cells(Cope et al., 2011). $IL\text{-}10^+IFN\text{-}ɣ^+$ double positive $CD4^+$ T cells have also been reported to arise from chronic stimulation in the presence of IL-27 and TGF-β (J. Chen & X. S. Liu, 2009; Saraiva et al., 2009). Furthermore, $CD4^+CD25^-FoxP3^-$ Th1 cells have been shown as a source of IL-10 in certain human disease conditions such as chronic cutaneous leishmaniasis characterised by strong early inflammatory signals (C. F. Anderson, Oukka, Kuchroo, & Sacks, 2007). However, it is also possible that the double positive cells observed in this study resulted from mechanisms other than chronic stimulation as these cells were detected as early as 24 hours.

Taken together, our data showed that CD3/CD55 costimulation resulted in a population of cells that were single positive for IL-10; negative for IFN-ɣ and IL-4 ($IL\text{-}10^{+}IFN\text{-}\gamma^{-}IL\text{-}4^{-}$). The contribution of TCR engagement via CD3 (alone) gave less than 1% of IL-10 single positive cells, compared to the 4-12% obtained with CD3/CD55 costimulation. This observation suggested that mobilisation of the TCR was necessary to prime the cells for CD55 costimulation resulting in expression of high amounts of IL-10.

Once differentiated, T cells adopt a committed phenotype and can be stimulated with a range of stimuli. In our studies, we adopted both broad spectrum nonspecific (PMA/I) stimulation and specific (via CD3/CD28 or CD3/CD55) costimulation in the study of cytokine profile of Tr1 cells in human peripheral blood. Plant lectin (e.g PHA), LPS or PMA/I are commonly used for stimulating T cells for cytokine evaluation assays. T cells respond to nonspecific stimulus such as PMA/I to secrete cytokines based on their differentiated phenotype. PMA directly activates Protein Kinase C (PKC), omitting the need for surface receptor stimulation, while Ionomycin, a calcium ionophore triggers calcium release needed for NFAT signalling. Stimulation with these compounds bypass the T cell membrane receptor complex resulting in activation of several intracellular signalling pathways, leading to T cell activation and production of a range of cytokines (Ai, Li, Song, Li, & Chen, 2013; Crawford, Jalbert, Ndhlovu, & Barbour, 2014). Our data showed that PMA/I stimulation of $CD4^{+}$ T cells reveal a distinct population of IL-10

single positive cells (about 3%) while a greater percentage of cells were positive for IFN-ɣ (about 8%) with very few cells double positive for IL-10 & IFN-ɣ (<1%). This indicates that PMA/I can stimulate both anti-inflammatory (IL-10) and pro-inflammatory (IFN-ɣ) arms of the immune system allowing cells to express cytokine profiles associated with their differentiated phenotype.

Like PMA/I stimulation, CD3/CD28 costimulation of $CD4^+$ T cells resulted is a similar pattern but higher levels of cytokine expression. The IL-10 expression observed in this study was in contrast with the differentiation of naïve $CD4^+$ T cells where CD28 costimulation failed to induce the expression of IL-10. CD3/CD28 costimulation has been reported to potently induce a Th1 phenotype in naïve T cells (J. H. Esensten et al., 2016) and ligation of the CD28 receptor provided enough stimulus to the IL-10 single positive population in human peripheral blood. CD55 costimulation on the other hand induced the expression of high levels of IL-10 but failed to induce significant levels of IFN-ɣ positive cells. These findings have biological significance in the sense that different co-stimuli to $CD4^+$ T cells reveal a distinct population of IL-10 single positive cells. Furthermore, a comparison between CD3/55 and CD3/28 costimulation of $CD4^+$ T cells showed CD55 costimulation preferentially stimulated IL-10 positive cells. In contrast, CD28 costimulation preferentially induced a Th1 phenotype by stimulating IFN-ɣ positive cells. Taken together, data suggested the presence of a differentiated IL-10 single positive population of cells in

the peripheral blood which can be readily stimulated by both nonspecific and costimulatory stimuli to produce IL-10.

To further characterise the IL-10 positive cells, co-expression of IL-4 by IL-10 single positive cells was measured. IL-4, initially identified as co-mitogen of B cells (Howard et al., 1982), and later shown to promote the growth and expansion of T cells (Junttila, 2018), induced the differentiation of naïve $CD4^+$ T cells into T_H2 cells. Upon activation, T_H2 cells secrete cytokines such as IL-4, IL-5, IL-13, and IL-10 (Berger, 2000). As IL-10 is known to be produced by T_H2 cells, co-expression of the T_H2 cytokine IL-4 (Chiricozzi, Maurelli, Peris, & Girolomoni, 2020; Egholm, Heeb, Impellizzieri, & Boyman, 2019; Lloyd & Snelgrove, 2018) was also assessed. It was interesting to note that across the different stimuli used (PMA/I, CD3/CD28 or CD55), IL-10 single positive cells did not co-express IL-4. This observation strongly supported the idea that the IL-10 single positive cells were not of the T_H2 lineage, in line with previous reports that Tr1 cells secrete high levels of IL-10 but little or no IL-4 (Battaglia, Gregori, et al., 2006; Norio Chihara, Asaf Madi, Katarzyna Karwacz, Amit Awasthi, & Vijay K. Kuchroo, 2016; Groux et al., 1997; Matsuda et al., 2019).

Many studies describe Tr1 cells as low proliferating or anergic cells and each study adopts a different approach of generating Tr1 cells. From the use of immature dendritic cells (Levings et al., 2005; Steinbrink, Graulich, Kubsch, Knop, & Enk, 2002), IL-27 (N. Chihara et al., 2016), IFN-α in the

presence of IL-10 (Levings et al., 2001) to CD3/CD46 (Meiffren, Flacher, Azocar, Rabourdin-Combe, & Faure, 2006). Contrary to these observations, our results from CD3/CD55 costimulation of $CD4^+$ T cells showed that IL-10^+ cells went through approximately three rounds of proliferation cycles in 72 hours, like the IL-10^- cells.

Therefore, we showed that these cells were a stable proliferative population of $CD4^+$IL-10^+IFN-γ^- IL-4^- Tr1-like cells. They can be stimulated by PMA/I and CD3/CD28 but preferentially via CD55 resulting in proliferation and IL-10 production. Our findings demonstrated that these IL-10 single positive Tr1-like cells were a differentiated population in human peripheral blood. It therefore remains unknown whether these cells were effector or memory T cells.

Chapter 5

Differentiation status of IL-10 secreting cells in human peripheral blood

5.1 Introduction:

T cells are responsible for the establishment of adaptive immune responses, homeostasis, and T cell memory(B. V. Kumar et al., 2018). Upon maturation and migration from thymus to the periphery, naïve T cells require the reorganization of signalling molecules at the T cell membrane into an immunological synapse with the TCR at the centre, making contact with MHC/peptide presented by the APCs(Smith-Garvin, Koretzky, & Jordan, 2009). Full activation of naïve $CD4^+$ T cells requires costimulatory signals that can come from a range of molecules, including CD40/CD40L(Grewal & Flavell, 1996; Munroe & Bishop, 2007), Ox40(Fu, Lin, Zhang, & Zhang, 2020; Redmond, Ruby, & Weinberg, 2009), 41BB/41BBL (Menk et al., 2018; Nguyen et al., 2020), CD46 (Astier, Trescol-Biémont, Azocar, Lamouille, & Rabourdin-Combe, 2000; Ni Choileain et al., 2017), CD55 via CD97 engagement(Capasso et al., 2006a; Dho, Lim, & Kim, 2018; Sutavani et al., 2013) and the classical CD28 engagement by CD80/CD86(L. Chen & Flies, 2013; Jonathan H. Esensten et al., 2016). Following activation, naïve $CD4^+$ T cells differentiate into effector cells including T_H1, T_H2, T_H17, T_H9, T follicular helper (T_{fh}), inducible T-regulatory cells (iTreg), and the T regulatory type 1 cells (Tr1). The differentiation of naïve $CD4^+$ T cells into different

subsets is dependent on a range of environmental conditions, including cytokine milieu. These factors influence transcription factor expression and epigenetic changes (Crotty, 2014; Luckheeram, Zhou, Verma, & Xia, 2012).

Following antigenic challenge, CD4 subsets are mobilised according to the type of immunological threat, and multiple subsets with overlapping or disparate functions may be mobilised during the clonal expansion phase. The involvement of each of the $CD4^+$ T cells subsets allows for a robust effector/regulatory cell response to immunological threats. Once the threat is eliminated, the majority of the expanded effector cells are lost by apoptosis during the contraction phase, leaving a small population of quiescent memory T cells. Memory T cells are antigen-specific cells that are long-lived after an infection has been eliminated and they confer enhanced secondary immune response to the host. These cells are quickly converted into large numbers of effector T cells upon re-challenge by specific antigen leading to a more rapid response by secondary effector cells with enhanced functionality (Garcia, DiSanto, & Stockinger, 1999; Gasper et al., 2014; McKinstry, Strutt, & Swain, 2008; Yamane & Paul, 2012).

Memory cells are characterized by expression of surface markers, adhesion molecules, cytokines, chemokines and transcription factors (Pepper & Jenkins, 2011). Changes in cell surface proteins: L selectin (CD62L), CD44 and CD45 are significant after antigen exposure and are

typically used to identify memory (cell) subsets. Chemokine receptor 7 (CCR7), which mediates homing of T cells to secondary lymphoid organs, is also commonly used as a marker of memory differentiation. Central memory cells (TCM) express CD62L and CCR7 at high levels as well as CD45RO and home to secondary lymphoid organs. Conversely, effector memory cells (TEM) lacking CCR7, express low levels of CD62L and high levels of CD45RO, a phenotype consistent with migration and homing to peripheral tissues(Campbell et al., 2001; B. V. Kumar et al., 2018; Sallusto, Geginat, & Lanzavecchia, 2004).

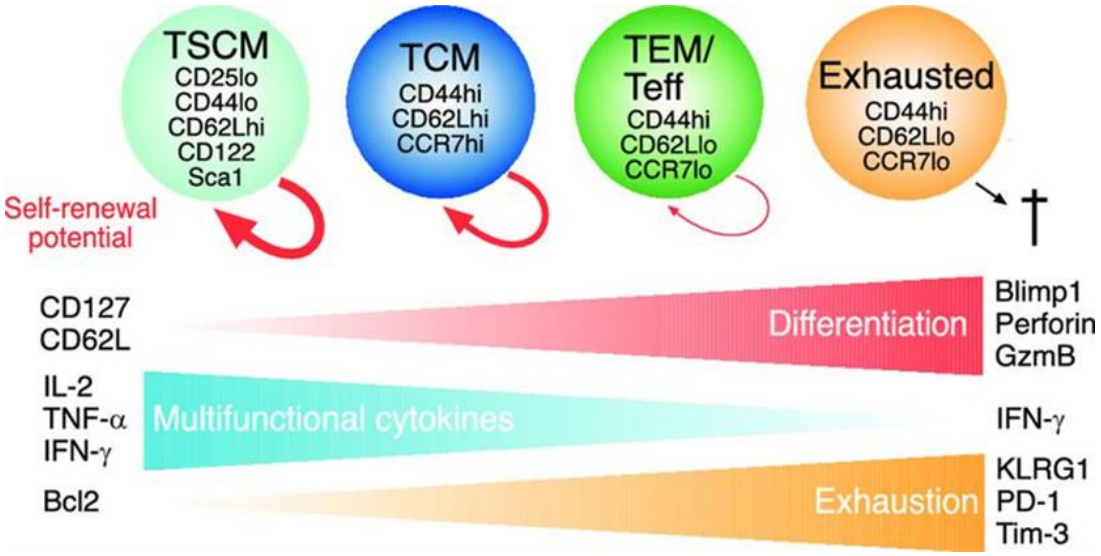


Figure 5.1: **Differentiation states for CD4 and CD8 memory development**

Memory CD4 and CD8 T cells progress through differentiation states in which they gradually lose the ability to proliferative and self-renewal and produce multifunctional cytokines (TNF-α and IL-2). The least differentiated stem-cell memory T cells (TSCMs) express low levels of CD44 and CD25, high levels of CD62L (and CCR7), like naïve T cells and are Sca-1^{+} and CD122^{+}; T central memory cells (TCM) upregulate CD44 expression while maintaining CD62L and CCR7 expression; T effector memory cells (TEM) and effectors (Teff) maintain high levels of CD44 expression while downregulating CD62L and CCR7 expression. (Adapted from Caserta, S., J.G. Borger, and R. Zamoyska, *Central and effector memory CD4 and CD8 T-cell responses to tumor-associated antigens.* Crit Rev Immunol, 2012) (Caserta, Borger, & Zamoyska, 2012).

Immunological memory has been well described for T_H1, T_H2, T_H17 and T_{fh} cells (Golubovskaya & Wu, 2016; Kim, Jay, & Williams, 2012). There are similar reports of long-lived memory cells for $FoxP3^+$ Treg cells (Brincks et al., 2013; Sanchez, Zhu, Huang, & Yang, 2012), but memory for T regulatory type 1 (Tr1) cells have not been widely reported. Therefore, since Tr1 cells play an important role in peripheral tolerance and are thought to be a differentiated population of cells within peripheral tissues, we hypothesised that Tr1-like cells maintained an immunological memory pool.

5.2: Results

5.2.1: Identifying naive, effector and memory populations following PMA/Ionomycin stimulation of $CD4^+$ T cells

The ability to respond to a previously encountered antigen remains the most powerful feature of the adaptive immune system. Recent studies reported that memory cells displayed superior efficacy and mediate primary immunosurveillance of peripheral tissues even against tumour (Q. Liu, Sun, & Chen, 2020). Lanzavecchia and Sallusto in their study reported that memory cells respond to 100-fold lower doses of antigen and more rapidly in the presence of costimulation compared to naïve cells (Lanzavecchia & Sallusto, 2001). Since memory cells with effector capabilities have lower thresholds for activation upon antigen challenge, we posited that there was a set of regulatory (potentially Tr1-like) cells that operated to resolve pro-inflammatory immune response once antigenic threats have been eliminated.

Optimisation and gating strategy for the determination of memory subsets (TCM and TEM) as well as IL-10 and IFN-ɣ dual cytokine secretion assay was established as shown in Figure 5.2. Unstimulated cells stained with antibodies against CD62L and CD45RO were used as a control to demarcate memory populations and define appropriate gates for stimulated conditions.

To determine the effector or memory status of these cells, $CD4^+$ T cells purified from human peripheral blood were stimulated with Phorbol 12-myristate 13-acetate/Ionomycin (PMA/I) for 24 hours. Memory expression was assessed by staining cells with antibodies against CD62L and CD45RO. The cell distribution pattern for PMA/I stimulation showed that most cells were effector or effector memory cells after 24 hours (Figure 5.3). There appeared to be a distinct lack/loss of CD62L expression in all donors treated with PMA/I. CD62L shedding has been reported in studies involving PMA/I stimulation (Crawford et al., 2014; Galkina et al., 2003). Consequently, CD3/CD28 was used as a stimulus to compare with CD3/CD55 costimulation in assessing memory marker distribution while CD3 (alone) stimulation was maintained as a positive control.

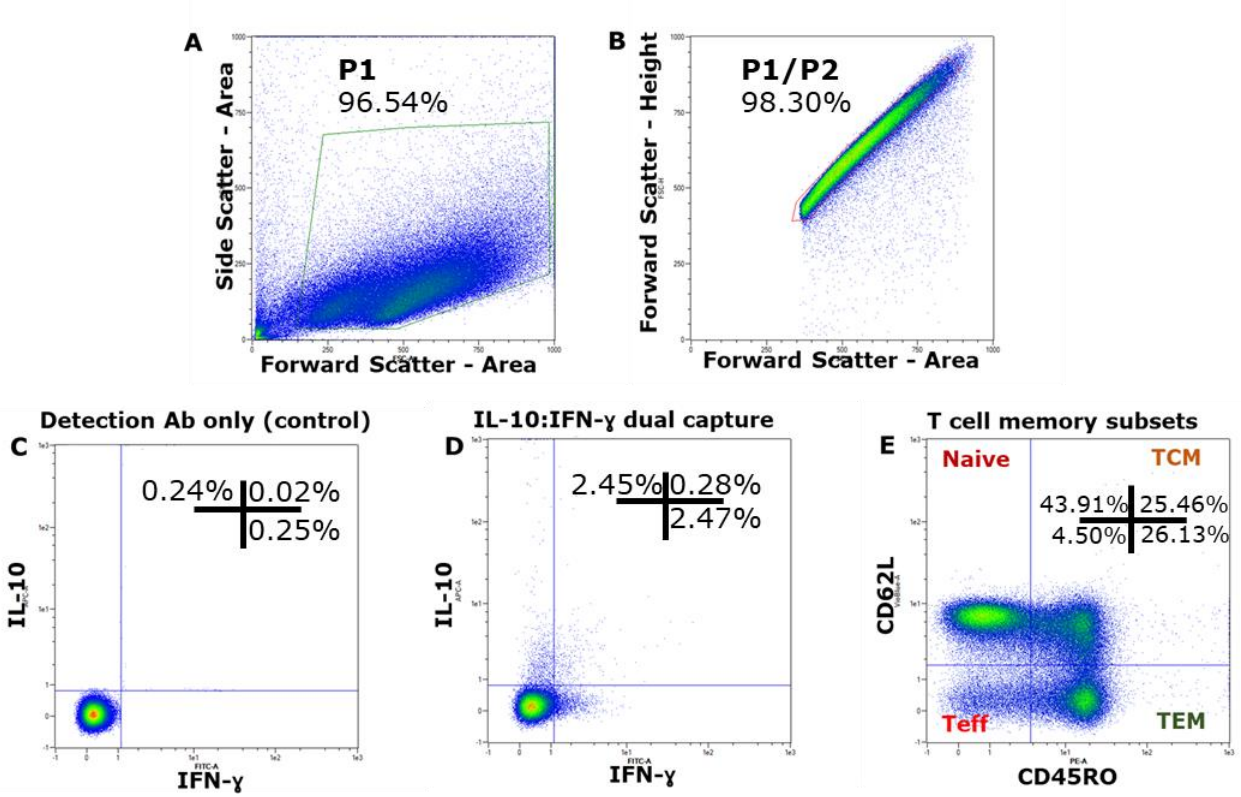


Figure 5.2: Determining the IL-10 and IFN-γ populations and memory subsets in CD4⁺ T cells

Gating for IL-10, IFN-γ and memory subsets set at the junction of cytokine-negative and cytokine-positive cell populations checked against control conditions. **A** Gating on the live cells was on forward scatter-area, side scatter – area. **B** doublet/aggregate discrimination was on forward scatter- area, forward scatter – height. **C** IL-10/IFN-γ dual capture with detection antibody only (control). **D** Assessment of cytokine expression (IL-10/IFN-γ) by cytokine secretion assay following 24 hours costimulation with CD3/CD55 or CD3/CD28. **E** CD62L/CD45RO demarcating the memory subsets, TCM (Central memory T cells) and TEM (Effector memory T cells) from naïve T cells and effector T cells (Teff) of un-stimulated CD4⁺ T cells. The gating strategy in Figure 5.2 (C&D) was to analyse data in Figure 5.6.

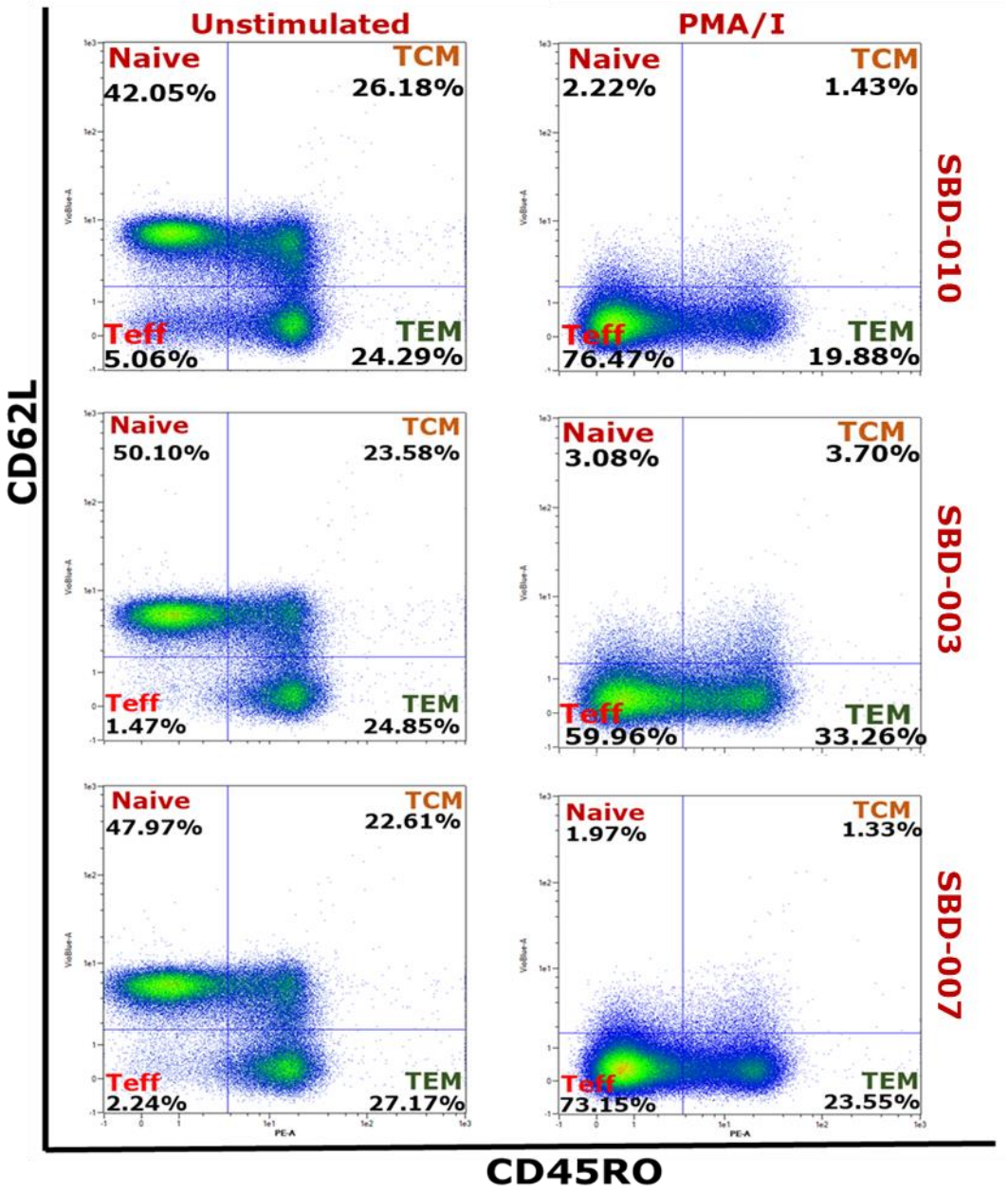


Figure 5.3: Distribution of memory phenotypes of CD4$^+$ T cells following PMA/I stimulation for 24 hours

CD4$^+$ T cells isolated from peripheral blood of healthy donors were stimulated with PMA/I for 24 hours. Cells were stained for memory markers, CD62L and CD45RO and data acquired by flow cytometry. Data shown are for three donors in three independent experiments.

5.2.2: Memory T cells display similar distribution with CD3/CD28 and CD3/CD55 costimulation

Studies have shown that costimulation plays a vital role in the function of memory cells. Alina C. and Peter D. reported that memory T cells need CD28 costimulation to 'remember', as costimulation provides synergistic signals to TCR-induced T cell activation (A. C. Boesteanu & P. D. Katsikis, 2009). Further studies have shown that CD28/B7 costimulatory pathway controls $CD4^+$ T cells memory (Ndejembi et al., 2006). Therefore, the memory T cells distribution for CD3/CD28 costimulation (pro-Th1) was determined and compared to CD3/CD55 costimulation (pro-Tr1).

$CD4^+$ T cells purified from human peripheral blood were stimulated with CD3 alone or costimulated with CD3/CD28 or CD3/CD55 for 24 hours and stained for CD62L and CD45RO. CD3 stimulation had little effect in altering any of the cell populations. CD3/CD28 however resulted in a greater population of effector cells (5-fold increase over unstimulated and CD3 stimulated cells), a 50% reduction in TCM and a small change in naïve cells at this 24-hour time point. Costimulation with CD3/CD55 gave similar changes to the CD3/CD28 costimulation but with a 3-fold increase in effector cells and a 30% reduction in TCM (Figure 5.4). Figure 5.5 A compared the distribution of cell subtypes and showed a significant difference ($p=0.0114$) in central memory T cells (TCM) when compared to unstimulated cells and significant increase (p-0.0438) in effector T cells (T_{eff}) following CD3/CD55 costimulation when compared

to unstimulated cells. Changes in average cell numbers (%) was also observed within cell subpopulations (Figure 5.5B).

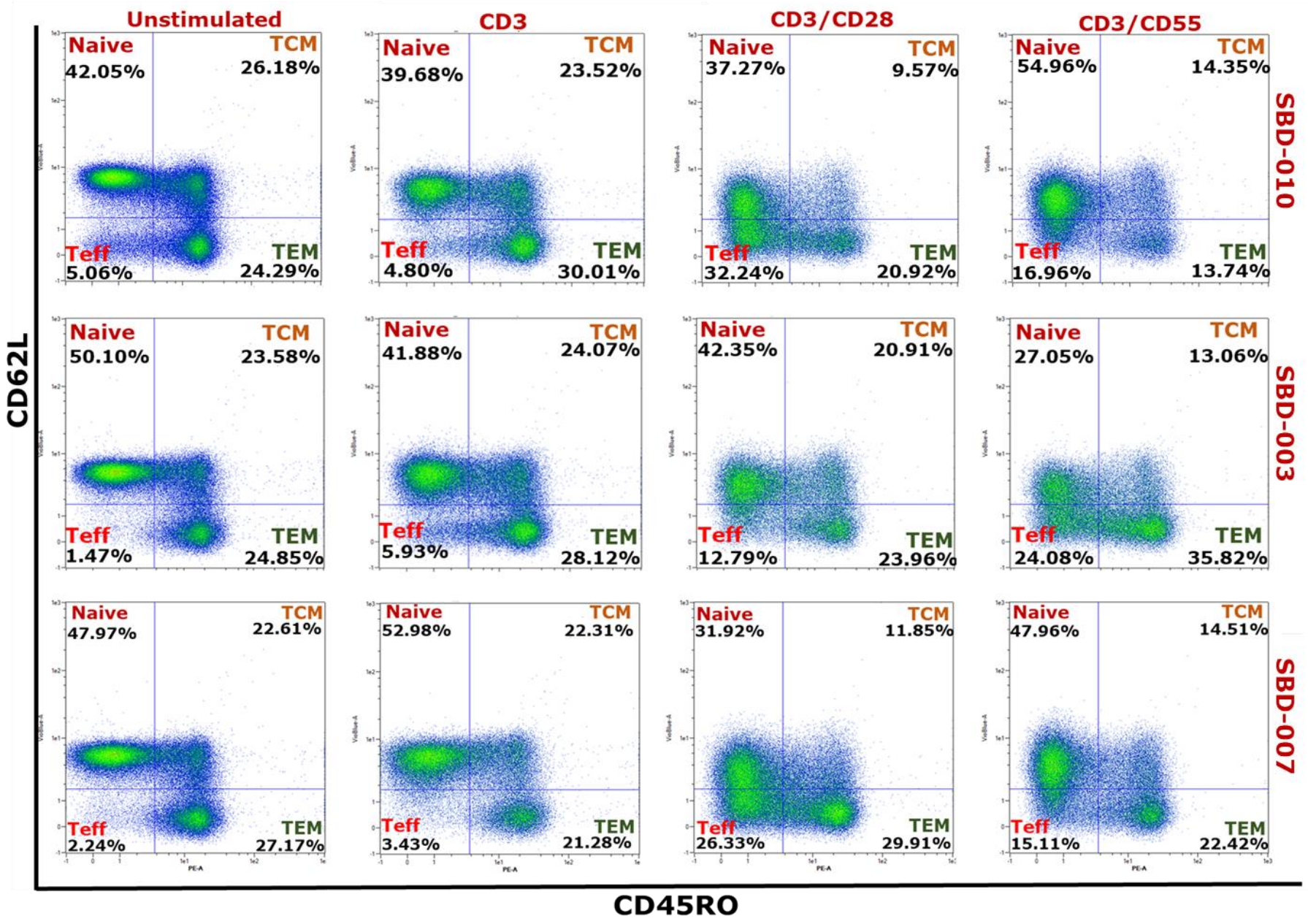
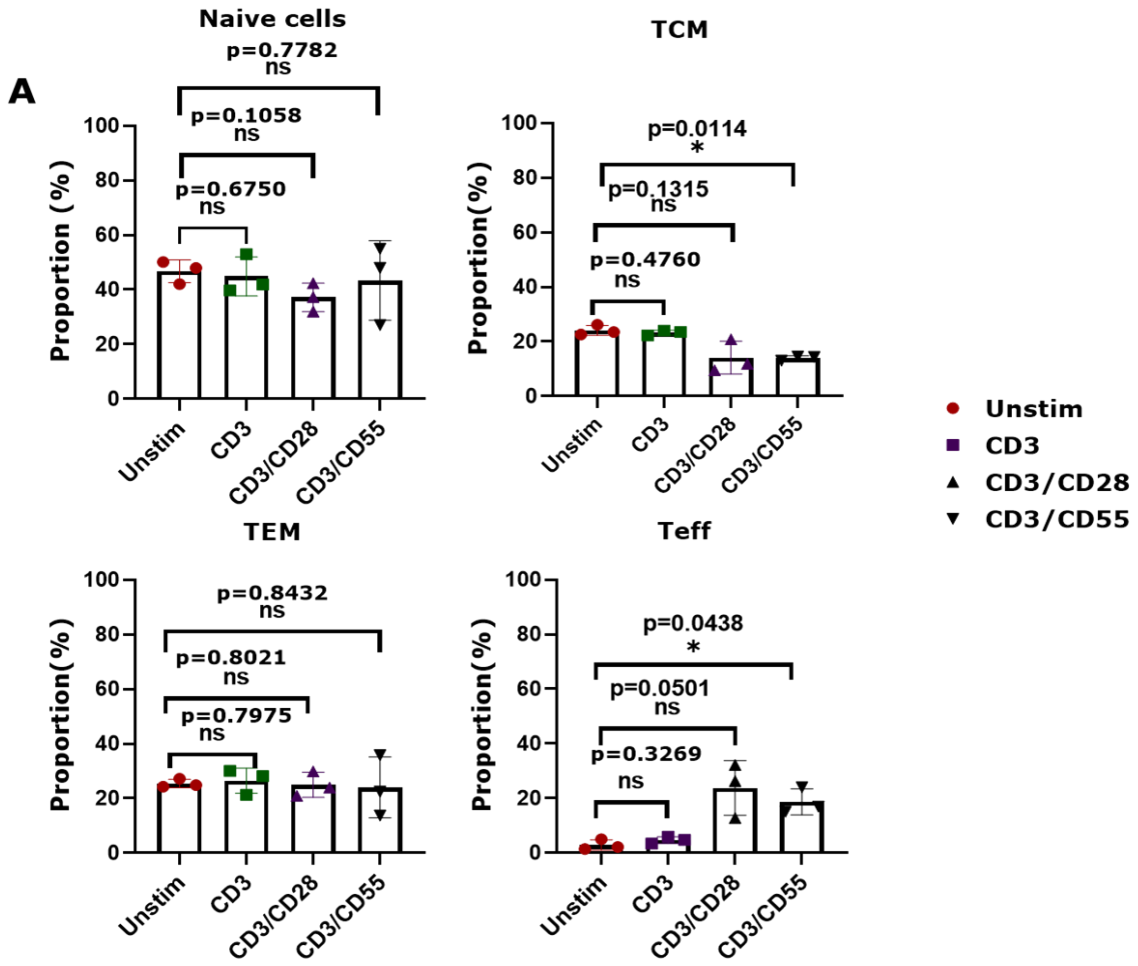

Unstimulated
CD3
CD3/CD28
CD3/CD55
SBD-010
SBD-003
SBD-007
CD62L
CD45RO
Naive 42.05%
TCM 26.18%
Teff 5.06%
TEM 24.29%
Naive 39.68%
TCM 23.52%
Teff 4.80%
TEM 30.01%
Naive 37.27%
TCM 9.57%
Teff 32.24%
TEM 20.92%
Naive 54.96%
TCM 14.35%
Teff 16.96%
TEM 13.74%
Naive 50.10%
TCM 23.58%
Teff 1.47%
TEM 24.85%
Naive 41.88%
TCM 24.07%
Teff 5.93%
TEM 28.12%
Naive 42.35%
TCM 20.91%
Teff 12.79%
TEM 23.96%
Naive 27.05%
TCM 13.06%
Teff 24.08%
TEM 35.82%
Naive 47.97%
TCM 22.61%
Teff 2.24%
TEM 27.17%
Naive 52.98%
TCM 22.31%
Teff 3.43%
TEM 21.28%
Naive 31.92%
TCM 11.85%
Teff 26.33%
TEM 29.91%
Naive 47.96%
TCM 14.51%
Teff 15.11%
TEM 22.42%

Figure 5.4: Distribution of memory phenotypes of $CD4^+$ T cells following CD3 stimulation, CD3/CD28 or CD55 costimulation

$CD4^+$ T cells isolated from peripheral blood of healthy donors were stimulated with CD3 alone, or costimulated with plate bound CD3/CD28 or CD3/CD55 for 24 hours. Unstimulated cells were maintained as negative control. Cells were stained with antibodies against CD62L & CD45RO, and data acquired by flow cytometry. Flow cytometry plots shown are for three donors in three independent experiments.



B

Population	mean (%)			
	Unstimulated	CD3	CD3/CD28	CD3/CD55
Naive	46.71	44.85	37.18	43.32
TCM	24.12	23.30	14.11	13.97
TEM	25.44	26.47	24.93	23.99
T_{eff}	2.92	4.72	23.79	18.72

Figure 5.5: Subpopulations of CD4$^+$ T cells following CD3 stimulation and CD3/C28 or CD3/CD55 costimulation of CD4$^+$ T cells

A Cells (%) within memory compartments defined by CD45RO/CD62L expression following stimulation of CD4$^+$ T cells with CD3 or costimulation with CD3/CD28 or CD3/CD55 for 24 hours. **B** Mean (%) changes following stimulation. Data shown are for three donors in three independent experiments summarized from Figure 5.4. Statistical significance was determined by Paired Student's *t* test, statistically significant at p≤0.05. ns= Not significant.

5.2.3 CD4$^+$ T cells secrete cytokines following CD3 stimulation, or costimulation with CD3/CD28 or CD3/CD55

To identify the memory phenotype of Tr1-like cells, CD4$^+$ T cells were stimulated, and cytokine secretion assays were performed for both IFN-γ and IL-10. These would then be combined with the effector/memory panel of markers to establish the presence of Tr1-like memory cells.

Purified CD4$^+$ T cells were isolated from human peripheral blood, stimulated with CD3 alone or costimulated with CD3/CD28 or CD3/CD55 for 24 hours, and assayed for IFN-γ and IL-10 expression by cytokine secretion assay. In Figure 5.6, results showed cytokine expression levels following the different stimuli. As with previous data, CD3 stimulation gave low amounts IFN-γ and IL-10 positive cells. This was increased with CD3/CD28 or CD3/CD55 costimulation. CD3/CD28 resulted in high amounts of IFN-γ expression, up to 9.57% and IL-10 expression up to 3.89%. The reverse trend was observed with CD3/CD55 costimulation as higher levels of IL-10 expression (up to 6.30%) was observed with lower (4.56%) IFN-γ expression. Data summarised in Figure 5.7 showed enhanced cytokine expression following CD3/CD28 and CD3/CD55 with no significant difference in levels of IL-10 expression, although CD3/CD28 costimulation resulted in a significantly higher ($p=0.0221$) IFN-γ$^+$ cells when compared to CD3 stimulation alone. However, the observed trend was consistent with data previously shown (Figure 4.8 and Figure 4.16).

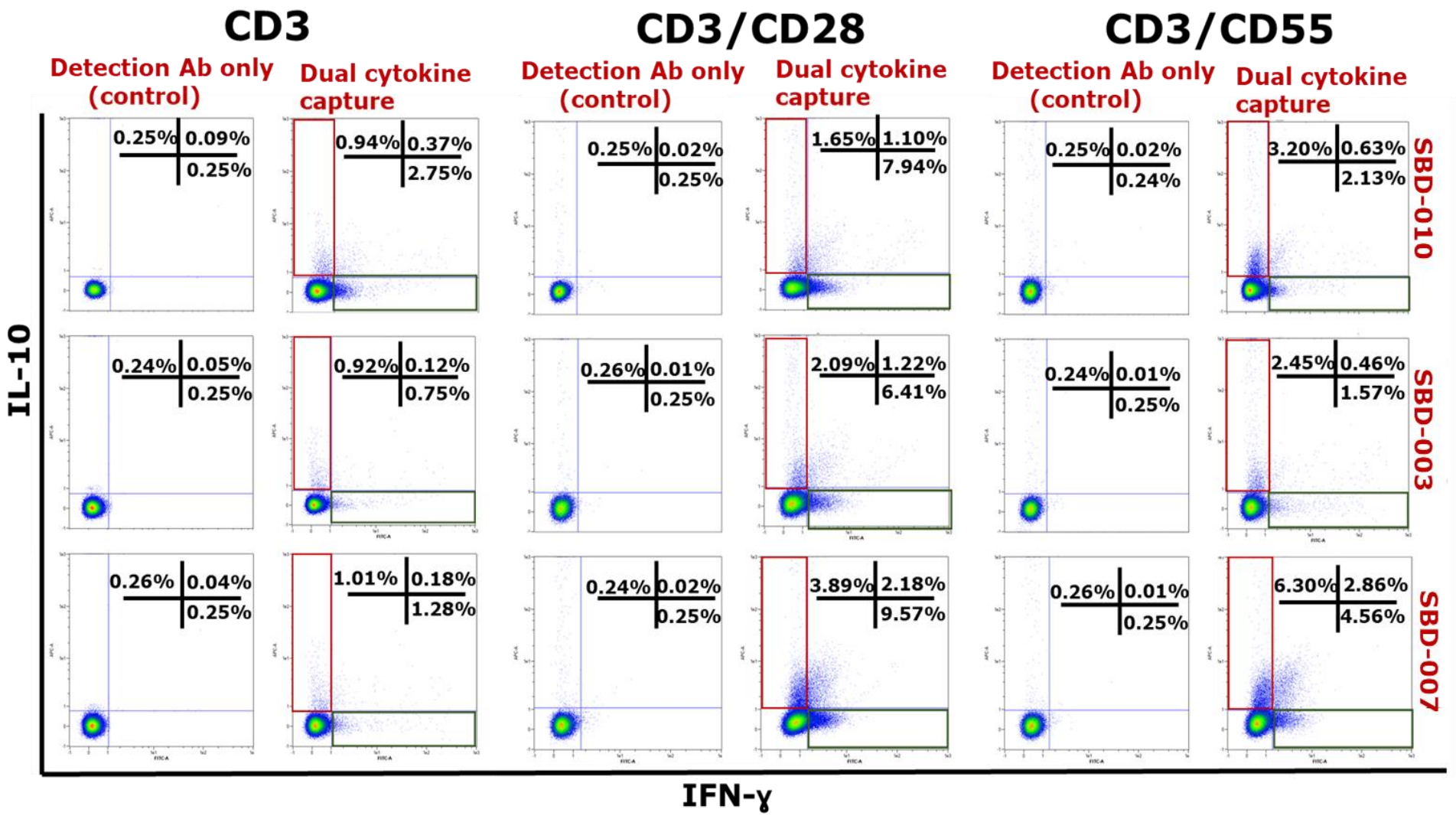

CD3
CD3/CD28
CD3/CD55
Detection Ab only (control)
Dual cytokine capture
Detection Ab only (control)
Dual cytokine capture
Detection Ab only (control)
Dual cytokine capture
IL-10
IFN-γ
SBD-010
SBD-003
SBD-007
0.25% 0.09% 0.25%
0.94% 0.37% 2.75%
0.25% 0.02% 0.25%
1.65% 1.10% 7.94%
0.25% 0.02% 0.24%
3.20% 0.63% 2.13%
0.24% 0.05% 0.25%
0.92% 0.12% 0.75%
0.26% 0.01% 0.25%
2.09% 1.22% 6.41%
0.24% 0.01% 0.25%
2.45% 0.46% 1.57%
0.26% 0.04% 0.25%
1.01% 0.18% 1.28%
0.24% 0.02% 0.25%
3.89% 2.18% 9.57%
0.26% 0.01% 0.25%
6.30% 2.86% 4.56%

Figure 5.6: Cytokine expression by $CD4^+$ T cells following CD3 stimulation and CD3/CD28 or CD3/CD55 costimulation

$CD4^+$ T cells isolated from peripheral blood of healthy donors were stimulated with plate bound anti-CD3, or costimulated with CD3/CD28 or CD3/CD55 for 24 hours at 1.5 x 10^5 cells/well in a 96 well plate. Flow cytometry plots showed IL-10, IFN-γ and double positive cells for the entire population as assessed by dual cytokine secretion assay. Stimulated cells stained with detection antibody (only) were used as control. Cells within the **RED** vertical rectangular box are IL-10 single positive while cells within the **GREEN** horizontal box are IFN-γ single positive cells. Data shown are for three donors in three independent experiments.

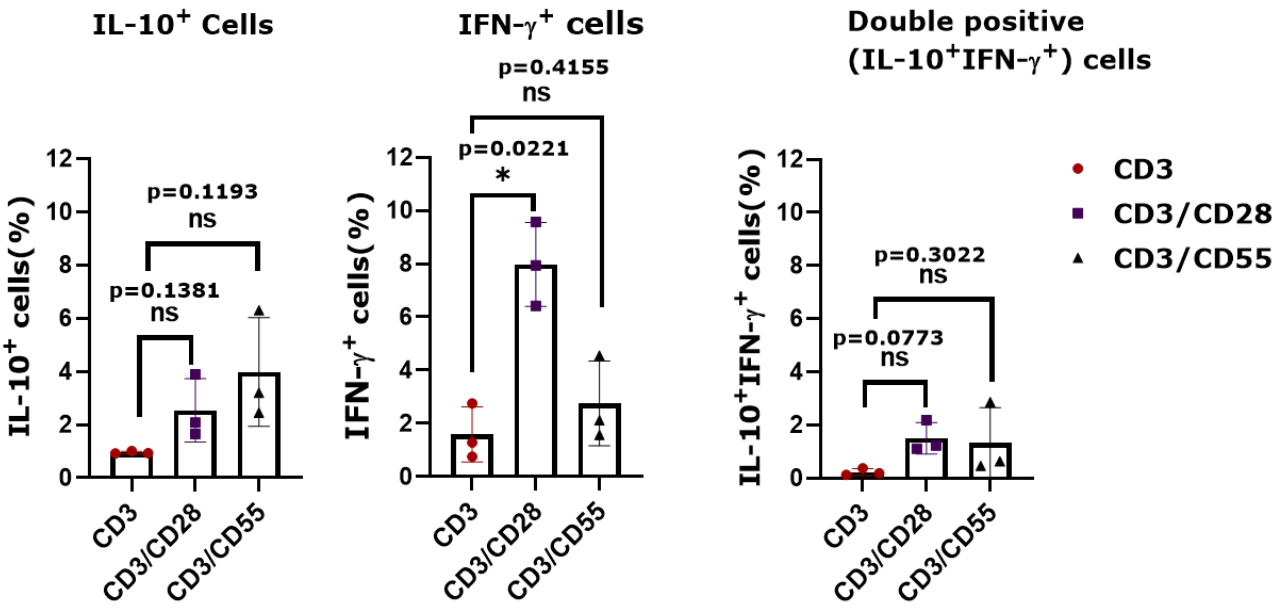


Figure 5.7: Cytokine positive CD4$^+$ T cells following CD3 stimulation, CD3/CD28 or CD3/CD55 costimulation

Cytokine positive cells (%) following stimulation of CD4$^+$ T cells with CD3 or costimulation with CD3/CD28 or CD3/CD55 for 24 hours. DP (cells double positive for IL-10 and IFN-γ). Data shown are for three donors in three independent experiments summarized from Figure 5.6. Statistical significance was determined by Paired Student's *t* test, statistically significant at p≤0.05. ns= Not significant

To determine the differentiation status of both IFN-γ and IL-10 positive cells following CD3 stimulation or CD3/CD28 or CD3/CD55 costimulation, $CD4^+$ T cells were assessed by dual cytokine secretion assay and simultaneously stained for CD62L and CD45RO.

Figure 5.8 shows percentage distribution of IFN-γ positive cells within the memory subtypes for the respective stimuli, while Figure 5.9 shows that a greater proportion (≈80%) of cells were in the effector memory population ($CD62L^{lo}CD45RO^+$) and cells subtypes displayed similar distribution across the different stimuli. This is despite either CD3 or CD3/CD55 costimulation resulting in half the number of IFN-γ positive cells.

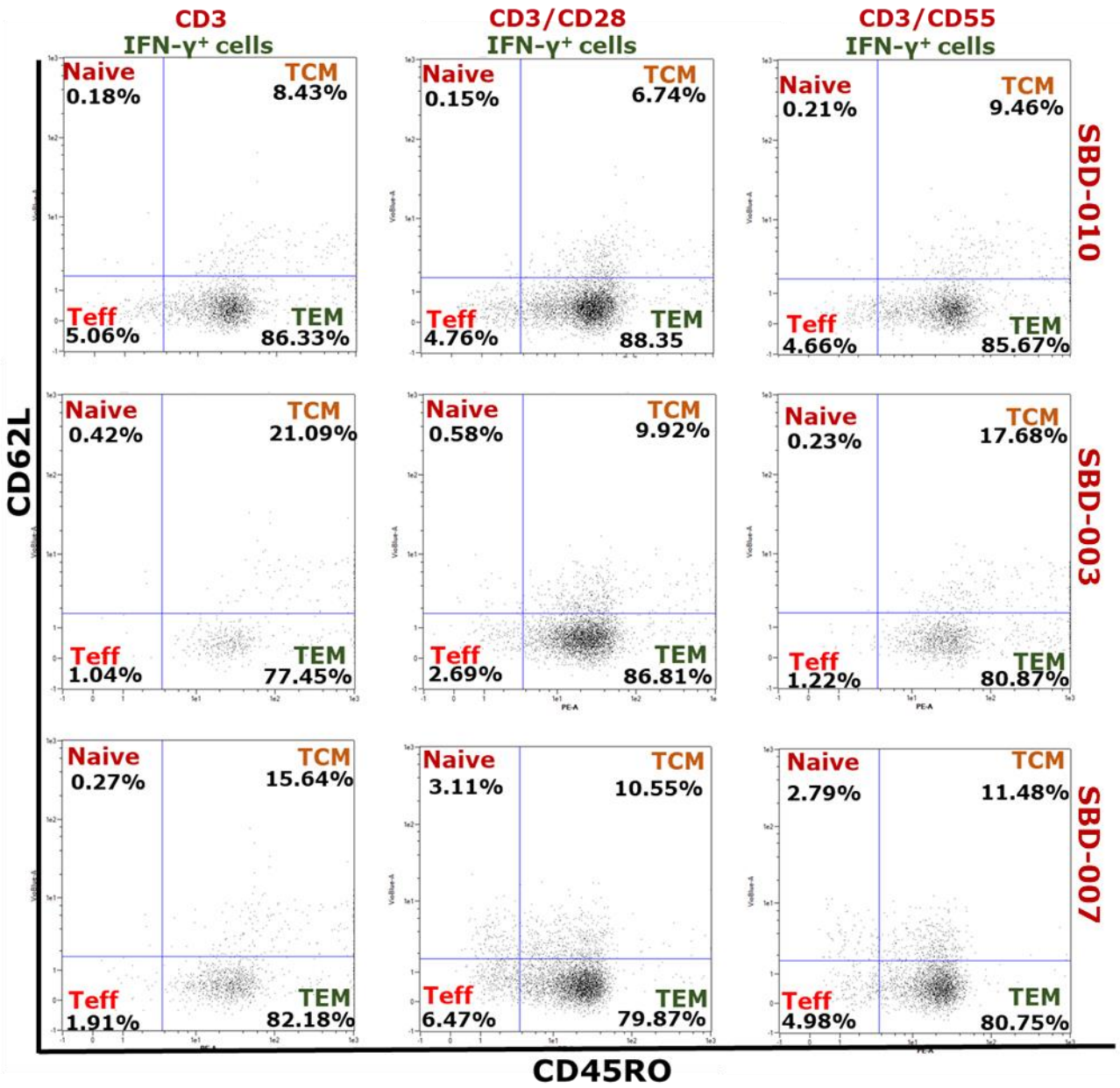


Figure 5.8: Distribution of IFN-ɣ$^+$ T cells following CD3 stimulation, CD3/CD28 or CD3/CD55 costimulation of CD4$^+$ T cells

CD4$^+$ T cells isolated from peripheral blood of healthy donors were stimulated with CD3, or costimulated with plate bound CD3/CD28 or CD3/CD55 for 24 hours. Cells were stained for memory markers (CD62L and CD45RO) and IFN-ɣ. IFN-ɣ$^+$ cells were measured by cytokine secretion assay, data acquisition by Flow cytometer, and IFN-ɣ$^+$ cells and CD62L$^+$ or CD45RO$^+$ cells determined based on gates from relevant controls. Percentage IFN-ɣ$^+$ cells are shown for three donors in three independent experiments.

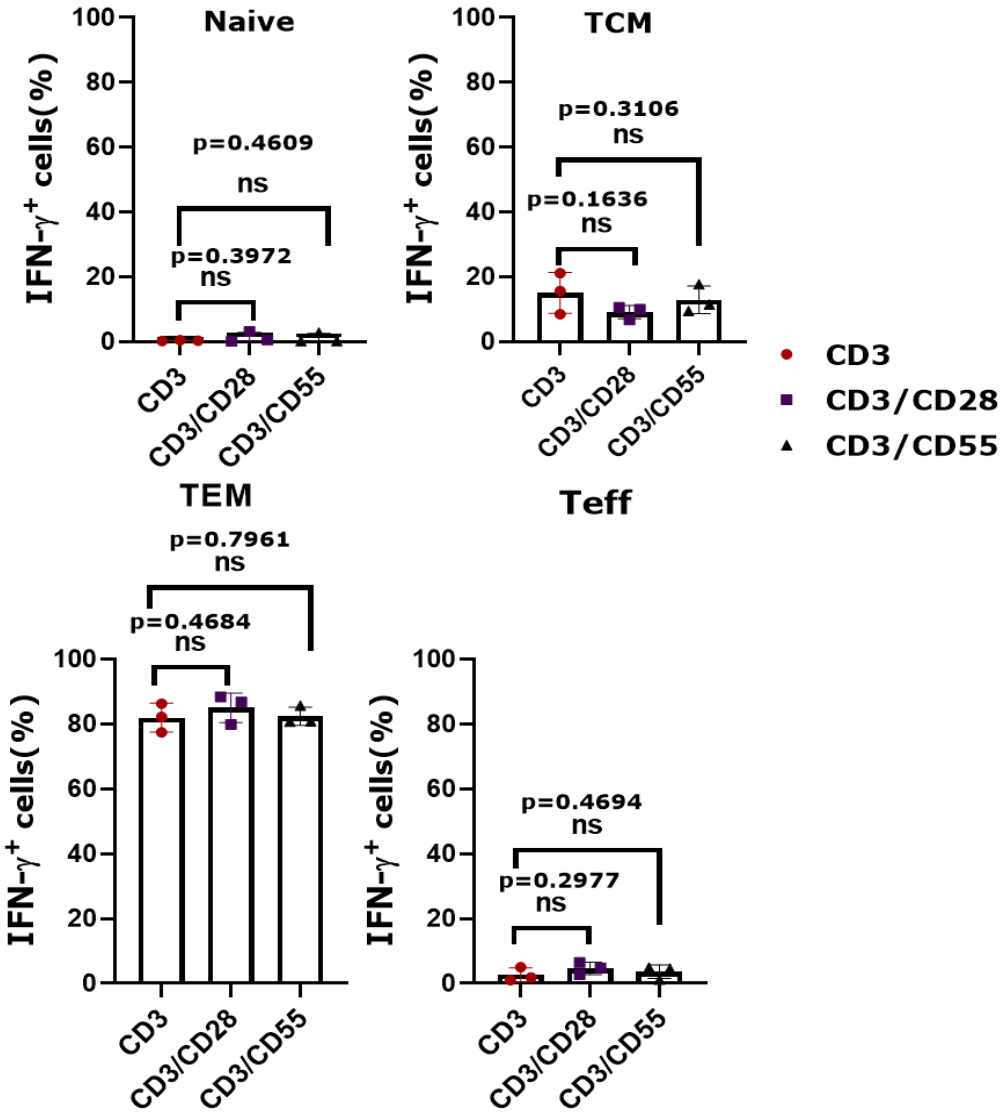


Figure 5.9: Distribution of IFN-ɣ⁺ T memory cells following CD3 stimulation, CD3/CD28 or CD3/CD55 costimulation

IFN-ɣ⁺ cells within memory compartments following stimulation of $CD4^+$ T cells with CD3 or costimulation with CD3/CD28 or CD3/CD55 for 24 hours. Data shown are for three donors in three independent experiments summarized from Figure 5.8. Statistical significance was determined by Paired Student's *t* test, statistically significant at p≤0.05. ns= Not significant

The distribution of the IL-10 positive cells within the effector memory pool showed a similar pattern to the IFN-γ population (Figure 5.10). Most cells (≈70%) were within the TEM pool (Figure 5.11) and data also showed that there was significant difference in IL-10^+ cells within TCM for CD3 stimulation compared to CD3/CD28 costimulation. This is despite the different number of cells stimulated to secrete either IFN-γ or IL-10 with the different stimuli (Figure 5.7). This would suggest that CD3/CD28 showed a preference for driving expansion of IFN-γ^+ cells and CD3/CD55 favoured the expansion of IL-10^+ cells, while both costimuli drove expansion of cells in the TEM pool in the 24 hours period assessed.

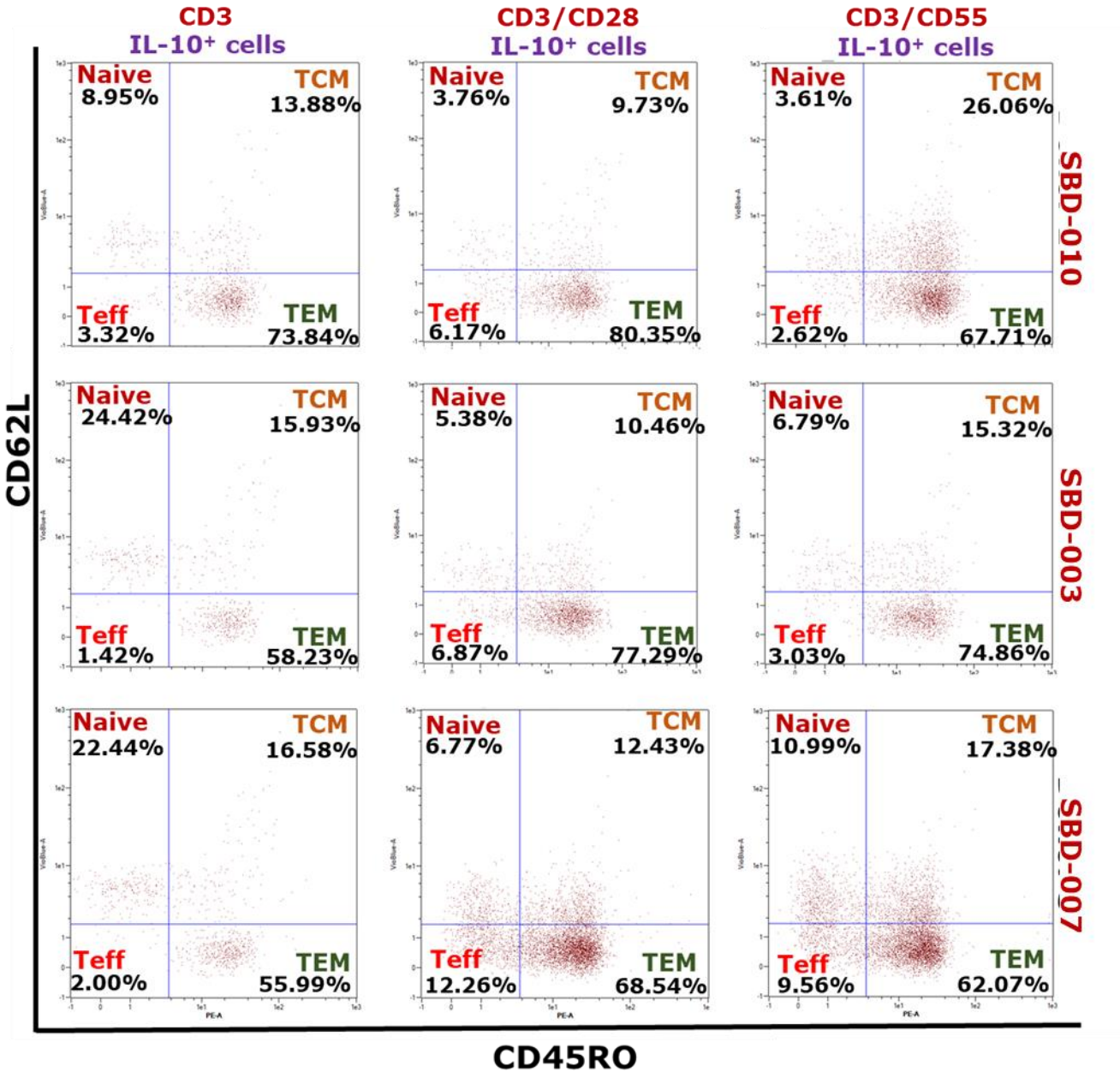


Figure 5.10: Distribution of IL-10$^+$ T cells following CD3 stimulation, CD3/CD28 or CD3/CD55 costimulation of CD4$^+$ T cells

CD4$^+$ T cells isolated from peripheral blood of healthy donors were stimulated with CD3, or costimulated with plate bound CD3/CD28 or CD3/CD55 for 24 hours. IL-10$^+$ cells were measured by cytokine secretion assay, data acquisition by Flow cytometer, and IL-10$^+$ cells and CD62L$^+$ or CD45RO$^+$ cells determined based on gates from relevant controls. Flow cytometer plots show percentage IL-10$^+$ cells for three donors in three independent experiments.

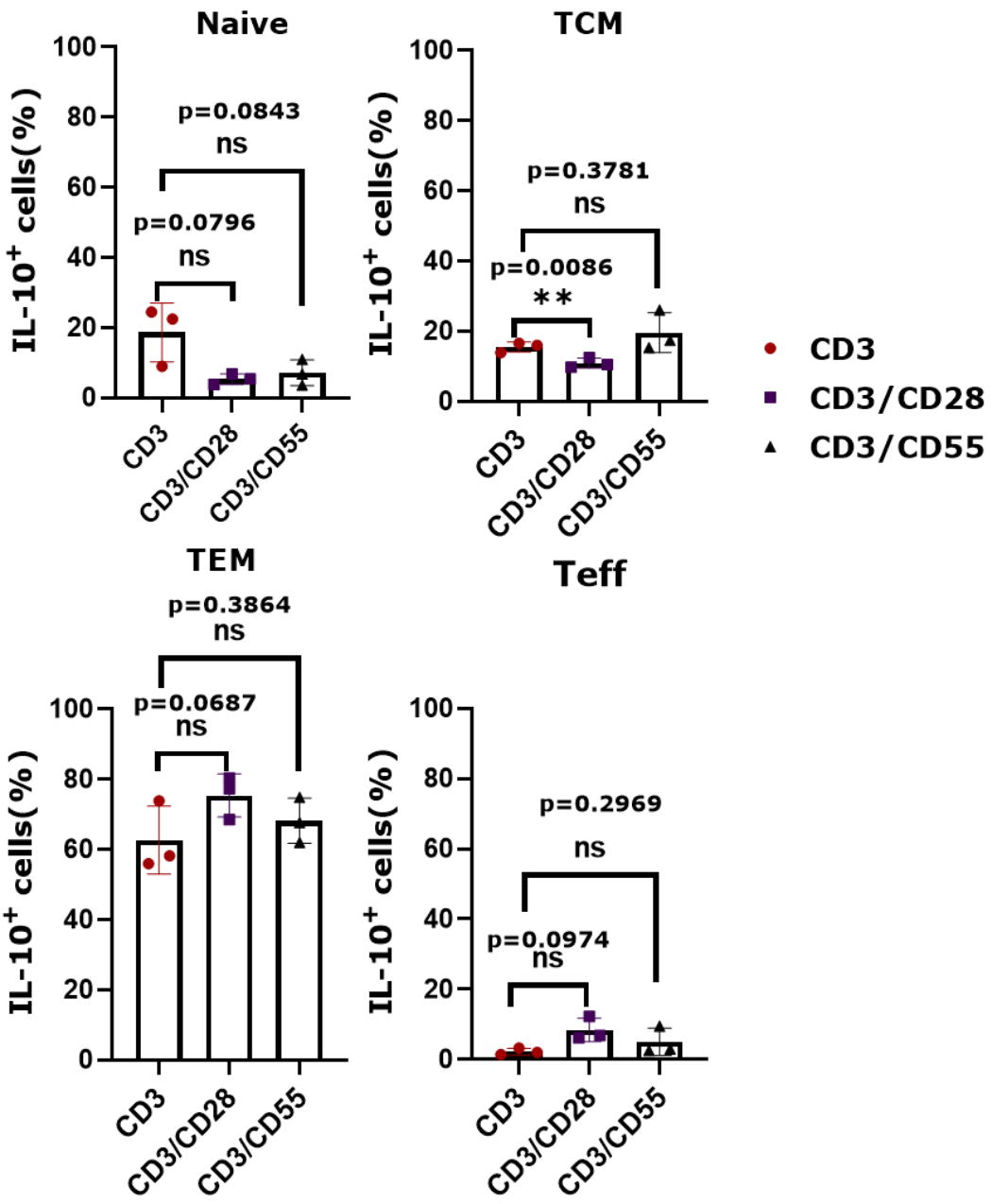


Figure 5.11: Distribution of IL-10$^+$ T memory cells among CD4$^+$ T memory subsets following CD3 stimulation, CD3/CD28 or CD3/CD55 costimulation

IL-10$^+$ cells within memory compartments following stimulation of CD4$^+$ T cells with CD3 or costimulation with CD3/CD28 or CD3/CD55 for 24 hours. Data shown are for three donors in three independent experiments summarized from Figure 5.10. Statistical significance was determined by Paired Student's *t* test, statistically significant at p≤0.05. ns= not significant

5.2.4 The kinetic profile of $CD4^+$ T cells subsets changes over 54 hours following CD3/CD55 costimulation

Having established that costimulation through either CD28 or CD55 resulted in activation of both IFN-ɣ and IL-10 secreting cells, with CD28 costimulation favouring the expansion of IFN-ɣ positive cells, and CD55 costimulation the expansion of IL-10 positive cells. It has been established that CD55 expanded IL-10 single positive cells are distributed in the same effector memory compartments as CD3/CD28 costimulated cells and in roughly the same proportions at 24 hours.

A kinetic analysis of $CD4^+$ T cells costimulated with CD3/CD55 revealed a reduction in naïve cells from 47.23% and an increase in cell numbers entering the memory pool (Figure 5.12). There was an initial doubling of cells entering the T effector pool which gradually declined back to baseline over 54 hours (Figure 5.13).

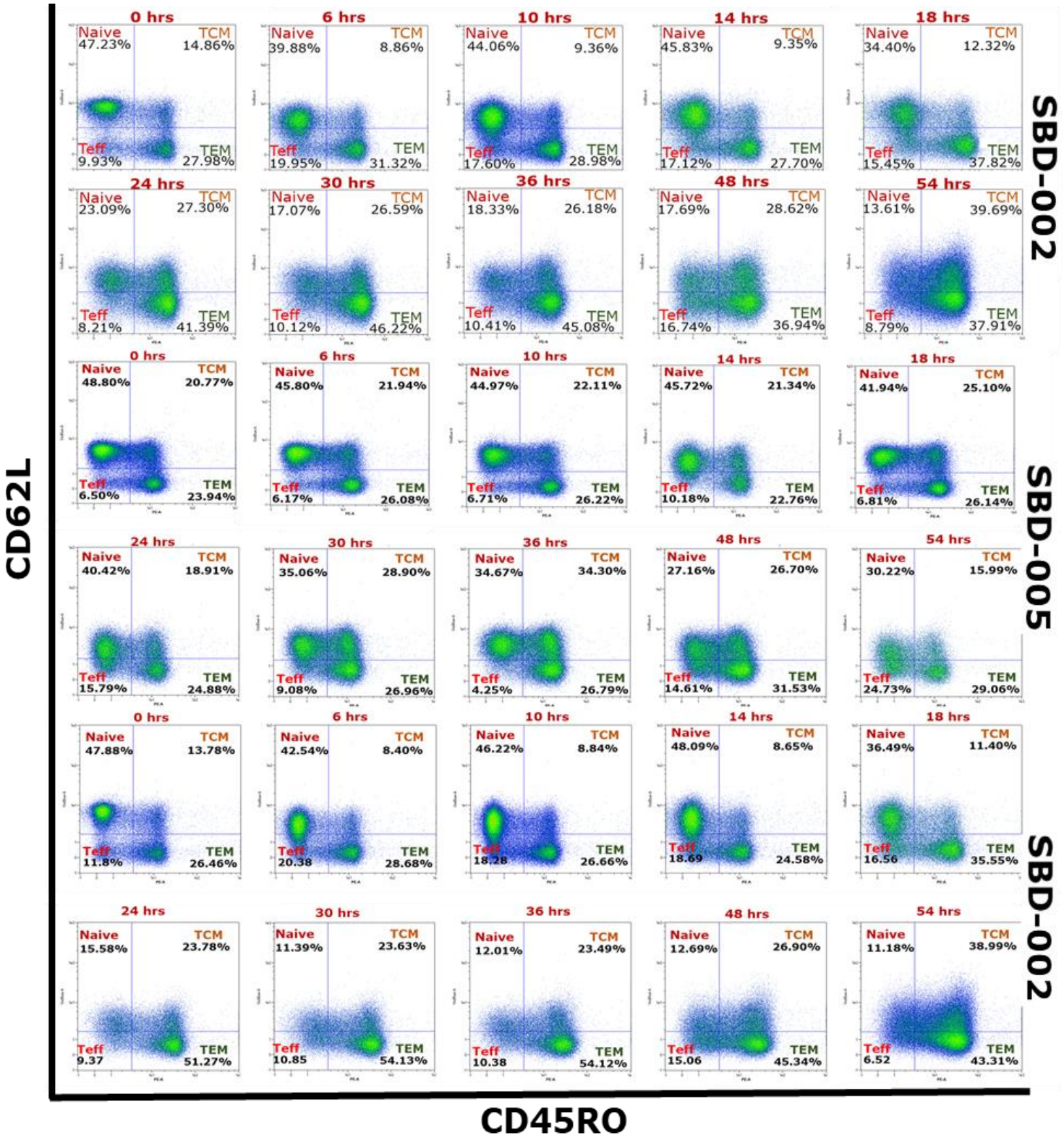

0 hrs
Naive 47.23%
TCM 14.86%
Teff 9.93%
TEM 27.98%
6 hrs
Naive 39.88%
TCM 8.86%
Teff 19.95%
TEM 31.32%
10 hrs
Naive 44.06%
TCM 9.36%
Teff 17.60%
TEM 28.98%
14 hrs
Naive 45.83%
TCM 9.35%
Teff 17.12%
TEM 27.70%
18 hrs
Naive 34.40%
TCM 12.32%
Teff 15.45%
TEM 37.82%
SBD-002
24 hrs
Naive 23.09%
TCM 27.30%
Teff 8.21%
TEM 41.39%
30 hrs
Naive 17.07%
TCM 26.59%
Teff 10.12%
TEM 46.22%
36 hrs
Naive 18.33%
TCM 26.18%
Teff 10.41%
TEM 45.08%
48 hrs
Naive 17.69%
TCM 28.62%
Teff 16.74%
TEM 36.94%
54 hrs
Naive 13.61%
TCM 39.69%
Teff 8.79%
TEM 37.91%
0 hrs
Naive 48.80%
TCM 20.77%
Teff 6.50%
TEM 23.94%
6 hrs
Naive 45.80%
TCM 21.94%
Teff 6.17%
TEM 26.08%
10 hrs
Naive 44.97%
TCM 22.11%
Teff 6.71%
TEM 26.22%
14 hrs
Naive 45.72%
TCM 21.34%
Teff 10.18%
TEM 22.76%
18 hrs
Naive 41.94%
TCM 25.10%
Teff 6.81%
TEM 26.14%
SBD-005
24 hrs
Naive 40.42%
TCM 18.91%
Teff 15.79%
TEM 24.88%
30 hrs
Naive 35.06%
TCM 28.90%
Teff 9.08%
TEM 26.96%
36 hrs
Naive 34.67%
TCM 34.30%
Teff 4.25%
TEM 26.79%
48 hrs
Naive 27.16%
TCM 26.70%
Teff 14.61%
TEM 31.53%
54 hrs
Naive 30.22%
TCM 15.99%
Teff 24.73%
TEM 29.06%
0 hrs
Naive 47.88%
TCM 13.78%
Teff 11.8%
TEM 26.46%
6 hrs
Naive 42.54%
TCM 8.40%
Teff 20.38
TEM 28.68%
10 hrs
Naive 46.22%
TCM 8.84%
Teff 18.28
TEM 26.66%
14 hrs
Naive 48.09%
TCM 8.65%
Teff 18.69
TEM 24.58%
18 hrs
Naive 36.49%
TCM 11.40%
Teff 16.56
TEM 35.55%
SBD-002
24 hrs
Naive 15.58%
TCM 23.78%
Teff 9.37
TEM 51.27%
30 hrs
Naive 11.39%
TCM 23.63%
Teff 10.85
TEM 54.13%
36 hrs
Naive 12.01%
TCM 23.49%
Teff 10.38
TEM 54.12%
48 hrs
Naive 12.69%
TCM 26.90%
Teff 15.06
TEM 45.34%
54 hrs
Naive 11.18%
TCM 38.99%
Teff 6.52
TEM 43.31%
CD62L
CD45RO

Figure 5.12: Kinetic profile of memory phenotype of $CD4^+$ T cells after costimulation with CD3/CD55 over 54 hours

$CD4^+$ T cells isolated from peripheral blood of healthy donors were stimulated with plate bound CD3/CD55. A 54hour time course assessment of cell phenotype was performed by staining for memory markers (CD62L and CD45RO). Data shown are for two donors in three independent experiments.

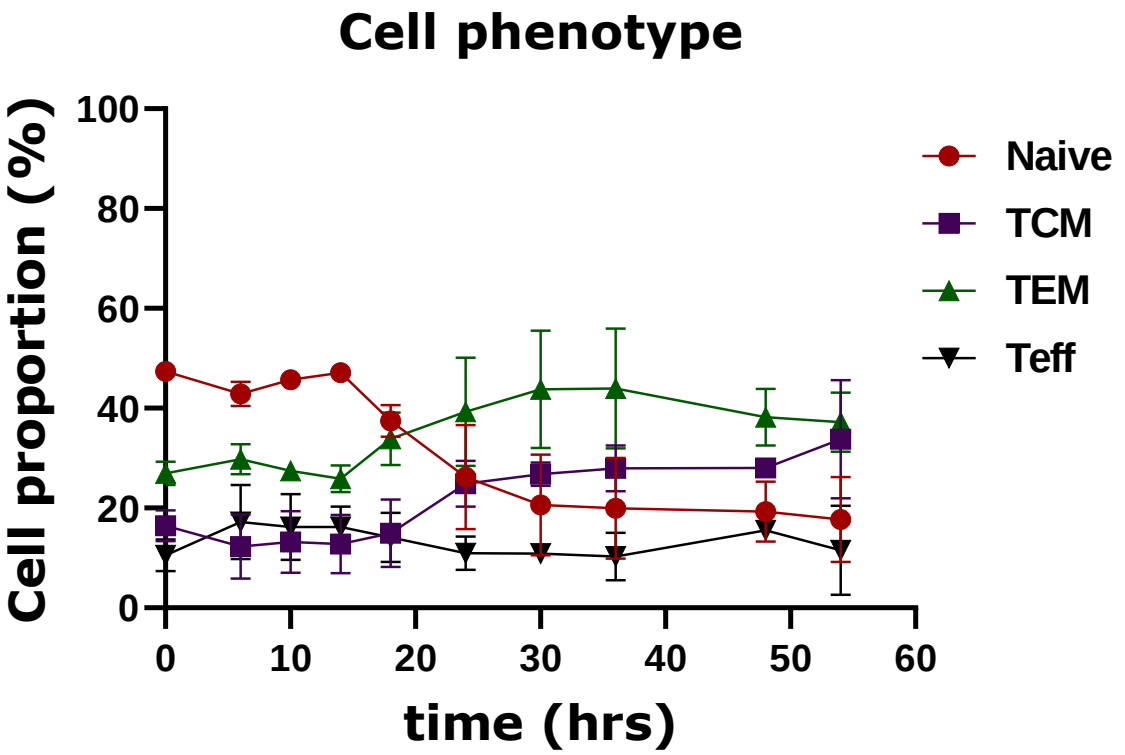


Figure 5.13: Changes in cell phenotype over 54hours time course following CD3/CD55 costimulation of $CD4^+$ T cells

Change in cell phenotype (%) over 54 hours following CD3/CD55 costimulation of $CD4^+$ T cells and staining for CD45RO and CD62L. Each coloured shape represents a timepoint for respective cell subpopulation (Naïve, TCM, TEM, Teff). Data shown are mean ± SD for two donors in three independent experiments (data from Figure 5.12).

5.2.5: Distribution of IFN-ɣ$^+$ and IL-10$^+$ T memory cells changes over time following CD3/CD55 costimulation of CD4$^+$ T cells

By analysing the memory phenotypes, it was possible to identify differences in the memory pools for both IFN-γ positive and IL-10 positive cells over time.

Both IFN-ɣ and IL-10 expressing cells showed similar profiles for the cells in each compartment. Cells within the naïve pool decreased while the TCM pool increased over 54 hours. Cells within the TEM pool increased up to 36 hours and returned to background levels at 54 hours. Teff cells in the IFN-ɣ positive Th1-like population showed a dip in numbers at 30 hours that then increased back to baseline levels at 56 hours. This was not the case for IL-10 positive Tr1-like cells that showed low T_{eff} cells throughout the experiment (Figure 5.16). The distribution of IFN-ɣ and IL-10 positive cells at 24 hours was maintained across multiple donors and comparison of TEM with other cell phenotypes: TEM with Naive ($P<0.0001$); TEM with TCM($P<0.0001$) and TEM with Teff($P<0.0001$) was significantly different (Figure 5.17).

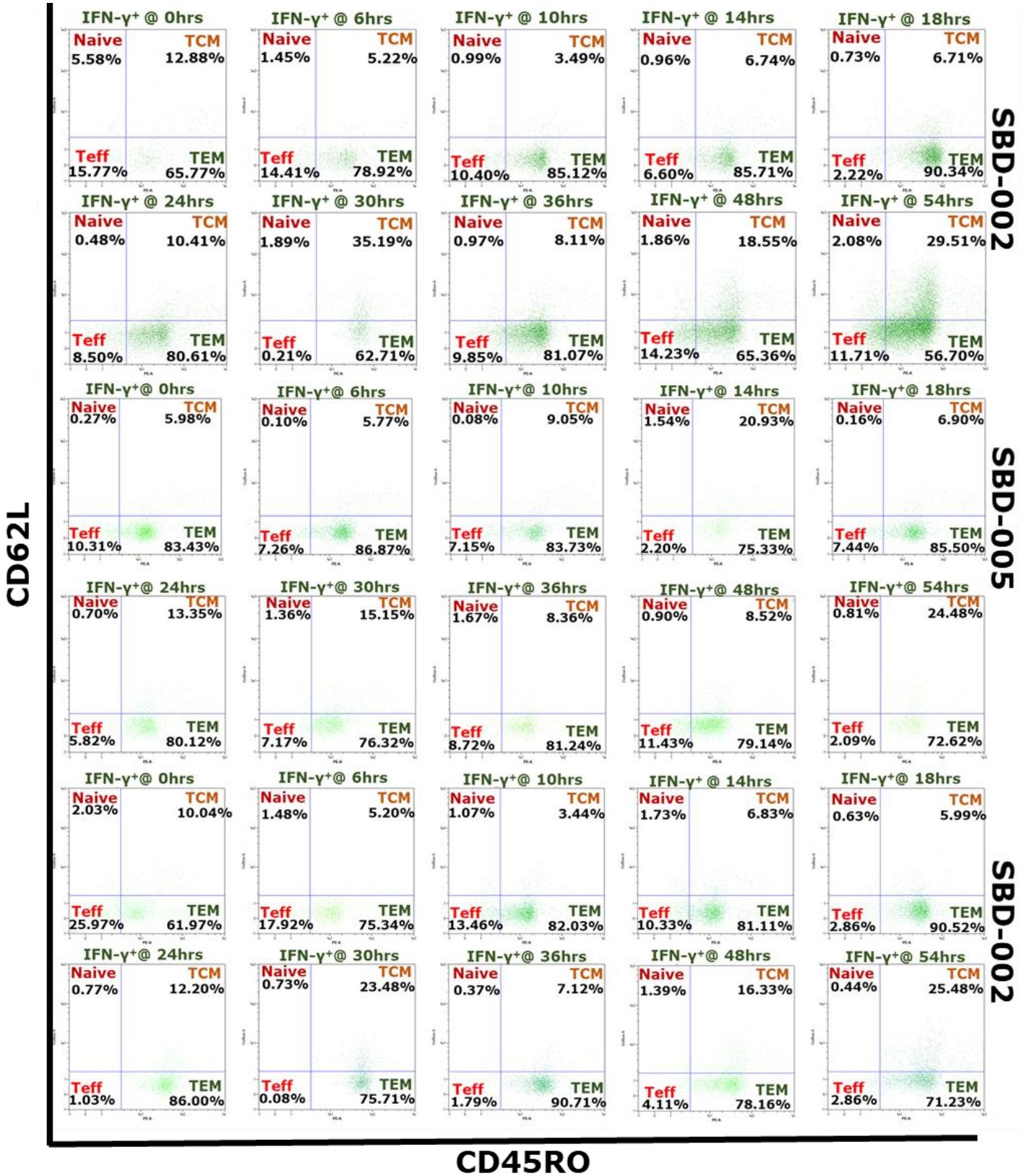

IFN-γ+ @ 0hrs
Naive 5.58%
TCM 12.88%
Teff 15.77%
TEM 65.77%
IFN-γ+ @ 6hrs
Naive 1.45%
TCM 5.22%
Teff 14.41%
TEM 78.92%
IFN-γ+ @ 10hrs
Naive 0.99%
TCM 3.49%
Teff 10.40%
TEM 85.12%
IFN-γ+ @ 14hrs
Naive 0.96%
TCM 6.74%
Teff 6.60%
TEM 85.71%
IFN-γ+ @ 18hrs
Naive 0.73%
TCM 6.71%
Teff 2.22%
TEM 90.34%
SBD-002
IFN-γ+ @ 24hrs
Naive 0.48%
TCM 10.41%
Teff 8.50%
TEM 80.61%
IFN-γ+ @ 30hrs
Naive 1.89%
TCM 35.19%
Teff 0.21%
TEM 62.71%
IFN-γ+ @ 36hrs
Naive 0.97%
TCM 8.11%
Teff 9.85%
TEM 81.07%
IFN-γ+ @ 48hrs
Naive 1.86%
TCM 18.55%
Teff 14.23%
TEM 65.36%
IFN-γ+ @ 54hrs
Naive 2.08%
TCM 29.51%
Teff 11.71%
TEM 56.70%
IFN-γ+@ 0hrs
Naive 0.27%
TCM 5.98%
Teff 10.31%
TEM 83.43%
IFN-γ+@ 6hrs
Naive 0.10%
TCM 5.77%
Teff 7.26%
TEM 86.87%
IFN-γ+@ 10hrs
Naive 0.08%
TCM 9.05%
Teff 7.15%
TEM 83.73%
IFN-γ+@ 14hrs
Naive 1.54%
TCM 20.93%
Teff 2.20%
TEM 75.33%
IFN-γ+@ 18hrs
Naive 0.16%
TCM 6.90%
Teff 7.44%
TEM 85.50%
SBD-005
IFN-γ+@ 24hrs
Naive 0.70%
TCM 13.35%
Teff 5.82%
TEM 80.12%
IFN-γ+@ 30hrs
Naive 1.36%
TCM 15.15%
Teff 7.17%
TEM 76.32%
IFN-γ+@ 36hrs
Naive 1.67%
TCM 8.36%
Teff 8.72%
TEM 81.24%
IFN-γ+@ 48hrs
Naive 0.90%
TCM 8.52%
Teff 11.43%
TEM 79.14%
IFN-γ+@ 54hrs
Naive 0.81%
TCM 24.48%
Teff 2.09%
TEM 72.62%
IFN-γ+@ 0hrs
Naive 2.03%
TCM 10.04%
Teff 25.97%
TEM 61.97%
IFN-γ+@ 6hrs
Naive 1.48%
TCM 5.20%
Teff 17.92%
TEM 75.34%
IFN-γ+@ 10hrs
Naive 1.07%
TCM 3.44%
Teff 13.46%
TEM 82.03%
IFN-γ+ @ 14hrs
Naive 1.73%
TCM 6.83%
Teff 10.33%
TEM 81.11%
IFN-γ+@ 18hrs
Naive 0.63%
TCM 5.99%
Teff 2.86%
TEM 90.52%
SBD-002
IFN-γ+@ 24hrs
Naive 0.77%
TCM 12.20%
Teff 1.03%
TEM 86.00%
IFN-γ+@ 30hrs
Naive 0.73%
TCM 23.48%
Teff 0.08%
TEM 75.71%
IFN-γ+@ 36hrs
Naive 0.37%
TCM 7.12%
Teff 1.79%
TEM 90.71%
IFN-γ+@ 48hrs
Naive 1.39%
TCM 16.33%
Teff 4.11%
TEM 78.16%
IFN-γ+@ 54hrs
Naive 0.44%
TCM 25.48%
Teff 2.86%
TEM 71.23%
CD62L
CD45RO

Figure 5.14: Changes in distribution of IFN-γ$^+$ T memory cells over 54 hours following CD3/CD55 costimulation of CD4$^+$ T cells

CD4$^+$ T cells, isolated from peripheral blood of healthy donors were stimulated with plate bound CD3/CD55. Time course assessment of IFN-γ$^+$ memory cells was done by staining for memory markers (CD62L and CD45RO) and IFN-γ. IFN-γ expression was measured by cytokine secretion assay. Data shown are for two donors in three independent experiments.

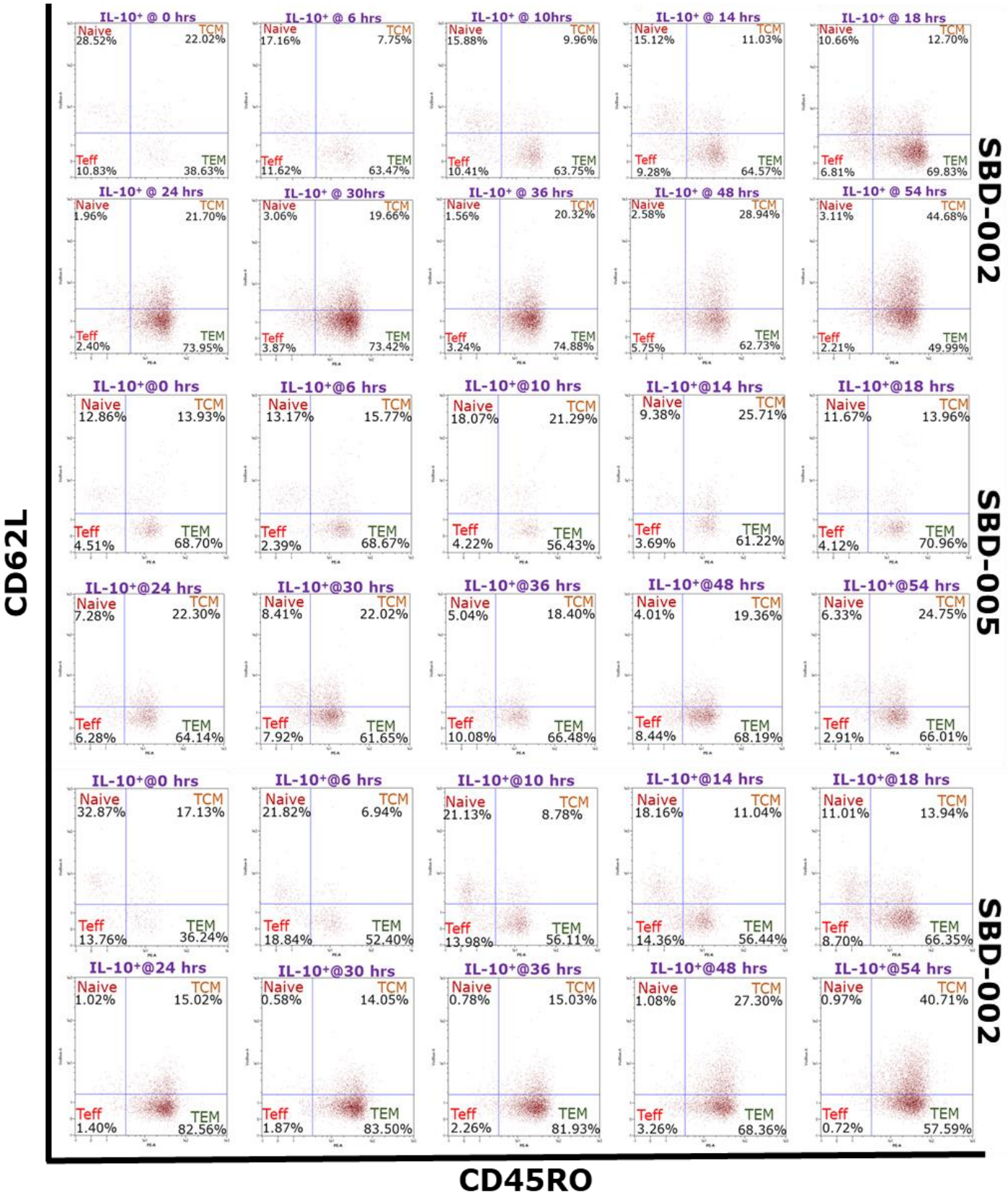

IL-10+ @ 0 hrs
Naive 28.52%
TCM 22.02%
Teff 10.83%
TEM 38.63%
IL-10+ @ 6 hrs
Naive 17.16%
TCM 7.75%
Teff 11.62%
TEM 63.47%
IL-10+ @ 10hrs
Naive 15.88%
TCM 9.96%
Teff 10.41%
TEM 63.75%
IL-10+ @ 14 hrs
Naive 15.12%
TCM 11.03%
Teff 9.28%
TEM 64.57%
IL-10+ @ 18 hrs
Naive 10.66%
TCM 12.70%
Teff 6.81%
TEM 69.83%
IL-10+ @ 24 hrs
Naive 1.96%
TCM 21.70%
Teff 2.40%
TEM 73.95%
IL-10+ @ 30hrs
Naive 3.06%
TCM 19.66%
Teff 3.87%
TEM 73.42%
IL-10+ @ 36 hrs
Naive 1.56%
TCM 20.32%
Teff 3.24%
TEM 74.88%
IL-10+ @ 48 hrs
Naive 2.58%
TCM 28.94%
Teff 5.75%
TEM 62.73%
IL-10+ @ 54 hrs
Naive 3.11%
TCM 44.68%
Teff 2.21%
TEM 49.99%
SBD-002
IL-10+@0 hrs
Naive 12.86%
TCM 13.93%
Teff 4.51%
TEM 68.70%
IL-10+@6 hrs
Naive 13.17%
TCM 15.77%
Teff 2.39%
TEM 68.67%
IL-10+@10 hrs
Naive 18.07%
TCM 21.29%
Teff 4.22%
TEM 56.43%
IL-10+@14 hrs
Naive 9.38%
TCM 25.71%
Teff 3.69%
TEM 61.22%
IL-10+@18 hrs
Naive 11.67%
TCM 13.96%
Teff 4.12%
TEM 70.96%
IL-10+@24 hrs
Naive 7.28%
TCM 22.30%
Teff 6.28%
TEM 64.14%
IL-10+@30 hrs
Naive 8.41%
TCM 22.02%
Teff 7.92%
TEM 61.65%
IL-10+@36 hrs
Naive 5.04%
TCM 18.40%
Teff 10.08%
TEM 66.48%
IL-10+@48 hrs
Naive 4.01%
TCM 19.36%
Teff 8.44%
TEM 68.19%
IL-10+@54 hrs
Naive 6.33%
TCM 24.75%
Teff 2.91%
TEM 66.01%
SBD-005
IL-10+@0 hrs
Naive 32.87%
TCM 17.13%
Teff 13.76%
TEM 36.24%
IL-10+@6 hrs
Naive 21.82%
TCM 6.94%
Teff 18.84%
TEM 52.40%
IL-10+@10 hrs
Naive 21.13%
TCM 8.78%
Teff 13.98%
TEM 56.11%
IL-10+@14 hrs
Naive 18.16%
TCM 11.04%
Teff 14.36%
TEM 56.44%
IL-10+@18 hrs
Naive 11.01%
TCM 13.94%
Teff 8.70%
TEM 66.35%
IL-10+@24 hrs
Naive 1.02%
TCM 15.02%
Teff 1.40%
TEM 82.56%
IL-10+@30 hrs
Naive 0.58%
TCM 14.05%
Teff 1.87%
TEM 83.50%
IL-10+@36 hrs
Naive 0.78%
TCM 15.03%
Teff 2.26%
TEM 81.93%
IL-10+@48 hrs
Naive 1.08%
TCM 27.30%
Teff 3.26%
TEM 68.36%
IL-10+@54 hrs
Naive 0.97%
TCM 40.71%
Teff 0.72%
TEM 57.59%
SBD-002
CD62L
CD45RO

Figure 5.15: Changes in distribution of IL-10$^+$ T memory cells over 54 hours following CD3/CD55 costimulation of CD4$^+$ T cells

CD4$^+$ T cells, isolated from peripheral blood of healthy donors were stimulated with plate bound CD3/CD55. Time course assessment of IL-10$^+$ cells within the memory compartments was done by staining for memory markers (CD62L and CD45RO) and IL-10. IL-10 expression was measured by cytokine secretion assay. Flow cytometry plots shown are for two donors in three independent experiments.

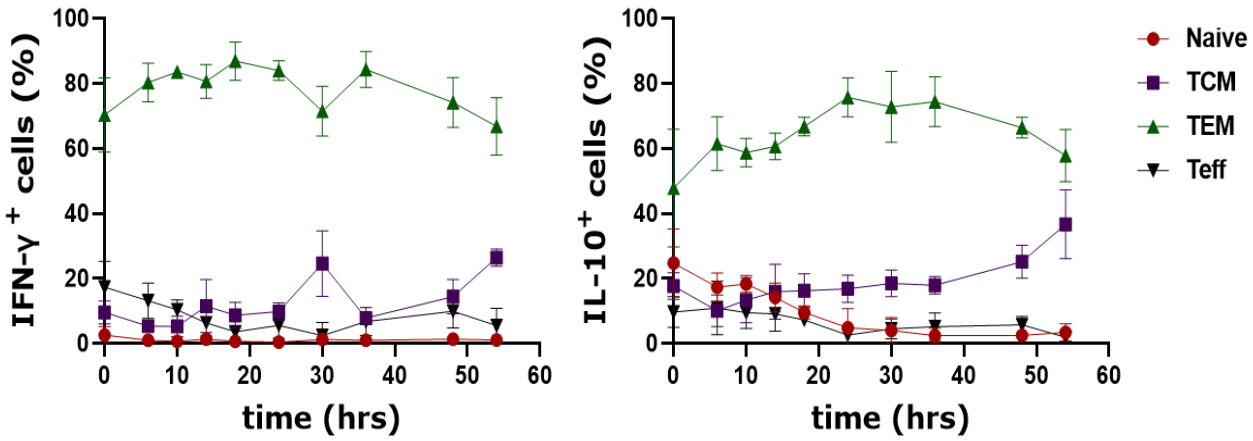


Figure 5.16: IFN-ɣ$^+$ and IL-10$^+$ expression by memory T cells over 54hours time course following CD3/CD55 costimulation of CD4$^+$ T cells

IFN-ɣ$^+$ and IL-10$^+$ expression by CD4$^+$ T cell subpopulations over 54 hours following CD3/CD55 costimulation. Each coloured shape represents a timepoint for respective cell subpopulation (Naïve, TCM, TEM, Teff). Data shown are mean ± SD for two donors in three independent experiments derived from Figures 5.14 and 5.15, respectively.

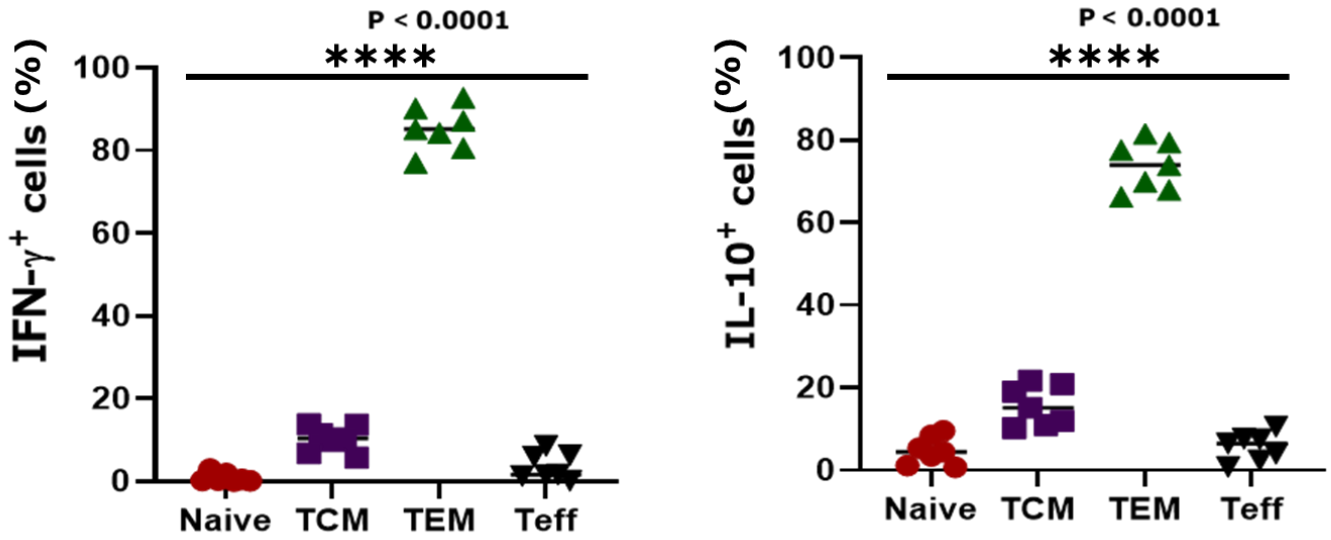


Figure 5.17: IFN-ɣ$^+$ and IL-10$^+$ cells are mainly within the effector memory pool across multiple donors following CD3/CD55 costimulation of CD4$^+$ T cells

Distribution of IFN-ɣ$^+$ and IL-10$^+$ cells (%) within memory compartments following CD3/CD55 costimulation of CD4$^+$ T cells for 24 hours. Measurement of IFN-ɣ and IL-10 positive cells by dual cytokine secretion assay together with the extracellular staining for CD62L and CD45RO. Data shown are for seven donors in seven independent experiments. Comparison of TEM with other cell phenotypes: TEM with Naive ($P<0.0001$); TEM with TCM($P<0.0001$) and TEM with Teff($P<0.0001$). Statistical significance was determined by Repeated Measures One-way Analysis of variance (ANOVA) followed by Dunnett's post hoc test and statistically significant at $p\leq0.05$.

5.3: Discussion

Costimulation of peripheral blood $CD4^+$ T cells with CD3/CD55 resulted in the activation, proliferation, and production of a small percentage of IL-10 positive cells. These cells also responded to stimulation of TCR via CD3 alone or costimulation by CD3/CD28. While the classical costimulators, CD3/CD28 gave a predominantly IFN-ɣ positive (Th1-like) cells, it also resulted in the stimulation of the IL-10 positive population. Costimulation with CD3/CD55 however gave a predominant IL-10 (Tr1-like) population but lower numbers of IFN-ɣ positive (Th1-like) cells. When examining the distribution of these cells in the naïve and effector/memory pools, the $IL\text{-}10^+$ Tr1-like cells in human peripheral blood were predominantly effector memory T cells and have similar T cell memory distribution compared to the well-established $IFN\text{-}\gamma^+$ Th1-like cells.

The differentiation status of the IL-10 positive, Tr1-like cells was assessed following either PMA/I, CD3 or by costimulation with CD3/CD28 or CD3/CD55. Although PMA/I stimulate a population of IL-10 cells (earlier reported in chapter 4), it proved difficult to characterize the memory status of $IL\text{-}10^+$ cells following PMA/I stimulation. This is because PMA/I stimulation leads to the shedding of CD62L selectin on T cells. It has been previously reported that T cells shed CD62L following activation by PMA/I (B. Gu, Bendall, & Wiley, 1998; Sengstake, Boneberg, & Illges, 2006; Yang, Liu, Wang, Rosenberg, & Morgan,

2011) and this process is thought to control the migration pathways of antigen-activated T cells by allowing cells to re-enter circulation to exert helper or effector functions (Galkina et al., 2003; Ivetic, Hoskins Green, & Hart, 2019). However, there was no reasonable level of CD62L shedding observed with CD3 stimulation alone or CD3/CD28 or CD3/CD55 costimulation. It is worth noting that while CCR7 is often used to distinguish the memory populations, the heterogeneity of CCR7 expression on memory T cells often possess a challenge. However, CD62L is much more evenly expressed and often a more reliable surface marker for distinguishing between T central memory (TCM) and T effector memory (TEM) phenotypes (Unsoeld & Pircher, 2005).

It has been established that upon TCR recruitment and costimulation, naïve $CD4^+$ T cells proliferate, differentiate, and acquire effector functions including cytokine secretion (Brenchley et al., 2002). The slight changes in overall cell phenotype observed upon stimulation of $CD4^+$ T cells with CD3 alone or costimulation with CD3/CD28 or CD3/CD55 compared with unstimulated cells, demonstrated the acquisition of effector functions by naïve cells and re-stimulated effector/memory pools. Of note was the fact that CD3 stimulation alone did not generate sufficient effector cells response from the memory pools. Furthermore, the high levels of cytokines (IL-10 and IFN-γ) expressed at 24 hours indicated that cells were predominantly effector/ memory T cells. CD3/CD28 costimulation of $CD4^+$ T cells revealed a predominantly IFN-γ positive population of cells with a small population

of IL-10 positive cell. This cytokine expression pattern was like that observed for CD3 but different from the expression pattern observed for CD3/CD55 costimulation, which showed a higher IL-10 positive cell population and a much lower population of Th1-like cells, compared to CD28. CD3/CD28 costimulation is regarded as the classical costimulation for Th1 responses for T cells and CD28 receptor ligation provides a critical second signal alongside TCR ligation for naive T cell activation, proliferation and differentiation(Alina C. Boesteanu & Peter D. Katsikis, 2009; Jonathan H. Esensten et al., 2016). Analysis of the IFN-γ and IL-10 positive cells following CD3/CD28 costimulation of $CD4^+$ T cells revealed that cells were mostly TEM cells. When compared to CD3/CD55 costimulation, it was noted that although CD55 stimulated a different cytokine profile, the resulting IFN-γ and IL-10 positive cells assumed a similar pattern of memory distribution, suggesting that the Tr1-like cells have followed a similar pathway of differentiation into effector and memory cells.

Memory T cells are antigen-specific cells that are long-lived after an infection has been eliminated and they confer enhanced secondary immune response to the host. These cells are quickly converted into large numbers of effector T cells upon re-challenge by specific antigen leading to a more rapid response by secondary effector cells with enhanced functionality (Garcia et al., 1999; Gasper et al., 2014; McKinstry et al., 2008; Yamane & Paul, 2012). $CD8^+$ effector/memory stimulation is reported to occur over a short period (6-24 hours) while

$CD4^+$ T cells require longer periods of antigen stimulation (3-4 days) for optimal expansion (Kaech & Ahmed, 2001; Obst, van Santen, Mathis, & Benoist, 2005; Ravkov & Williams, 2009).

This study reported a robust memory response over 54 hours of stimulation of $CD4^+$ T cells and two possible scenarios may account for the early memory expansion observed. It is possible that these cells were predominantly effector or memory cells with enhanced ability for robust recall response upon stimulation or a strong secondary stimulus may have driven the early response observed. Nonetheless, $CD4^+$ T cell effector cell restimulation has been reported to depend in part on the strength of the antigenic stimulus (Gett, Sallusto, Lanzavecchia, & Geginat, 2003; Swain et al., 2006) and a robust secondary challenge improved the stability, size and early acquisition of increased effector functions by Th1 memory cells (H. D. Marshall et al., 2011).

IFN-γ has been reported to play a vital role in the generation but not maintenance of Th1 memory(Kim et al., 2012; Sallusto et al., 2004). Additionally, primary Th1 memory cells are reported to decline as the cells acquire heightened sensitivity to antigen (Williams, Ravkov, & Bevan, 2008). This might support the rapid expansion of IFN-γ^+ TEM cells followed by a decline over the time course seen in this study. This observation is consistent with the reporting that TEM cells are protective memory cells and migrate to inflamed peripheral tissues and display rapid effector functions, and therefore sustain a repertoire of high

avidity responder cells upon antigenic re-challenge (Sallusto et al., 2004). Conversely, the rapid decline in IFN-γ^+ TCM population upon stimulation and gradual increase in cell population supports the idea that central memory cells are reactive in nature, and home to secondary lymphoid organs, possessing no effector functions but readily become effector cells upon restimulation. It is important to note that the kinetics of IL-10^+ TEM and TCM populations were like that observed for IFN-γ^+ memory cells, essentially showing evidence for a Tr1-like memory with similar memory distribution like the Th1 memory. Furthermore, these cells were predominantly within the TEM pool which showed they possessed a pronounced capacity for immediate effector functions within non-lymphoid tissues and inflammation sites.

This study showed a population of IL-10^+ Tr1-like memory cells that displayed similar distribution pattern to the well-established IFN-γ^+ Th1-like memory cells. These cells were predominantly effector memory cells upon CD3/CD55 costimulation and compared to CD3 stimulation alone or CD3/CD28 costimulation. This is the first study that attempted to characterize Tr1 memory cells, although it is not yet known whether these cells expressed surface markers and transcription factors commonly associated with Tr1 cells.

Chapter 6

IL-10^{+} cells from human peripheral blood express markers and transcription factors commonly associated with Tr1 cells

6.1: Introduction

Evidence suggests that active suppression by T regulatory (Tr) cells is essential for induction and maintenance of peripheral tolerance to both self and foreign antigens (Groux, 2003; Satoguina et al., 2002; Hanyu Zeng et al., 2015). Based on experimental models, many distinct types of Tr1 cells have been described(Kemper et al., 2003; Levings et al., 2005; Levings et al., 2001); however, many aspects of the mechanisms which control their differentiation and function remain to be elucidated due to lack of definite phenotypic markers for both murine and human. Current descriptions of Tr1 cells include a variety of phenotypic markers including cytokines, cell surface receptors and transcription factors.

Since discovery (Groux et al., 1997), several studies have put forward a range of markers to fully characterise Tr1 cells. Gagliani et.al., suggested that co-expression of LAG-3 and CD49b along with CD226 identified Tr1 cells (Gagliani et al., 2013), whereas White et al., suggested TIM-3 and PD-1 expression better describes Tr1 cells(White & Wraith, 2016). Additionally, studies have reported that Tr1 cells can kill effector and myeloid cells through the Perforin-Granzyme B pathway (Norio Chihara et al., 2016), but no transcription factor has yet been characterized as the master regulator for Tr1 cells.

The presence of these markers is dependent on a range of environmental factors. Most phenotypic immune markers change in response to different stimuli and often with different kinetics. Combined, these provide a timely and measured response of any cellular population to an insult or trigger.

Thus, the manner of stimuli and the point at which markers were measured may well provide variation in the reporting of any single marker and may be even more varied for multiple markers. This has, for Tr1 cells, made them poorly defined. Tr1 cells mediate anti-inflammatory role through their production of interleukin 10 (IL-10), which dampens the function of both antigen-presenting cells and antigen-specific effector T cells. Therefore, the most common description of Tr1 cells is the expression of high amounts of IL-10 (G. Andolfi et al., 2012; Battaglia, Gianfrani, Gregori, & Roncarolo, 2004; Cook et al., 2019), combined with absence of IL-4 (Levings et al., 2001; Roncarolo et al., 2014). This is often reported with variations in a range of cells surface markers and transcription factors.

In this study, IL-10 expression was used as the starting point to evaluate other reported phenotypic markers and transcription factors associated with Tr1 cells and therefore we hypothesized that IL-10^{+} cells from CD3/CD55 costimulation of human peripheral blood $CD4^{+}$ T cells expressed markers and transcription factors associated with Tr1 cells.

6.2 Results:

6.2.1: High proportion of IL-10$^+$ cells from human peripheral CD4$^+$ T cells express CD226 but not CD49b and LAP

CD49b, LAP and CD226 have been commonly described as being representative markers of Tr1 cells.

Co-expression of CD49b and LAG-3 has been reportedly used to characterise both human and murine Tr1 cells (Gagliani et al., 2013), although further studies demonstrated that co-expression of CD49b and LAG3 was not restricted to the Foxp3$^-$ Tr1 cells, but was also observed in Foxp3$^+$ T regulatory (Treg) cells and CD8$^+$ T cells that produced IL-10 (W. Huang, Solouki, Carter, Zheng, & August, 2018). CD4$^+$ T cells expressing LAP plays an important role in the modulation of immune responses(Ochi et al., 2006; Tran et al., 2009). Scurr et al., reported that tumour infiltrating lymphocytes (TILs) that were CD4$^+$LAP$^+$ and co-expressed LAG-3 and CD25 were more potent supressing effector T cells compared with conventional FoxP3$^+$ Tregs (Scurr et al., 2014b). It has also been reported that expression of CD226 and TIGIT on human CD4$^+$Foxp3$^+$ Treg cells were related to their stability and function (Lozano, Dominguez-Villar, Kuchroo, & Hafler, 2012) and was also involved in the killing of myeloid antigen presenting cells by human Tr1 cells (Magnani et al., 2011).

Since these surface markers (CD49b, LAP and CD226) were reported to mediate crucial functions of Tr1 cells, their expression by IL-10$^+$ Tr1-like cells following CD3/CD55 costimulation of human CD4$^+$ T cells was assessed.

CD4$^+$ T cells were purified from human peripheral blood and costimulated with CD3/CD55. Cells were assessed for purity, gated for single cells and with isotypes for surface, markers and intracellular staining (Figure 6.1). IL-10 cytokine secretion assay was used to define IL-10 positive cells and cells were co-stained simultaneously for expression of CD49b, LAP and CD226. The percentage of IL-10 positive population that were also positive for each of the markers is shown in the upper right quadrant (Figure 6.2).

It can be seen that only 12-20% of the IL-10$^+$ cells were positive for CD49b (Figure 6.2A row 1 and Figure 6.2B). Similarly, an average of about 20% of the IL-10$^+$ cells were positive for LAP (Figure 6.2A row 2 and Figure 6.2B) while approximately 80% of IL-10$^+$ cells also expressed CD226.

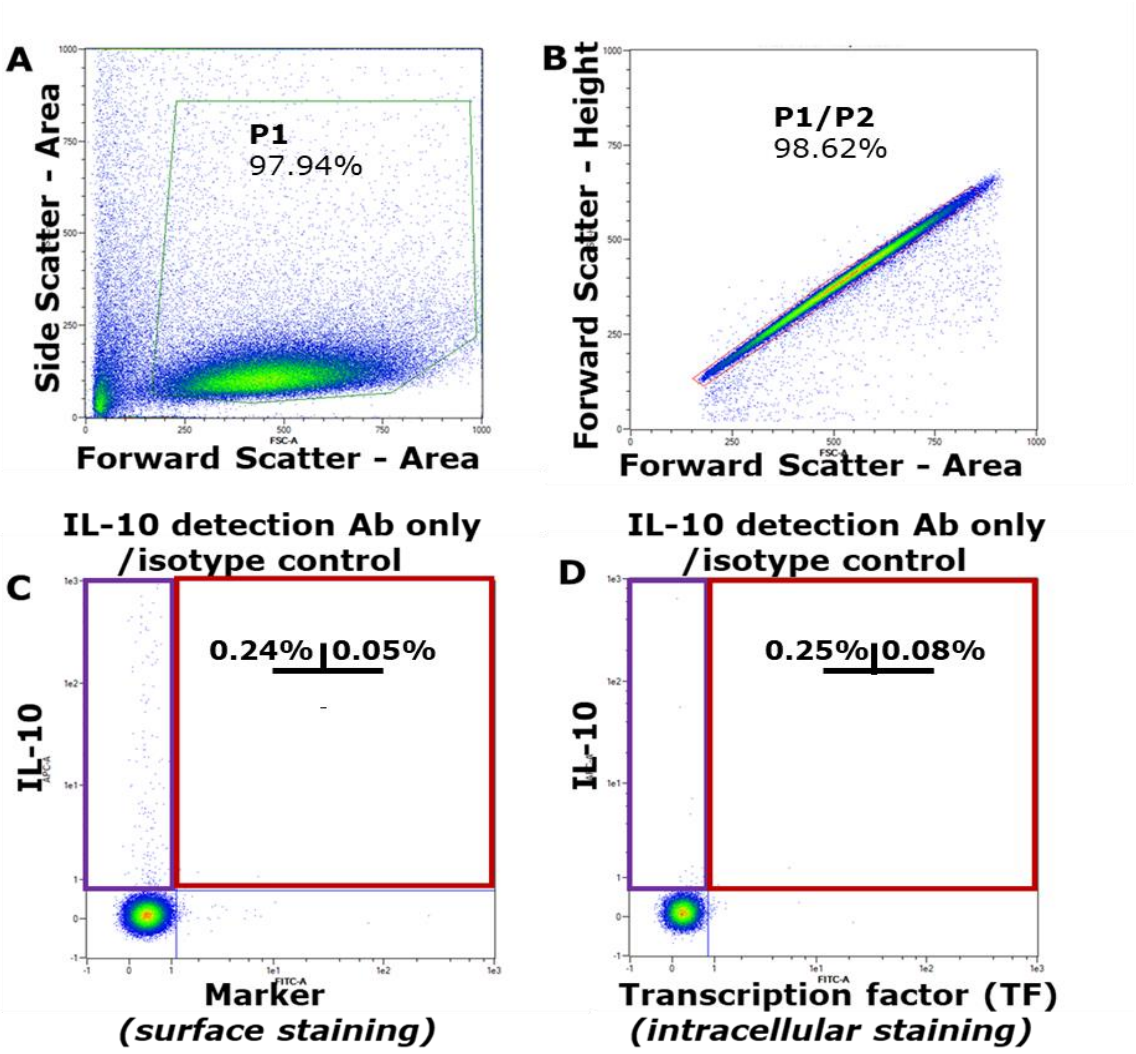


Figure 6.1: Determining the IL-10 and marker/transcription factor (TF) positive populations in CD4$^+$ T cells

Gating for IL-10 positive cells, IL-10/Marker positive and IL-10/ Transcription Factors (TFs) positive cells set at the junction of negative and positive cell populations checked against control conditions on purified CD4$^+$ T cells. **A** Live cell inclusion on forward scatter-area, side scatter – area. **B** doublet discrimination and cell clumps exclusion by forward scatter- area, forward scatter – height. **C** IL-10/Marker dual capture with detection antibody only (IL-10) and isotype (Marker) controls. **D** IL-10/TFs (intracellular) dual capture with detection antibody only (IL-10) and isotype (TFs) controls.

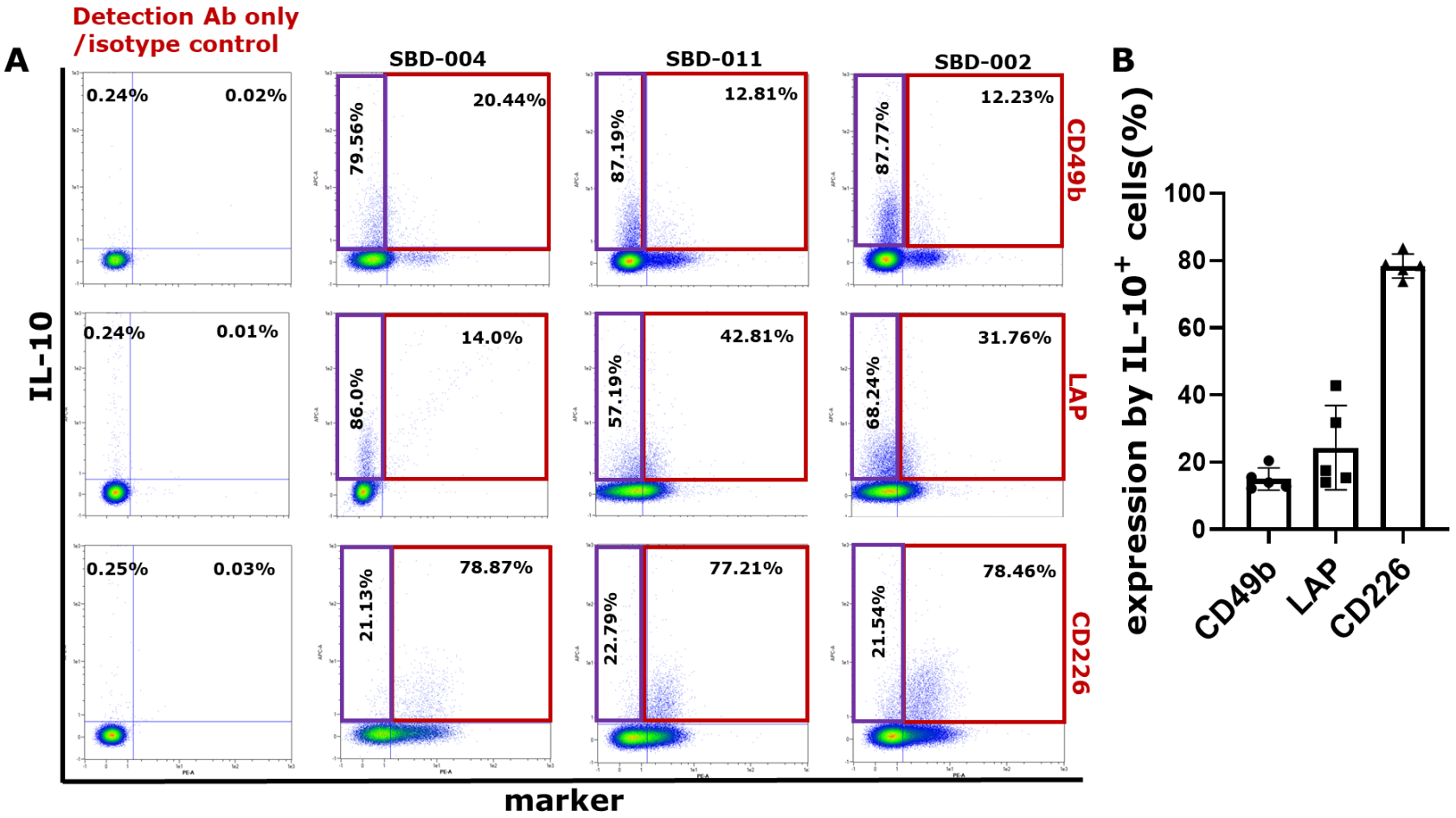

A
Detection Ab only
/isotype control
SBD-004
SBD-011
SBD-002
CD49b
LAP
CD226
IL-10
marker
0.24%
0.02%
79.56%
20.44%
87.19%
12.81%
87.77%
12.23%
0.24%
0.01%
86.0%
14.0%
57.19%
42.81%
68.24%
31.76%
0.25%
0.03%
21.13%
78.87%
22.79%
77.21%
21.54%
78.46%
B
expression by IL-10+ cells(%)
100
80
60
40
20
0
CD49b
LAP
CD226

Figure 6.2: CD3/CD55 costimulation of $CD4^+$ T cells induced the expression of Tr1 markers CD226, CD49b and LAP

A $CD4^+$ T cells isolated from peripheral blood of healthy donors were costimulated with CD3/CD55 for 24 hours. Cells were simultaneously assessed for IL-10 (cytokine secretion assay) and CD49b, LAP and CD226 (extracellular staining) and data captured by flow cytometry. IL-10 and marker expression were evaluated against detection antibody only control and isotype control, respectively. Cells single positive for IL-10 are within the **PURPLE** box (left) while cells positive for both IL-10 and marker are within the **RED** box (right). Percentages shown are of $IL\text{-}10^+$ cells for three donors, representative of five donors. **B** Marker expression by $IL\text{-}10^+$ cells following CD3/CD55 costimulation of $CD4^+$ T cells. Data shown are percentages of overall $IL\text{-}10^+$ cells also positive for CD49b, LAP or CD226. Results are from five donors.

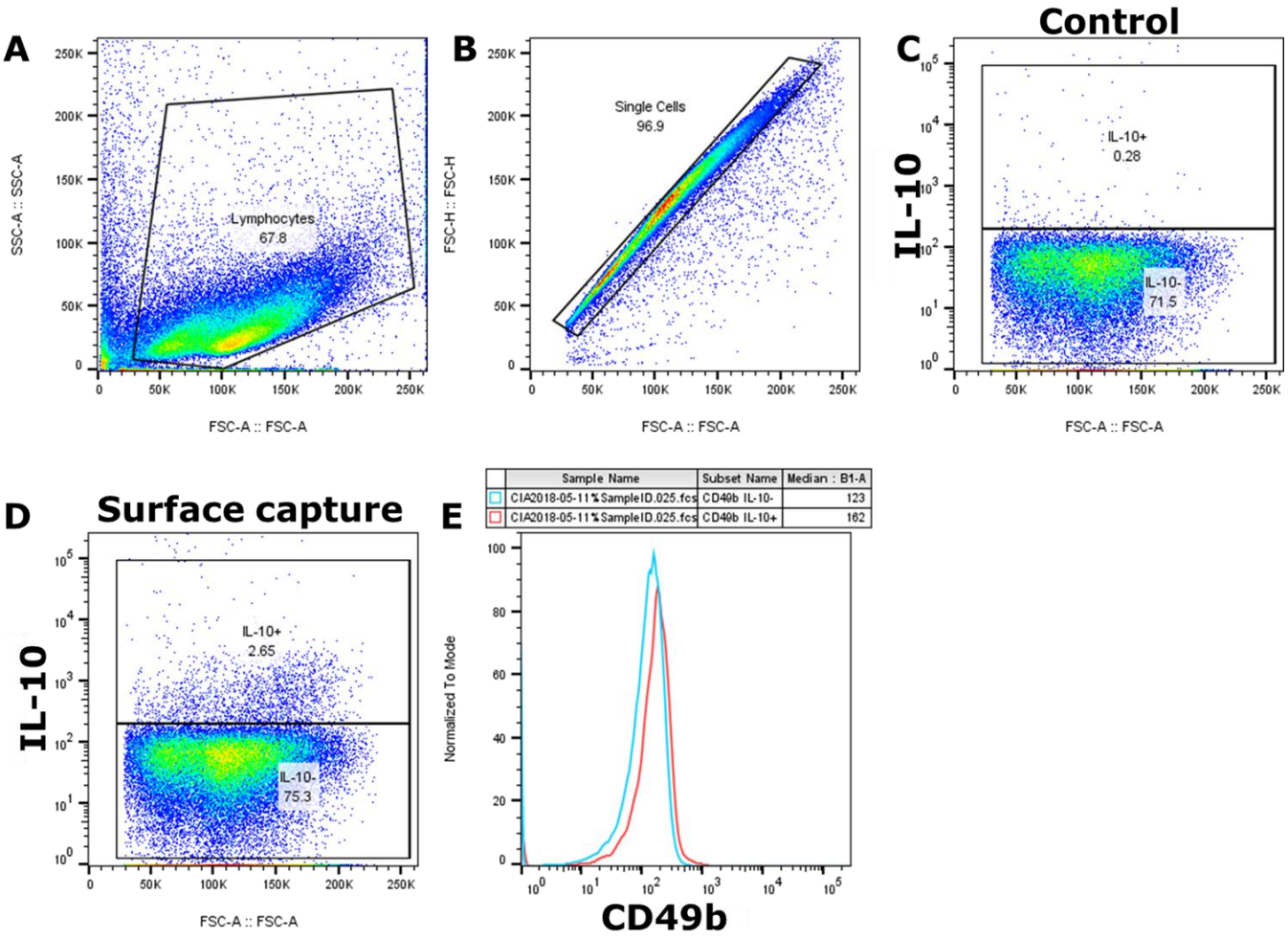


Figure 6.3: Determining the Median Fluorescence Intensity (MFI) for IL-10$^+$ and IL-10$^-$ cells expressing CD49b

Gating for IL-10$^+$ and IL-10$^-$ cells expressing marker CD49b following costimulation of CD4$^+$ T cells. **A** Live cell inclusion on forward scatter-area, side scatter – area. **B** Doublet discrimination by forward scatter-Area, Forward Scatter – Height. **C** Control gate on IL-10$^+$ and IL-10$^-$ cells. **D** Control gates applied to actual IL-10 surface capture. **E** Histogram of CD49b expression in IL-10$^+$ and IL-10$^-$ cells. This strategy was used to determine the MFI for IL-10$^+$ and IL-10$^-$ cells for all Tr1 markers/transcription factors analysed.

The intensity of marker expression (Median Fluorescent Intensity, MFI) for CD49b, LAP and CD226 were assessed across the IL-10^+ and IL-10^- cell populations. The intensity of CD49b and LAP were similar in IL-10^+ or IL-10^- $CD4^+$ T cell populations (Figure 6.4). Further examination of the expression levels by analysis of the histogram of each of the markers within the IL-10^+ and IL-10^- populations revealed no difference in expression for CD49b (Figure 6.4 A & B) or LAP (Figure 6.4 C & D). The intensity of CD226 expression was significantly higher within the IL-10^+ population when compared to the distribution in the IL-10^- population (Figure 6.4 E & F).

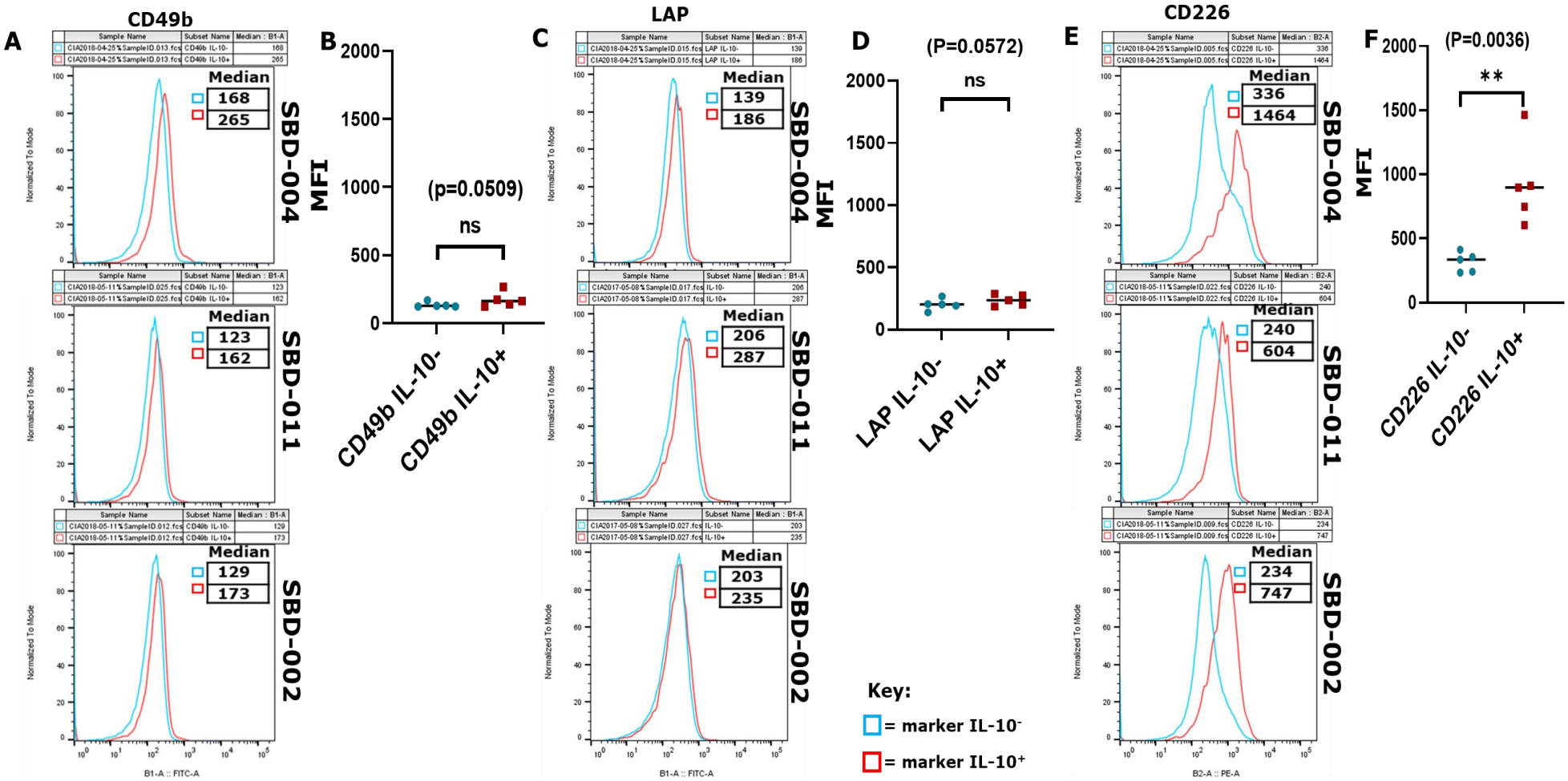

A
CD49b
Median
168
265
SBD-004
123
162
SBD-011
129
173
SBD-002
Normalized To Mode
B1-A :: FITC-A
B
MFI
(p=0.0509)
ns
CD49b IL-10-
CD49b IL-10+
C
LAP
139
186
206
287
203
235
D
(P=0.0572)
LAP IL-10-
LAP IL-10+
Key:
= marker IL-10⁻
= marker IL-10⁺
E
CD226
336
1464
240
604
234
747
B2-A :: PE-A
F
(P=0.0036)
**
CD226 IL-10-
CD226 IL-10+

Figure 6.4: Intensity of marker expression following CD3/CD55 costimulation of $CD4^+$ T cells

MFI for markers on entire IL-10^- and IL-10^+ cells population following CD3/CD55 costimulation for 24 hours; extracellular staining for markers and cytokine secretion assay for IL-10 from Figure 6.2A. Histogram for marker expression in IL-10^+ and IL-10^- was determined by gating on respective controls and applying gates to actual samples. **A, C & E** CD49b, LAP and CD226, respectively (n=3). **B, D & F** MFI for CD49b, LAP and CD226, respectively (n=5), **BLUE** dot (IL-10^- cells), **RED** square (IL-10^+ cells). Statistical significance was determined by Paired Student *t* test at P≤0.05. ns=not significant

6.2.2 IL-10$^+$ cells co-express immune checkpoint inhibitors CTLA-4, PD-1 and LAG-3

The costimulatory receptors often associated with regulatory function have also been reported to be markers of Tr1 cells. Cytotoxic T lymphocyte-antigen-4 (CTLA-4), Programmed cell Death protein 1 (PD-1) and Lymphocyte Activation Gene 3 (LAG-3) have all been reportedly expressed by Tr1 cells as checkpoint inhibitors(Roncarolo et al., 2018). CTLA-4 functions in inhibiting T cells responses by competing with CD28 for ligands, CD80/CD86 (Hosseini, Gharibi, Marofi, Babaloo, & Baradaran, 2020; Mitsuiki, Schwab, & Grimbacher, 2019). On the other hand, PD-1 binds to its ligands PD-L1 and PD-L2 and these interactions control the induction and maintenance of peripheral T cell tolerance(Sharpe, Wherry, Ahmed, & Freeman, 2007). LAG-3 is reported to be expressed by Tr1 cells having a negative regulatory effect on T cell receptor-mediated signal transduction in both human and murine models(H. Zeng, R. Zhang, B. Jin, & L. Chen, 2015). Owing to their inhibitory effects on immune responses, CTLA-4, PD-1 and LAG-3 have been reported as markers of Tr1 cells and their expression by IL-10$^+$ Tr1-like population in human peripheral blood CD4$^+$ T cells was assessed.

CD4$^+$ T cells purified from human peripheral blood and costimulated with CD3/CD55 were analysed for IL-10 expression together with CTLA-4, PD-1 and LAG-3 expression. Results showed that CTLA-4 was expressed at modest levels by up to 40% of IL-10$^+$ in multiple donors

(Figure 6.5A row 1 and Figure 6.5B). PD-1 however was expressed by about 95% of IL-10^{+} cells in all donors tested (Figure 6.5A row 2 and Figure 6.5B) while LAG-3 was expressed by about 60% of the IL-10^{+} population (Figures 6.5A row 3 and 6.5B)

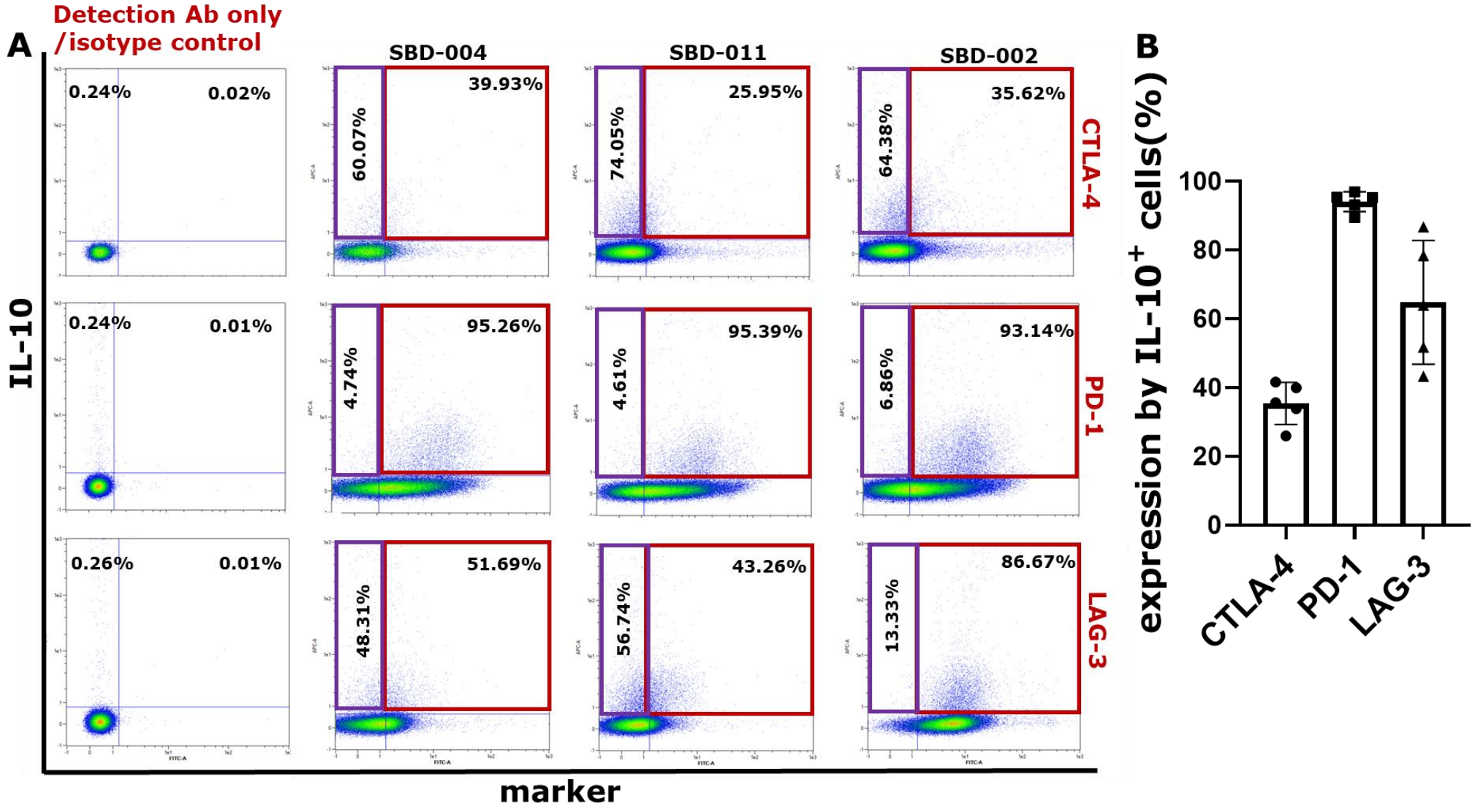

A
Detection Ab only
/isotype control
SBD-004
SBD-011
SBD-002
CTLA-4
PD-1
LAG-3
IL-10
marker
0.24%
0.02%
60.07%
39.93%
74.05%
25.95%
64.38%
35.62%
0.24%
0.01%
4.74%
95.26%
4.61%
95.39%
6.86%
93.14%
0.26%
0.01%
48.31%
51.69%
56.74%
43.26%
13.33%
86.67%
B
expression by IL-10+ cells(%)
100
80
60
40
20
0
CTLA-4
PD-1
LAG-3

Figure 6.5: CD3/CD55 costimulation CD4$^+$ T cells gave an IL-10$^+$ population with a high percentage expressing LAG-3 and PD-1 but not CTLA-4

A CD4$^+$ T cells isolated from peripheral blood of healthy donors were costimulated with CD3/CD55 for 24 hours. Cells were simultaneously assessed for IL-10 (cytokine secretion assay) and CTLA-4, PD-1 and LAG-3 (extracellular staining) and data captured by flow cytometry. Secreted IL-10 and marker expression were evaluated against detection antibody only control and isotype control, respectively. Cells single positive for IL-10 are within the **PURPLE** box (left) while cells positive for both IL-10 and marker are within the **RED** box (right). Percentages shown are of IL-10$^+$ cells for three donors, representative of five donors. **B** Marker expression by IL-10$^+$ cells following CD3/CD55 costimulation of CD4$^+$ T cells. Data shown are percentages of overall IL-10$^+$ cells also positive for CTLA-4, PD-1 or LAG-3. Results are from five donors.

Further analysis showed that the intensity of CTLA-4 expression was significantly higher within the IL-10^{+} population when compared to the IL-10^{-} population (Figure 6.6 A & B). The intensity of PD-1 expression was also significantly higher in the IL-10^{+} population than seen in the IL-10^{-} population (Figure 6.6 C & D) while LAG-3 expression was similar in both IL-10+ and IL-10^{-} populations (Figure 6.6 E & F).

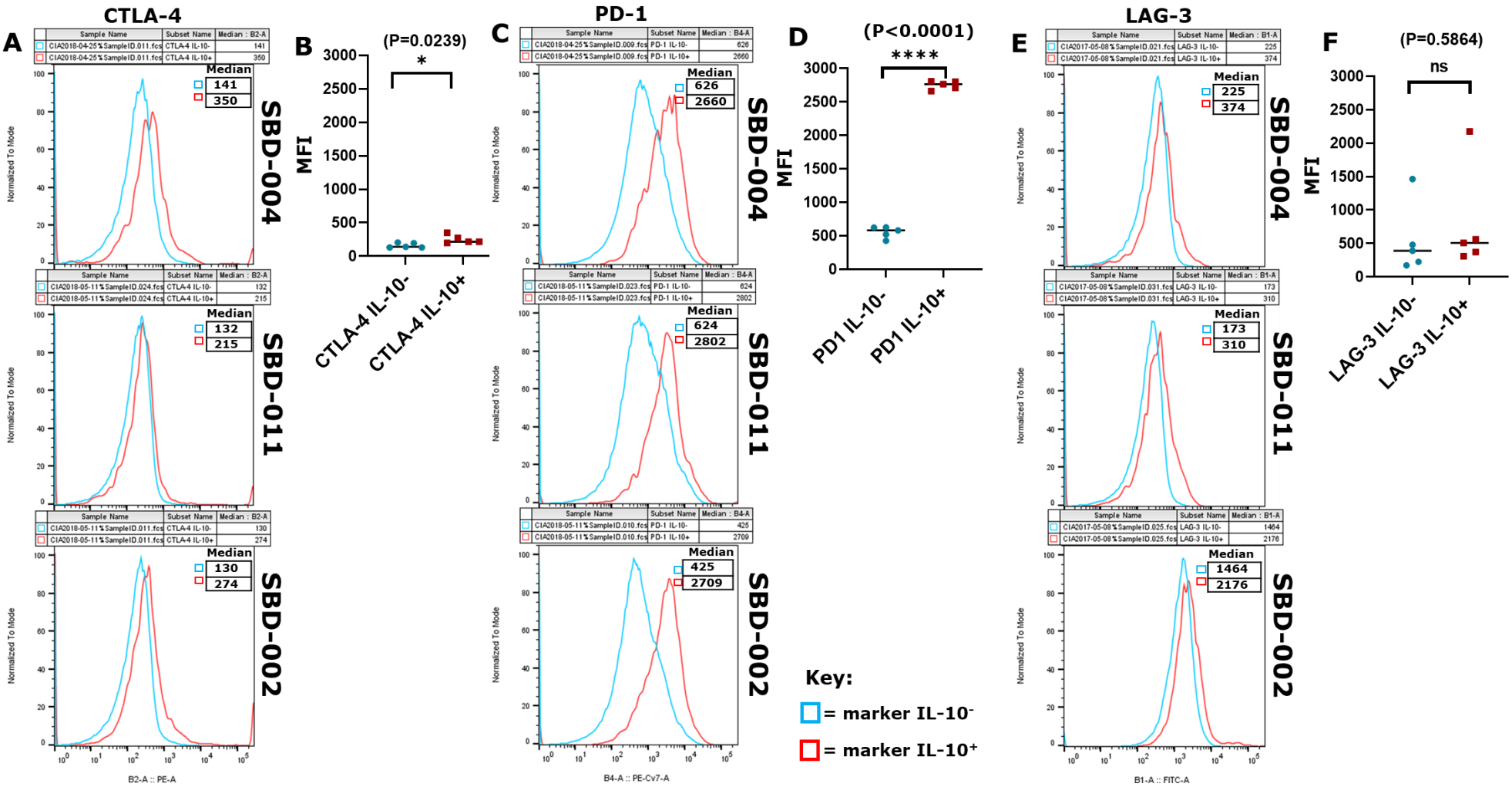

CTLA-4
A
Sample Name | Subset Name | Median : B2-A
CIA2018-04-25%SampleID.011.fcs | CTLA-4 IL-10- | 141
CIA2018-04-25%SampleID.011.fcs | CTLA-4 IL-10+ | 350
Median
141
350
SBD-004
CIA2018-05-11%SampleID.024.fcs | CTLA-4 IL-10- | 132
CIA2018-05-11%SampleID.024.fcs | CTLA-4 IL-10+ | 215
Median
132
215
SBD-011
CIA2018-05-11%SampleID.011.fcs | CTLA-4 IL-10- | 130
CIA2018-05-11%SampleID.011.fcs | CTLA-4 IL-10+ | 274
Median
130
274
SBD-002
Normalized To Mode
B2-A :: PE-A
B
(P=0.0239)
*
MFI
CTLA-4 IL-10-
CTLA-4 IL-10+
C
PD-1
Sample Name | Subset Name | Median : B4-A
CIA2018-04-25%SampleID.009.fcs | PD-1 IL-10- | 626
CIA2018-04-25%SampleID.009.fcs | PD-1 IL-10+ | 2660
Median
626
2660
SBD-004
CIA2018-05-11%SampleID.023.fcs | PD-1 IL-10- | 624
CIA2018-05-11%SampleID.023.fcs | PD-1 IL-10+ | 2802
Median
624
2802
SBD-011
CIA2018-05-11%SampleID.010.fcs | PD-1 IL-10- | 425
CIA2018-05-11%SampleID.010.fcs | PD-1 IL-10+ | 2709
Median
425
2709
SBD-002
Normalized To Mode
B4-A :: PE-Cv7-A
D
(P<0.0001)
****
MFI
PD1 IL-10-
PD1 IL-10+
Key:
= marker IL-10-
= marker IL-10+
E
LAG-3
Sample Name | Subset Name | Median : B1-A
CIA2017-05-08%SampleID.021.fcs | LAG-3 IL-10- | 225
CIA2017-05-08%SampleID.021.fcs | LAG-3 IL-10+ | 374
Median
225
374
SBD-004
CIA2017-05-08%SampleID.031.fcs | LAG-3 IL-10- | 173
CIA2017-05-08%SampleID.031.fcs | LAG-3 IL-10+ | 310
Median
173
310
SBD-011
CIA2017-05-08%SampleID.025.fcs | LAG-3 IL-10- | 1464
CIA2017-05-08%SampleID.025.fcs | LAG-3 IL-10+ | 2176
Median
1464
2176
SBD-002
Normalized To Mode
B1-A :: FITC-A
F
(P=0.5864)
ns
MFI
LAG-3 IL-10-
LAG-3 IL-10+

Figure 6.6: Intensity of CTLA-4, PD-1 and LAG-3 expression following CD3/CD55 costimulation of $CD4^+$ T cells

MFI for markers on entire $IL\text{-}10^-$ and $IL\text{-}10^+$ cells population following CD3/CD55 costimulation for 24 hours; extracellular staining for markers and cytokine secretion assay for IL-10 from Figure 6.5A. Histogram for marker expression in $IL\text{-}10^+$ and $IL\text{-}10^-$ was determined by gating on respective controls and applying gates test samples. **A, C & E** CTLA-4, PD-1 and LAG-3, respectively (n=3). **B, D & F** MFI for CTLA-4, PD-1 and LAG-3, respectively (n=5), **BLUE** dot ($IL\text{-}10^-$ cells), **RED** square ($IL\text{-}10^+$ cells). Statistical significance was determined by Paired Student *t* test at $P \leq 0.05$. ns=not significant

6.2.3: IL-10$^+$ Tr1-like cells highly expressed c-MAF; but not FoxP3 or Helios

To date, there has not been a clear candidate for a master regulator transcription factor for Tr1 cells. This may be due in part, to the way Tr1 cells have been isolated or induced. Expression of c-MAF, FoxP3 and Helios have been associated with regulatory T cell functions. c-MAF is reported to transactivate IL-10 promoter (Xu et al., 2009) and interacts with aryl hydrocarbon receptor (AhR) to promote the secretion of IL-10 and IL-21 (Apetoh et al., 2010), and is involved in Tr1 differentiation (Barroso et al., 2021; Imbratta et al., 2020). On the other hand, transient expression of Foxp3 by Tr1 cells has been reported, however, not as a prerequisite for suppressive functions (W. Lin et al., 2007; J. Wang et al., 2007). Helios is thought to enhance Treg cells function in cooperation with FoxP3 by repressing pro-apoptotic genes in Tregs and contributing to the development of follicular Treg cells (Sebastian et al., 2016). Therefore, the expression of c-MAF, FoxP3 and Helios by IL-10$^+$ cells following CD3/CD55 costimulation of peripheral blood CD4$^+$ T cells was evaluated.

Purified CD4$^+$ T cells from human peripheral blood were costimulated with CD3/CD55 and analysed for IL-10 expression as well as c-MAF, FoxP3 and Helios. When stimulated with CD3/CD55, about 75% of IL-10$^+$ cells expressed c-MAF in four of the five donors, with exception of one donor with about 30% c-MAF expression (Figures 6.7A row 1 and

6.7B). This observation contrasted with the approximately 20% of FoxP3 and Helios expressed by IL-10^{+} cells (Figures 6.7 A & B).

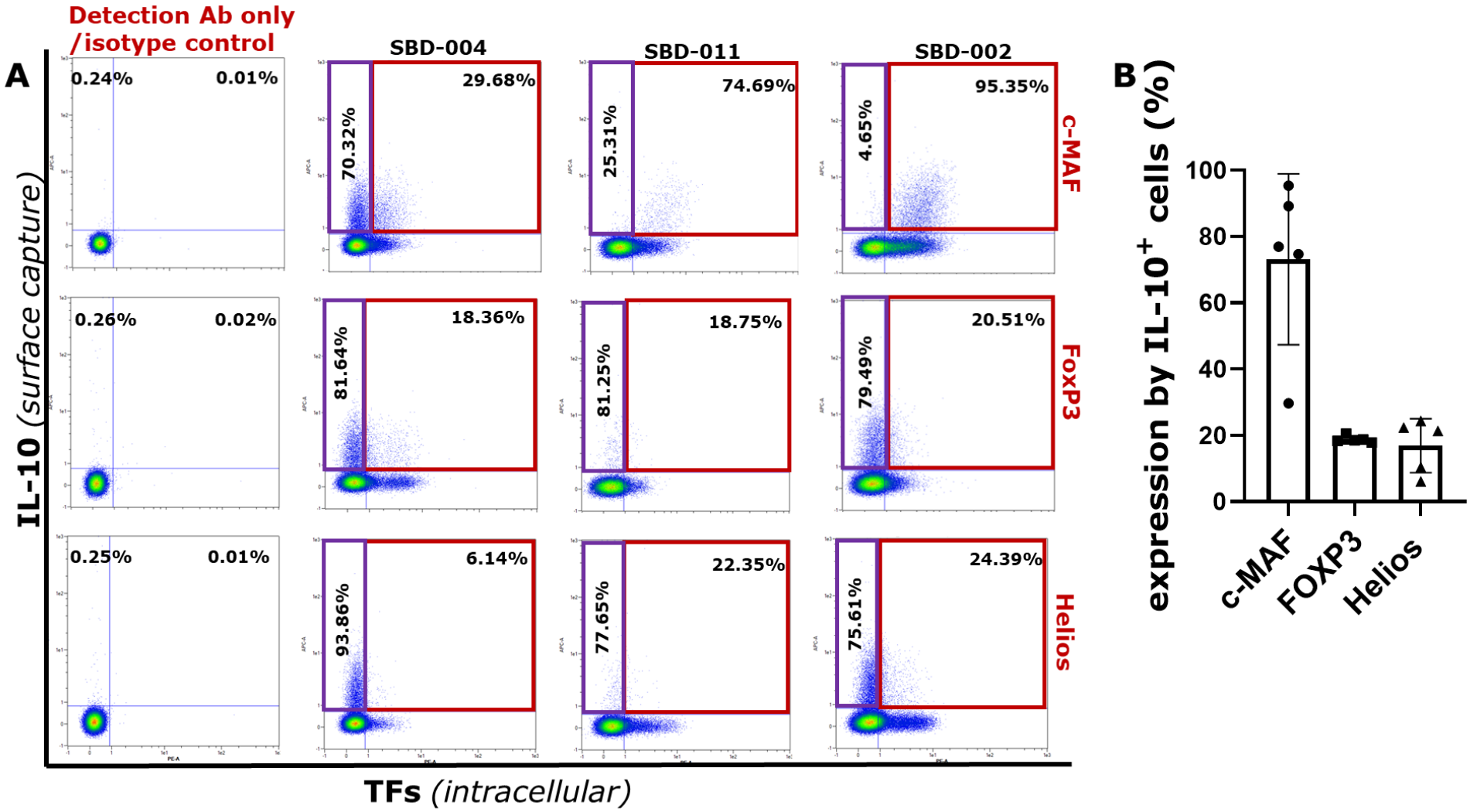

A
Detection Ab only
/isotype control
SBD-004
SBD-011
SBD-002
c-MAF
FoxP3
Helios
0.24%
0.01%
70.32%
29.68%
25.31%
74.69%
4.65%
95.35%
0.26%
0.02%
81.64%
18.36%
81.25%
18.75%
79.49%
20.51%
0.25%
0.01%
93.86%
6.14%
77.65%
22.35%
75.61%
24.39%
IL-10 (surface capture)
TFs (intracellular)
B
expression by IL-10+ cells (%)
100
80
60
40
20
0
c-MAF
FOXP3
Helios

Figure 6.7: CD3/CD55 costimulation of $CD4^+$ T cells revealed high expression of transcription factor c-MAF; but low FoxP3 and Helios

A $CD4^+$ T cells isolated from peripheral blood of healthy donors were costimulated with CD3/CD55 for 24 hours. Cells were simultaneously assessed for IL-10 (cytokine secretion assay); c-MAF, FoxP3 and Helios (intracellular staining) and data captured by flow cytometry. Secreted IL-10 and transcription factors (TFs) were evaluated against detection antibody only control and isotype control, respectively. Cells single positive for IL-10 are within the **PURPLE** box (left) while cells positive for both IL-10 and TFs are within the **RED** box (right). Percentages shown are of $IL\text{-}10^+$ cells for three donors, representative of five donors. **B** TF expression by $IL\text{-}10^+$ cells following CD3/CD55 costimulation of $CD4^+$ T cells. Data shown are percentages of overall $IL\text{-}10^+$ cells also positive for c-MAF, FoxP3 or Helios. Results are from five donors

The intensity of c-MAF expression was observed to be significantly higher within the IL-10^{+} population than the IL-10^{-} population (Figure 6.8 A & B). Further analysis showed no difference in FoxP3 expression intensity (Figure 6.8 C & D) or Helios expression (data not shown) by IL-10^{+} and IL-10^{-} cells.

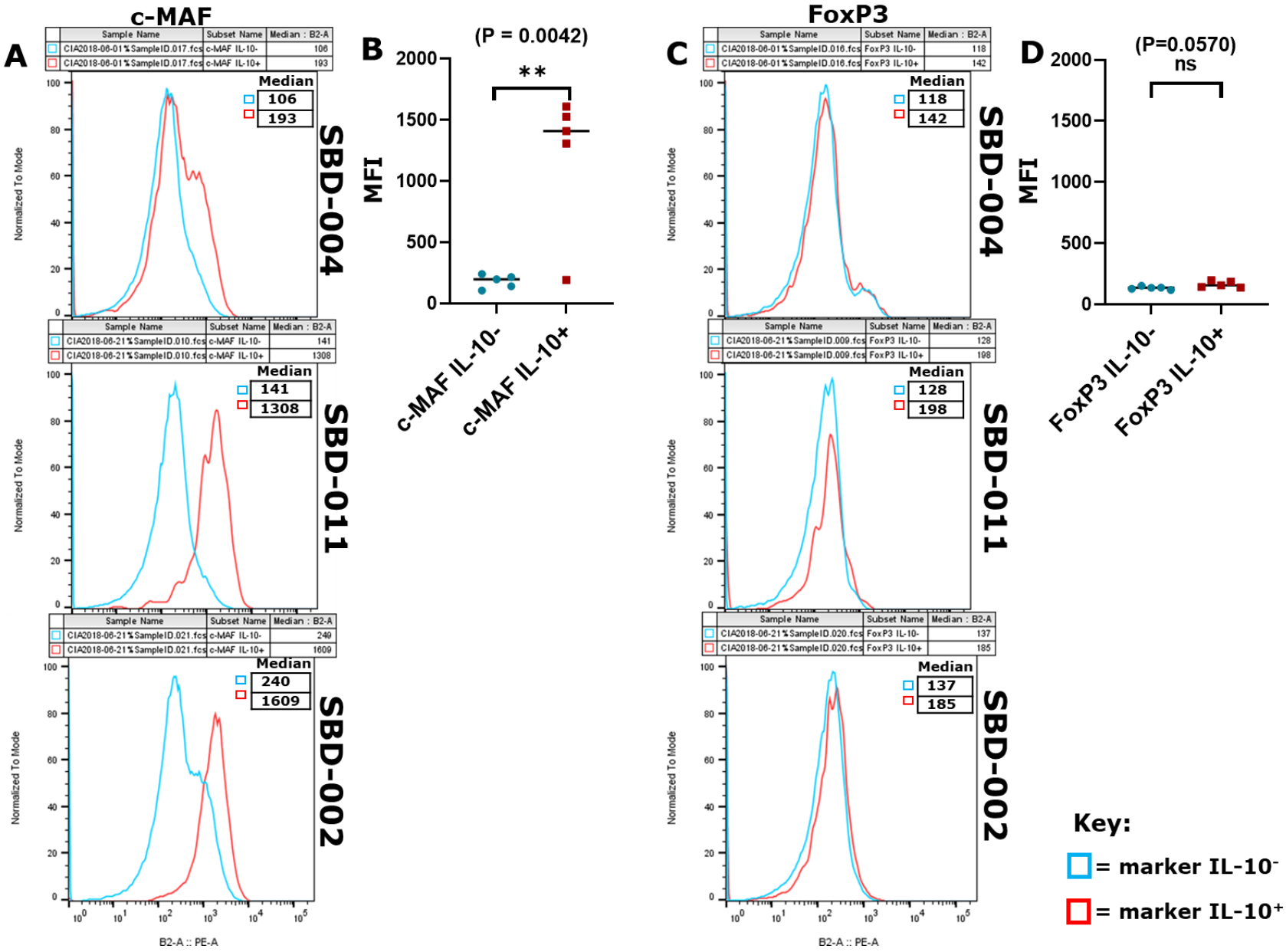

A
c-MAF
Sample Name | Subset Name | Median : B2-A
CIA2018-06-01%SampleID.017.fcs | c-MAF IL-10- | 106
CIA2018-06-01%SampleID.017.fcs | c-MAF IL-10+ | 193
Median
106
193
SBD-004
CIA2018-06-21%SampleID.010.fcs | c-MAF IL-10- | 141
CIA2018-06-21%SampleID.010.fcs | c-MAF IL-10+ | 1308
Median
141
1308
SBD-011
CIA2018-06-21%SampleID.021.fcs | c-MAF IL-10- | 240
CIA2018-06-21%SampleID.021.fcs | c-MAF IL-10+ | 1609
Median
240
1609
SBD-002
Normalized To Mode
B2-A :: PE-A
B
(P = 0.0042)
**
MFI
c-MAF IL-10-
c-MAF IL-10+
C
FoxP3
CIA2018-06-01%SampleID.016.fcs | FoxP3 IL-10- | 118
CIA2018-06-01%SampleID.016.fcs | FoxP3 IL-10+ | 142
Median
118
142
SBD-004
CIA2018-06-21%SampleID.009.fcs | FoxP3 IL-10- | 128
CIA2018-06-21%SampleID.009.fcs | FoxP3 IL-10+ | 198
Median
128
198
SBD-011
CIA2018-06-21%SampleID.020.fcs | FoxP3 IL-10- | 137
CIA2018-06-21%SampleID.020.fcs | FoxP3 IL-10+ | 185
Median
137
185
SBD-002
D
(P=0.0570)
ns
FoxP3 IL-10-
FoxP3 IL-10+
Key:
= marker IL-10-
= marker IL-10+

Figure 6.8: High intensity of c-MAF expression for IL-10$^+$ cells following CD3/CD55 costimulation of CD4$^+$ T cells

MFI for c-MAF and FoxP3 on entire IL-10$^-$ and IL-10$^+$ cells population following CD3/CD55 costimulation for 24 hours; intracellular staining for TFs and cytokine secretion assay for IL-10 from Figure 6.7A. Histogram for marker expression in IL-10$^+$ and IL-10$^-$ was determined by gating on respective controls and applying gates test samples. **A & C** c-MAF and FoxP3, respectively (n=3). **B & D** MFI for c-MAF and FoxP3, respectively (n=5), **BLUE** dot (IL-10$^-$ cells), **RED** square (IL-10$^+$ cells). Statistical significance was determined by Paired Student *t* test at P≤0.05. ns=not significant

6.2.4: IL-10$^+$ Tr1-like cells express T Bet and RoRɣ(t); but not GATA-3

Initially thought to be expressed exclusively by Th1 cells, T Bet is now reported to be extensively expressed by both innate and adaptive immune cells during type 1 immune response (Kallies & Good-Jacobson, 2017) as well as regulatory T cells (Levine et al., 2017). RoRγ(t) is a useful target in treating autoimmune conditions associated with Th17 gene mutations and although predominantly pro-inflammatory in nature, it is reportedly expressed by a population of regulatory T cells in the colon (Hegazy & Powrie, 2015). GATA-3 controls Th2 development by inhibiting Th1 differentiation through the repression of the IL-12 receptor beta2-chain expression and augmenting its own expression via positive feedback autoregulation(M. Zhou & Ouyang, 2003). Therefore, the expression of T Bet, RoRγ(t) and GATA-3 by IL-10$^+$ Tr1-like cells following CD3/CD55 costimulation of CD4$^+$ T cells was evaluated.

Analysis of IL-10$^+$ cells was performed simultaneously with the evaluation of expression of T-Bet, RoRγ(t) and GATA-3 following CD3/CD55 costimulation of purified CD4$^+$ T cells from human peripheral blood for 24 hours. Results showed that more than 90% of IL-10$^+$ cells expressed T-Bet, approximately 80% expressed RoRγ(t), while less than 5% expressed GATA-3 (Figures 6.9A & B). Analysis of distribution of transcription factor expression between IL-10$^+$ and IL-10$^-$ cells revealed

no difference in distribution for T-Bet (Figure 6.10 A & B), RoRγ(t) (Figure 6.10 C&D) and GATA-3 (data not shown).

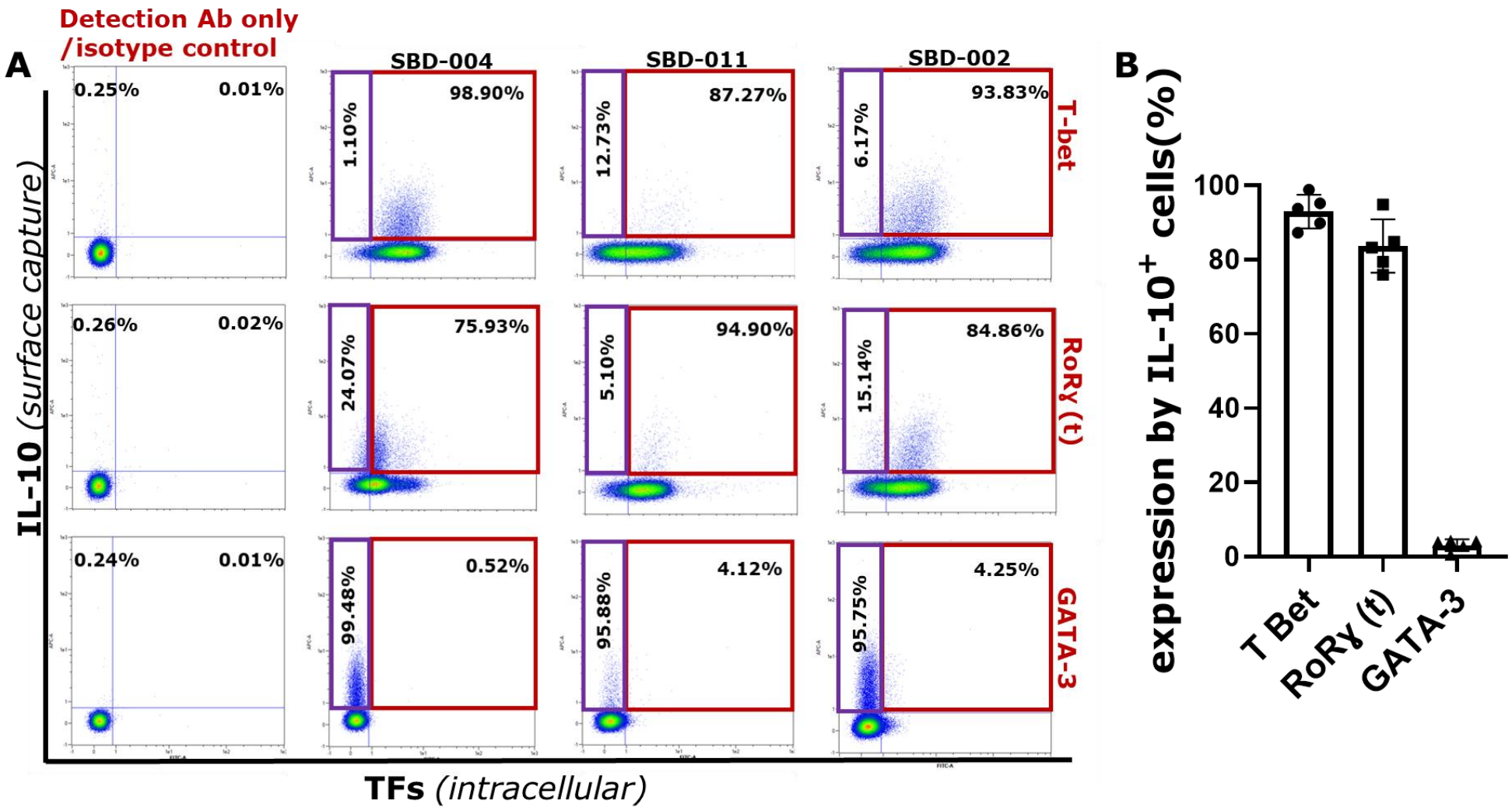

A
Detection Ab only
/isotype control
SBD-004
SBD-011
SBD-002
T-bet
RoRγ (t)
GATA-3
0.25%
0.01%
1.10%
98.90%
12.73%
87.27%
6.17%
93.83%
0.26%
0.02%
24.07%
75.93%
5.10%
94.90%
15.14%
84.86%
0.24%
0.01%
99.48%
0.52%
95.88%
4.12%
95.75%
4.25%
IL-10 (surface capture)
TFs (intracellular)
B
expression by IL-10+ cells(%)
100
80
60
40
20
0
T Bet
RoRγ (t)
GATA-3

Figure 6.9: CD3/CD55 costimulation of $CD4^+$ T cells revealed high expression of transcription factors T-Bet and RoRɣ(t); but no GATA-3

A $CD4^+$ T cells isolated from peripheral blood of healthy donors were costimulated with CD3/CD55 for 24 hours. Cells were simultaneously assessed for IL-10 (cytokine secretion assay); T Bet and RoRɣ (t) and GATA-3 (intracellular staining) and data captured by flow cytometry. IL-10 and transcription factors (TFs) expression were evaluated against detection antibody only control and isotype control, respectively. Cells single positive for IL-10 are within the **PURPLE** box (left) while cells positive for both IL-10 and TFs are within the **RED** box (right). Percentages shown are of $IL\text{-}10^+$ cells for three donors, representative of five donors. **B** TF expression by $IL\text{-}10^+$ cells following CD3/CD55 costimulation of $CD4^+$ T cells. Data shown are percentages of overall $IL\text{-}10^+$ cells also positive for T Bet, RoRɣ (t) or GATA-3. Results are from five donors

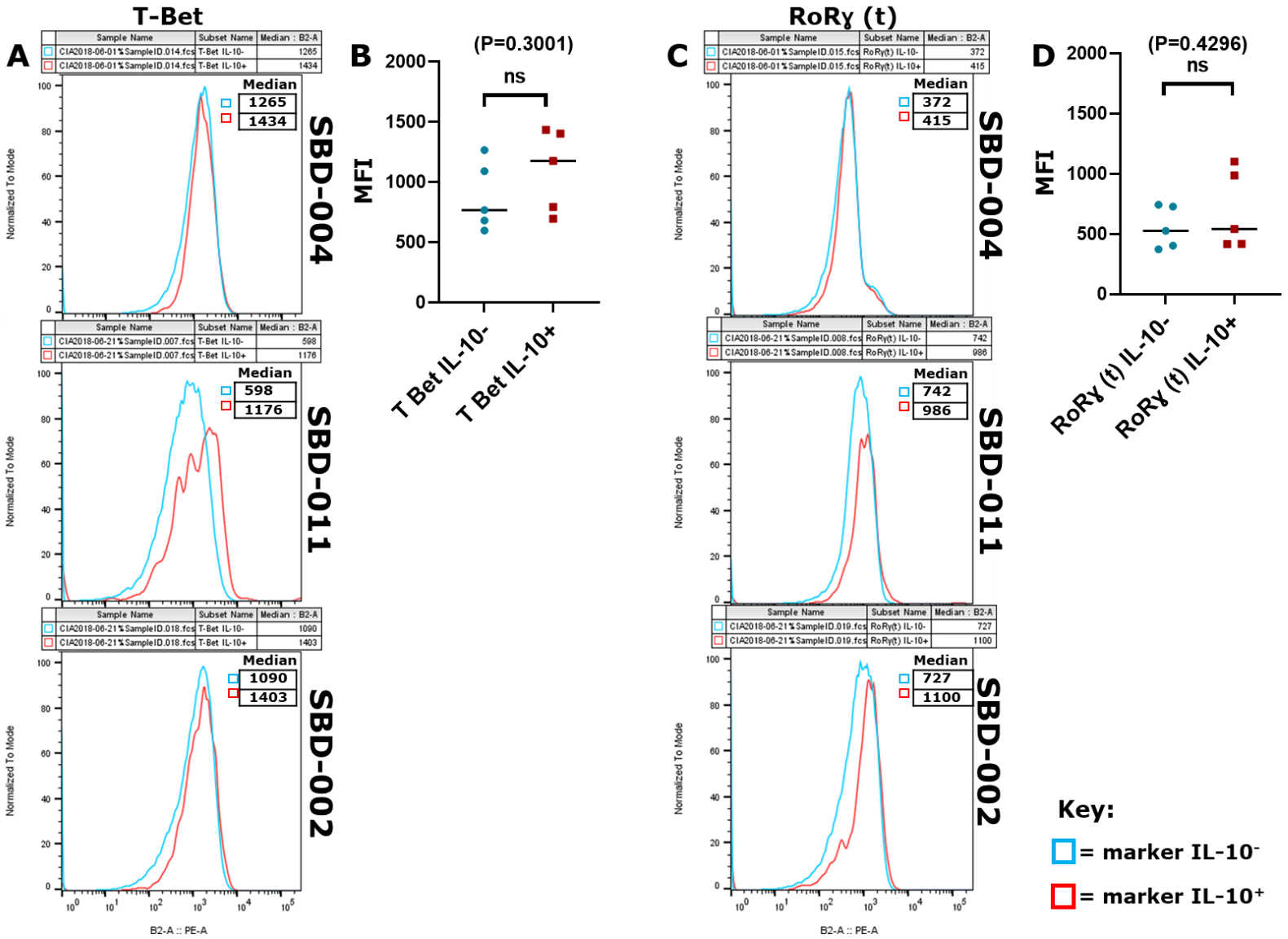

T-Bet
A
Sample Name | Subset Name | Median : B2-A
CIA2018-06-01%SampleID.014.fcs | T-Bet IL-10- | 1265
CIA2018-06-01%SampleID.014.fcs | T-Bet IL-10+ | 1434
Median
1265
1434
SBD-004
CIA2018-06-21%SampleID.007.fcs | T-Bet IL-10- | 598
CIA2018-06-21%SampleID.007.fcs | T-Bet IL-10+ | 1176
Median
598
1176
SBD-011
CIA2018-06-21%SampleID.018.fcs | T-Bet IL-10- | 1090
CIA2018-06-21%SampleID.018.fcs | T-Bet IL-10+ | 1403
Median
1090
1403
SBD-002
Normalized To Mode
B2-A :: PE-A
B
(P=0.3001)
ns
MFI
T Bet IL-10-
T Bet IL-10+
RoRγ (t)
C
CIA2018-06-01%SampleID.015.fcs | RoRγ(t) IL-10- | 372
CIA2018-06-01%SampleID.015.fcs | RoRγ(t) IL-10+ | 415
372
415
CIA2018-06-21%SampleID.008.fcs | RoRγ(t) IL-10- | 742
CIA2018-06-21%SampleID.008.fcs | RoRγ(t) IL-10+ | 986
742
986
CIA2018-06-21%SampleID.019.fcs | RoRγ(t) IL-10- | 727
CIA2018-06-21%SampleID.019.fcs | RoRγ(t) IL-10+ | 1100
727
1100
D
(P=0.4296)
ns
RoRγ (t) IL-10-
RoRγ (t) IL-10+
Key:
= marker IL-10⁻
= marker IL-10⁺

Figure 6.10: No difference in intensity of T-Bet or RoRɣ (t) expression for IL-10$^-$ and IL-10$^+$ cells following CD3/CD55 costimulation of CD4$^+$ T cells

MFI for T-Bet and RoRɣ (t) on entire IL-10$^-$ and IL-10$^+$ cells population following CD3/CD55 costimulation for 24 hours; intracellular staining for TFs and cytokine secretion assay for IL-10 from Figure 6.9A. Histogram for marker expression in IL-10$^+$ and IL-10$^-$ was determined by gating on respective controls and applying gates test samples. **A & C** T-Bet and RoRɣ (t), respectively (n=3). **B & D** MFI for T-Bet and RoRɣ (t), respectively (n=5), **BLUE** dot (IL-10$^-$ cells), **RED** square (IL-10$^+$ cells). Statistical significance was determined by Paired Student *t* test at P≤0.05. ns=not significant

6.2.5: IL-10$^+$ Tr1-like cells possess cytotoxic potentials by expressing Granzyme B and Perforin

Granzyme/perforin pathway represents the major mechanism by which cells of the innate (Natural killer cells) and adaptive (e.g cytotoxic T lymphocytes) immune systems mediate the killing of intracellular pathogens and tumour cells(Kam et al., 2000; Lieberman & Stroup, 2003). Tr1 cells have been reported to express granzyme B and its killing ability is not MHC/TCR-dependent; rather perforin-dependent (William J. Grossman et al., 2004; W. J. Grossman, J. W. Verbsky, B. L. Tollefsen, et al., 2004). Recent studies showed that Tr1 cells prevented the activation of antigen specific T cells by eliminating APCs of myeloid origin through the secretion of granzyme B and perforin (Magnani et al., 2011). Furthermore, Tr1 cells expressing CD226 and granzyme B(S. Gregori et al., 2012) and Foxp3$^+$ Treg cells have been shown to kill myeloid APCs through the secretion of granzymes A and B, and perforin (W. J. Grossman, J. W. Verbsky, W. Barchet, et al., 2004; Vignali, Collison, & Workman, 2008). Therefore, we evaluated the cytotoxic potential of IL-10$^+$ Tr1-like cells from human peripheral blood following CD3/CD55 costimulation.

$CD4^+$ T cells purified from human peripheral blood and costimulated with CD3/CD55 for 24 hours, were analysed for IL-10 expression by cytokine secretion followed by intracellular staining for granzyme B and perforin. Results showed that about 40% of $IL\text{-}10^+$ cells co-expressed Granzyme B and about 20% co-expressed perforin (Figure 6.11 A & B). Analysis of the Median Fluorescence Intensity of populations revealed that the intensity of granzyme B expression between $IL\text{-}10^+$ and $IL\text{-}10^-$ cells was significantly different (Figure 6.12). There was no difference in intensity of perforin expression between $IL\text{-}10^+$ and $IL\text{-}10^-$ cells (data not shown).

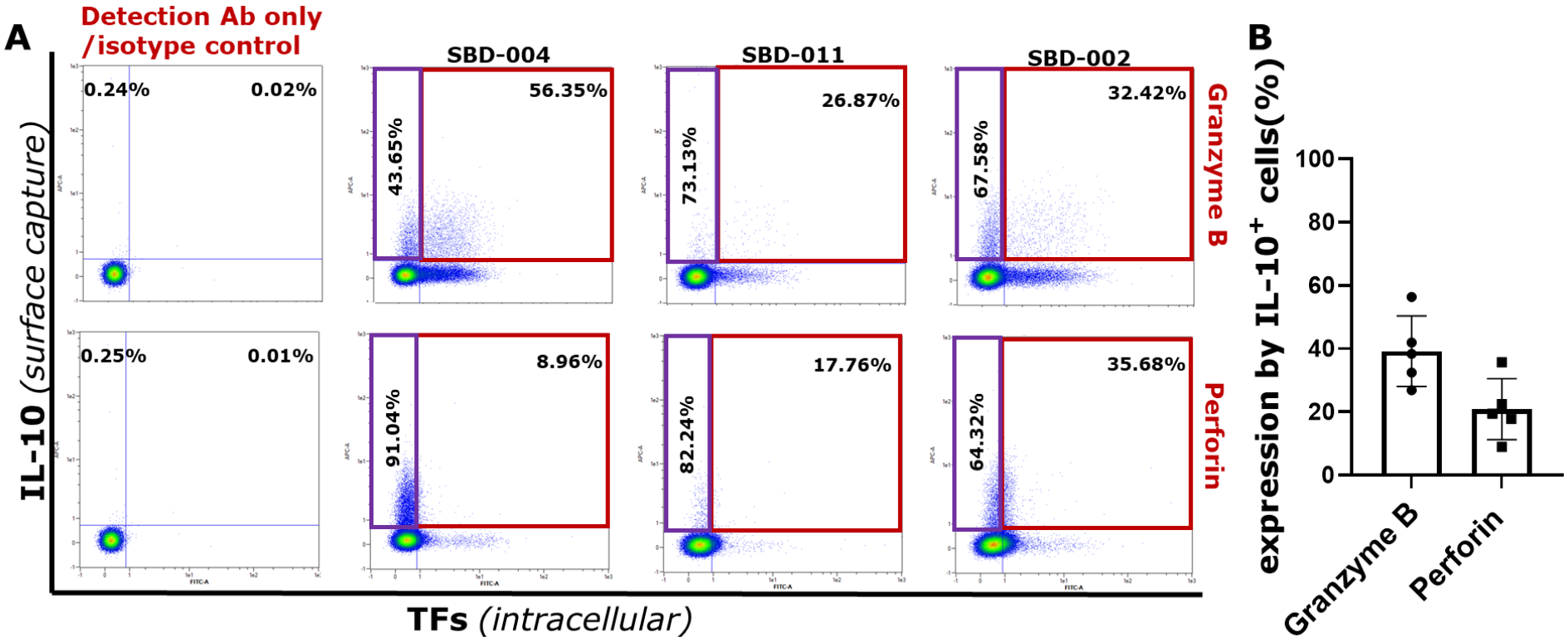

A
Detection Ab only
/isotype control
SBD-004
SBD-011
SBD-002
Granzyme B
Perforin
0.24%
0.02%
43.65%
56.35%
73.13%
26.87%
67.58%
32.42%
0.25%
0.01%
91.04%
8.96%
82.24%
17.76%
64.32%
35.68%
IL-10 (surface capture)
TFs (intracellular)
B
expression by IL-10+ cells(%)
100
80
60
40
20
0
Granzyme B
Perforin

Figure 6.11: CD3/CD55 costimulation of $CD4^+$ T cells showed expression of Granzyme B and Perforin in a small population of $IL\text{-}10^+$ cells

$CD4^+$ T cells isolated from peripheral blood of healthy donors were costimulated with CD3/CD55 for 24 hours. Cells were simultaneously assessed for IL-10 (cytokine secretion assay); Granzyme B and Perforin (intracellular staining) and data captured by flow cytometry. IL-10 and protein expression levels were evaluated against detection antibody only control and isotype control, respectively. Cells single positive for IL-10 are within the **PURPLE** box (left) while cells positive for both IL-10 and marker are within the **RED** box (right). Percentages shown are of $IL\text{-}10^+$ cells for three donors, representative of five donors. **B** Protein expression by $IL\text{-}10^+$ cells following CD3/CD55 costimulation of $CD4^+$ T cells. Data shown are percentages of overall $IL\text{-}10^+$ cells also positive for Granzyme B or Perforin. Results are from five donors.

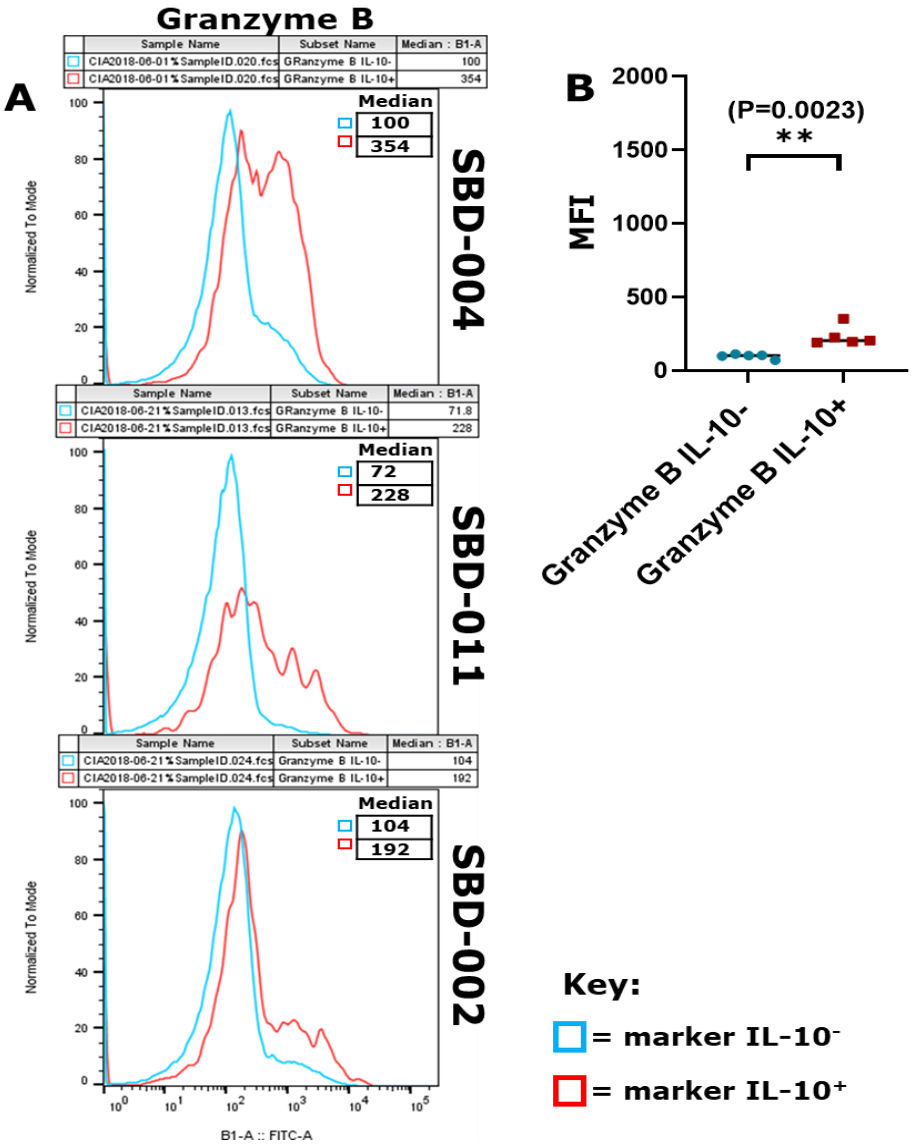


Figure 6.12: High intensity of Granzyme B expression for IL-10$^+$ cells following CD3/CD55 costimulation of CD4$^+$ T cells

MFI for Granzyme B on entire IL-10$^-$ and IL-10$^+$ cells population following CD3/CD55 costimulation for 24 hours; intracellular staining for Granzyme B and cytokine secretion assay for IL-10 from Figure 6.11A. Histogram for marker expression in IL-10$^+$ and IL-10$^-$ was determined by gating on respective controls and applying gates test samples. **A** Histogram of marker MFI for individual donors (n=3). **B** Summary marker MFI plot (n=5). **BLUE** dot (IL-10$^-$ cells), **RED** square (IL-10$^+$ cells). Statistical significance determined by Paired Student *t* test at P≤0.05.

6.3: Discussion

The expression of Tr1 associated cell surface markers and transcription factors by IL-10$^+$ Tr1-like cells following CD3/CD55 costimulation of CD4$^+$ T cells was studied. These cells were IL-10$^+$ cells and expressed all the major Tr1-associated cell surface makers including CD49b, CD226, LAG-3, PD-1 and CTLA-4. Tr1-like cells also expressed high levels of c-MAF, as well as granzyme B. However, it is important to note that none of these cell surface markers or transcription factors were expressed exclusively by the Tr1-like cells. CD3/CD55 costimulation assay revealed the presence of IL-10 positive Tr1-like cells but also showed another population of cells that were equally stimulated, some of which produced IFN-ɣ. These populations gave a good internal comparison for the marker and transcription factor expression.

Costimulation of CD4$^+$ T cells purified from human peripheral blood of healthy donors revealed varying levels of expression of Tr1 associated markers by IL-10$^+$ Tr1-like cells, including CD49b, LAP, CD226, CTLA-4, PD-1 and LAG-3. CD226, LAG-3 and PD-1 were expressed by majority of IL-10$^+$ cells while CD49b, LAP and CTLA-4 were expressed by a minority of the IL-10$^+$ Tr1-like population. Majority of these cells also expressed transcription factors c-MAF and T- Bet while RoRγ(t), FoxP3 as well as Granzyme B were expressed by a minority of Tr1-like cell population. Strength of marker expression is summarised in Table 6.1.

Table 6.1: Summary of expression levels for Tr1 associated cells surface markers, transcription factors and intracellular markers by IL-10^+ and IL-10^- cells following CD3/CD55 costimulation of $CD4^+$ T cells from human peripheral blood. [Key: **+** (low expression), **++** (moderate expression); **+++** (high expression)]

Tr1 associated cell surface marker	**Expression levels (IL-10^+ cells)**	**Expression levels (IL-10^- cells)**
CD49b	**+**	**+**
LAP	**+**	**+**
CD226	**+++**	**+**
CTLA-4	**++**	**+**
PD-1	**+++**	**+**
LAG-3	**++**	**++**
Tr1 associated transcription factors/other markers		
c-MAF	**+++**	**+**
FoxP3	**+**	**+**
Helios	**+**	**+**
T Bet	**+++**	**+++**
RoRγ (t)	**++**	**++**
GATA-3	**-**	**-**
Granzyme B	**++**	**+**
Perforin	**+**	**+**

The ability of Tr1 cells to suppress adaptive immune responses is well known, but how these cells regulate innate immunity is less defined. The study of Tr1 cells has been enigmatic since discovery and the application of Tr1 therapies in clinical settings has had minimal success due to the difficulty in purifying and fully characterizing these cells (Groux et al., 1997). This is in part due to the lack of exclusive set of cell surface markers that identify this cell population or a master regulator that drives their transcriptional events. The situation is further complicated by the different approaches for Tr1 induction used by many groups. This results in groups reporting different set of markers and transcription factors depending on their model. For instance, Galiani et al., differentiating Tr1 clones in the presence of IL-10 demonstrated that co-expression of LAG-3, CD49b and CD226 as cell surface markers was sufficient to distinguish IL-10^+ Tr1 cells from T helper or Treg cells in human and mouse (Gagliani et al., 2013). However, recent studies showed that co-expression of LAG-3 and CD49b does not uniquely identify Tr1 cells; but also identify IL-10 producing non-Tr1 cells subset including $CD4^+Foxp3^+$, $CD4^+Foxp3^-$ and even $CD8^+$ T cells in both human and mouse (W. Huang et al., 2018).

Our data showed that IL-10^+ Tr1-like cells co-expressed LAP and CD49b but the distribution was similar to the non-Tr1 (IL-10^-) cells. Recent studies have shown that CD49b provides little contribution to the differentiation and function of Tr1 cells (Gagliani et al., 2013) and that might explain the low expression of CD49b by Tr1-like cells observed in

our studies. The high expression of CD226 by IL-10$^+$ Tr1-like supported the idea of enhanced ability of these cells to kill myeloid APCs as interactions between CD226 on Tr1 cells and their ligands on myeloid APCs define Tr1-cell target specificity (Magnani et al., 2011). Furthermore, high CD226 expression may account in part for immunosuppressive role of Tr1 cells as CD226 has emerged as a checkpoint inhibitor in a CD8$^+$ T cell study (Bi, 2021). Our study also showed that LAP was expressed at low levels by Tr1-like cells. A similar observation was made by Yu Yao et al., where they observed that low LAP expressing Tr1 cells, but not Foxp3$^+$ Treg cells suppressed NLRP3 inflammasome activation in an IL-10-dependent manner (Yao et al., 2015). In another study, Scurr et al., found that LAP was only expressed on very small populations of T cells in the peripheral blood, and that LAP expression by T cells was strongly associated with the tumour microenvironment as they were more prevalent than the FoxP3 expressing nTreg and iTreg cells, particularly among early-stage Dukes' A tumours. They further reported that CD4$^+$LAP$^+$ Tr1-like TILs were ~50-fold more suppressive than Foxp3$^+$ Treg cells (Scurr et al., 2014b).

This study also revealed a Tr1-like population that co-expressed CTLA-4, PD-1 and LAG-3, an important set of checkpoint inhibitors commonly associated with Tr1 cells. CTLA-4, PD-1 and LAG-3 are not expressed on naive T cells but expressed on activated CD4$^+$ and CD8$^+$ (Okazaki et al., 2011; Triebel et al., 1990), and their expression by regulatory cells has great implication in the treatment of many cancer types. For instance,

CTLA-4 downregulates T cells activation and induces tolerance by competing with CD28 for its ligands CD80/CD86. CTLA-4 expression correlates with suppressor functions and Tr1 cells have been reported to inhibit T cell responses *via* CTLA-4/CD80 (M. Akdis et al., 2004). On the other hand, PD-1 engagement by its ligands (PD-L1/PD-L2) regulates both central and peripheral tolerance through the induction of inhibitory cytokine synthesis, cytotoxicity and reduction in cell proliferation (Mazanet & Hughes, 2002). It achieves this by down-regulating self-reactive T cells during antigen presentation by dendritic cells (Probst, McCoy, Okazaki, Honjo, & van den Broek, 2005), directly promoting IL-10 secretion (H. Dong et al., 2003) and inhibiting dendritic cells maturation (Kuipers et al., 2006) and PD-1/PD-L1 interaction within the tumour microenvironment have been reported to aid the evasion of immunosurveillance by tumour (Park et al., 2012). Therefore, the very high PD-1 expression by Tr1-like cells reported in this study confirms the fact that the Tr1 cells may be an important target for cancer immunotherapies. Furthermore, high expression levels of LAG-3 by Tr1-like cells have been reported to strongly correlate with the inhibitory function of LAG-3(Maeda, Sugiura, Okazaki, Maruhashi, & Okazaki, 2019). Although LAG-3 expression was also detected on the IL-10^{-} cell population, it is thought to play a crucial role in the induction and function of Tr1 cells, as LAG-3 expression confers a suppressive activity and blocking LAG-3 inhibits the suppressive function of Tr1 cells *in vitro* and *in vivo* (C. T. Huang et al., 2004). Furthermore, the

crosslinking of LAG-3 on T cells has been shown to induce functional unresponsiveness, reversible via IL-2 (Hannier, Tournier, Bismuth, & Triebel, 1998).

The primary mechanisms of immune suppression by Tr1 cells have been the secretion of high levels of IL-10 and the ability to inhibit T cell proliferation and proinflammatory cytokine production (Magnani et al., 2011). However, several other immune cell populations have been shown to produce IL-10 but are unable to mediate immune suppression. In fact, depending on the polarizing conditions, other immune cell subtypes have been reportedly differentiated into Tr1 cells, thus raising the question about the lineage specificity of Tr1 cells and the overarching transcriptional events that drive such differentiation. The observation that IL-10^{+} Tr1-like cells from CD3/CD55 costimulation expressed several transcription factors commonly associated with other cell types suggested that multiple transcription factors may be required for Tr1 regulation.

The high levels of c-MAF expression suggested that c-MAF plays a crucial role in regulating IL-10 secretion as it has already been reported to promote the secretion of IL-10 and IL-21 (Apetoh et al., 2010), as well as in Tr1 differentiation (Barroso et al., 2021; Imbratta et al., 2020). Tr1 cells do not constitutively express FoxP3 (Yudoh, Matsuno, Nakazawa, Yonezawa, & Kimura, 2000) but only transiently, upon their activation and in smaller amounts than $CD25^{+}$ $FoxP3^{+}$ regulatory

cells(Silvia Gregori, Bacchetta, Battaglia, & Roncarolo, 2012). FoxP3 is not required for Tr1 induction nor for its function(Silvia Gregori, Kevin S. Goudy, & Maria Grazia Roncarolo, 2012) and FoxP3 mutations in patients with IPEX syndrome did not result in the loss of function by Tr1 cells (Passerini et al., 2011). Therefore, the transiently low expression of FoxP3 by Tr1-like cells observed in this study may have been to stabilize the cells; FoxP3 has been reported to play essential role in the stability and function of natural Treg cells(W. Liu et al., 2006; Miyara et al., 2009). Furthermore, Tr1 cells have been reported to produce IL-10 and have a suppressor function independent of FoxP3 expression(Roncarolo et al., 2018).

It was interesting to observe that IL-10^{+} Tr1-like cells expressed T-Bet and RoRγ(t), transcription factors reported as master regulators for Th1 and Th17 cells, respectively. Although the expression distribution for T-Bet and RoRγ(t) by Tr1-like cells was like the IL-10^{-} (non-Tr1 cells), it nonetheless raises the possibility of the plasticity of these cells. It is possible that CD3/CD55 costimulation may have reprogrammed Th1 cells or Th17 to adopt a Tr1 phenotype. Studies have shown that Th1 cells (Meiron, Zohar, Anunu, Wildbaum, & Karin, 2008), Th2 cells (Heinemann et al., 2014; Pacciani et al., 2010; Pellerin et al., 2018), Th17 cells (Heinemann et al., 2014), memory CD4^{+} T cell (Yao et al., 2015) as well as naïve CD4^{+} T cells (Bacchetta et al., 2010; Sutavani et al., 2013) can been differentiate into Tr1 cells. It is therefore unclear whether the phenotype and transcriptional events for the naïve CD4^{+} T

cells differentiated Tr1 cells are fixed and distinct from those derived from other T helper cell populations. However, the fact that c-MAF was significantly expressed on the Tr1-like cells suggests that CD3/CD55 costimulation may have induced high IL-10 production via the c-MAF pathway which inhibited T helper functions, making the cells acquire Tr1 phenotype.

CD3/CD55 costimulation of $CD4^+$ T cells revealed a Tr1-like cell population that produced high levels of IL-10 and highly expressed CD226, LAG-3, PD-1, and c-MAF and granzyme B. These were examined at 24 hours and whether marker expression levels or phenotypes changed over time, remains unknown. However, the signalling mechanisms that lead from CD3/CD55 to IL-10 production and c-MAF involvement are not fully understood. Recent study showed that crosslinking of CD44 on memory $CD4^+$ T cells induced Tr1 phenotype through p38MAPK and ERK1/2 signalling (Bollyky et al., 2011). Mitogen-activated protein kinase (MAPK) signalling pathway has been reported to control several cellular processes and it remains unknown whether IL-10 production by Tr1-like cells following CD3/CD55 costimulation is driven by the p38MAPK or other MAPK signalling pathways.

Chapter 7

Understanding the IL-10 signalling pathway for Tr1-like cells following CD3/CD55 costimulation of CD4$^+$ T cells

7.1: Introduction

Inducible T regulatory cells (e.g. Tr1s) have been implicated in cancers where they mediate immune suppression by secreting high levels of IL-10(Bergmann et al., 2008; Groux et al., 1997; Locafaro et al., 2017; H. Zeng et al., 2015) and decrease in number or impaired function of Tr1 cells have been reported in autoimmune conditions(Jia et al., 2019; Pan, Wang, Liu, Guo, & Yang, 2020; Caroline Pot, Lionel Apetoh, & Vijay K. Kuchroo, 2011). Thus, regulation of IL-10 expression by Tr1 cells could enhance the clearance of cancerous cells. Our group previously reported that costimulation of naïve CD4$^+$ T cells via CD55 results in a rare population of IL-10 single positive, Tr1-like cells (Figure 3.17) and we showed in this study that these cells were stimulated in peripheral blood via CD55 (Figure 4.14). Understanding the link between CD55 costimulation and IL-10 secretion could lead to a mechanism of control of IL-10 production by these cells through the Mitogen-activated protein kinase (MAPK) signalling pathways.

MAPK cascades are key signalling pathways that phosphorylate their own dual serine and threonine residues (autophosphorylation), or those

found on their substrates, to activate or de-activate their target (Johnson & Lapadat, 2002; Peti & Page, 2013). They regulate a wide variety of cellular processes such as proliferation, differentiation, apoptosis, and stress responses under both normal and pathological conditions(Arthur & Ley, 2013; Guo et al., 2020). In mammals, the MAP kinases can be grouped into three main families, the ERK1/2 (extracellular-signal-regulated kinases), the c-JUN N-terminal kinase 1, 2 and 3 (JNK1/2/3) (Jun amino-terminal kinases), and the p38 MAPK α, β, δ, and γ pathways (Morrison, 2012; Y. Zhang & Dong, 2007). ERK1/2 is activated in response to growth factors, hormones and proinflammatory stimuli, while JNK1/2/3 and p38 MAPK α, β, δ, and γ are activated by cellular and environmental stresses, in addition to proinflammatory stimuli (Kyriakis & Avruch, 2012; Owens & Keyse, 2007). Upon activation MAPKKK, which is nearest to the signal source, phosphorylates MAPKK, which in turn phosphorylates MAPK which then triggers downstream signalling events, leading to an immune response(Munshi & Ramesh, 2013). MAPKs regulate several key immune responses including the production of immunomodulatory cytokines, such as TNFα, IL-1, IL-10, and IL-12 (Arthur & Ley, 2013; Guindi et al., 2018; Strnisková, Barancík, & Ravingerová, 2002). These pathways have been exploited in the treatment and management of many disease conditions, including cancers(Dhillon, Hagan, Rath, & Kolch, 2007; Roberts & Der, 2007), kidney injury (Cuarental et al., 2019) among others, in which case small molecule kinase inhibitors are

utilised. Kinase inhibitors are commonly used to target specific molecules of the MAPK cascade(Cohen, 2002). Some kinase inhibitors used in this study are highly specific while others have been reported to display some off target effects. SL-0101 is highly specific for inhibiting both RSK1/2. BIRB-0796 is a dual inhibitor of p38/JUN N-terminal kinase (JNK) MAPK and potently inhibits p38α and p38β even at low concentrations but requires a high concentration to block the activation of SAPK3/p38γ complex. VX-745 is an exquisite inhibitor of p38α MAPK while SB/GW-681323 is a potent, selective and a reversible inhibitor of p38α MAPK. SB-747651A is a MSK 1 shown *in vitro* to have some off target inhibitions, including inhibition of PRK2 (double-stranded-RNA-dependent protein kinase 2), RSK1 (ribosomal S6 kinase 1), $p70^{s6k}$ and ROCK II (Rho-associated protein kinase 2). PF-3644022 is a freely reversible ATP-competitor and potent MK2 inhibitor. PD-184352 is a highly potent inhibitor of MEK (MAPK/ERK kinase) and is reported to induce cytotoxicity in chronic myeloid leukaemia (CML) cells.

Several signalling molecules have been identified to induce and maintain IL-10 expression in a variety of cells, including STAT3 (Cevey, Penas, Alba Soto, Mirkin, & Goren, 2019), p38 MAP kinase(Zhao, Pan, Tang, & Lin, 2018) and ERK1/2 (Qian et al., 2006). Meanwhile in macrophages, MSK 1/2 have been shown to regulate dectin-1 induced IL-10 production by phosphorylating the transcription factor, CREB (Elcombe et al., 2013) and inhibitors of p38 MAPK shown to reduce IL-10 production by LPS-stimulated primary human macrophages(Page,

Brown, Timms, Foxwell, & Ray, 2010). Franks, H.A., et al., reported that in myeloid DCs, inhibition of p38 MAPK/MK-2 pathway inhibits RSK, resulting in enhanced secretion of IL-12 while inhibiting IL-10 production (Franks et al., 2014). Furthermore, in Foxp3^{+} Treg cells, it is reported that IL-10 signalling sustains IL-10 production via STAT3 activation (Schmetterer & Pickl, 2017). However, in IL-27 induced Tr1 cells, STAT3 activation plays a role but is reportedly dispensable whereas p38 MAPK is vital for the maintenance of IL-10 secretion (Brockmann et al., 2017). CD55 is a surface located protein that is tethered to the plasma membrane by a glycosyl-phosphatidyl-inositol (GPI) anchor. It is in regions of membrane that are enriched in cholesterol, glycosphingolipids and GPI-anchored proteins and are often referred to as lipid rafts / microdomains (Horejsí et al., 1999; Stefanová et al., 1991). During T cell activation there is a coalescing of the rafts, which associate with the immunological synapse and are thought to potentiate T cell activation. On the inner side of these rafts are located other lipid anchored proteins such as Src family kinases. Crosslinking of CD55 has been shown to result in the activation of two members of this family, Fyn and Lck, which are also known to activate the downstream MAP kinase pathways (L. S. Davis, Patel, Atkinson, & Lipsky, 1988a; J. R. Hwang et al., 2020; Loertscher & Lavery, 2002; Shenoy-Scaria et al., 1992b). The proposed pathways and inhibitor targets are shown in Figure 7.1 and we hypothesised that CD55 costimulation of CD4^{+} T cells

influenced the secretion of IL-10 in Tr1 cells through the MAPK signalling pathway.

Therefore, the current study utilised SL-0101 (RSK1/2 inhibitor), BIRB-0796, VX-745 and SB/GW-681323 (p38 MAPK inhibitors), PF-3644022 (MK2 inhibitor), SB-747651A (MSK1 inhibitor) and PD-184352 (MEK inhibitor) in a first line screen of the effects on IL-10 secretion by Tr1-like cells, summarised in Table 7.1 and preparation of kinase inhibitors shown in section 2.5.1.

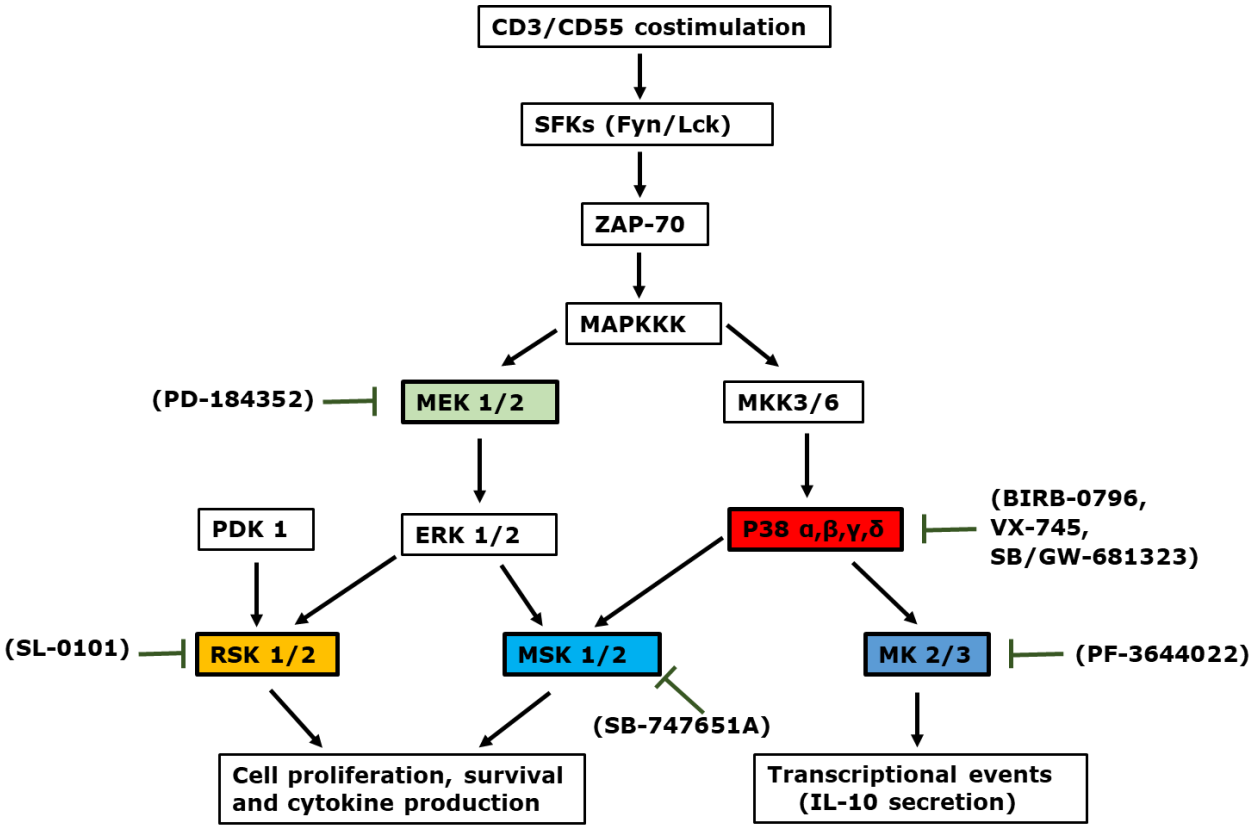


Figure 7.1: Proposed pathways and inhibitor targets for IL-10 production following CD3/CD55 costimulation of $CD4^+$ T cells

SFKs Src family kinases, ZAP-70 Zeta-chain-associated protein kinase 70, MAPKKK Mitogen Activated Protein (MAP) kinase kinase kinase, MEK 1/2 Mitogen-activated protein kinase kinase 1/2, MKK3/6 Mitogen-activated protein kinase kinase 3/6, PDK 1 Phosphoinositide-dependent kinase-1, ERK 1/2 Extracellular signal-regulated protein kinase 1/2, p38 MAPKs *p38* mitogen-activated protein kinases, RSK 1/2 p90 ribosomal S6 kinases 1/2, MSK 1/2 Mitogen- and stress-activated kinases 1/2, MK2 MAP kinase-activated protein kinase 2.

→ Activation ⊣ Inhibition

7.2: Results

7.2.1 Kinase inhibitors block IL-10 production; but not IFN-ɣ or overall cell proliferation following CD3/CD55 costimulation of $CD4^+$ T cells

Studies have shown that inhibition of IL-10 signalling can lead to better control and faster clearance of intracellular infections, resulting in enhanced adaptive immune response by $CD4^+$ T cells (Sabat et al., 2010). Phosphorylation of certain kinases and transcription factor upregulation are responsible for IL-10 secretion by cells and these kinases can be targeted using certain molecules in order to elucidate pathways for cytokine production in response to different stimuli (Shankar Subramanian Iyer & Gehong Cheng, 2012). The aim of this study was to target possible pathways for IL-10 production by Tr1-like cells following CD3/CD55 costimulation of $CD4^+$ T cells.

Purified $CD4^+$ T cells from human peripheral blood were pre-treated for 30 minutes with a dose titration of different inhibitors and then costimulated with CD3/CD55 for 24 hours. The effect of inhibitors on cell proliferation was measured by tritiated thymidine uptake and cytokine secretion by IL-10/IFN-ɣ ELISA. For cell proliferation following tritiated thymidine uptake by $CD4^+$ T cells, treatment with SL-0101 (RSK1/2 inhibitor), BIRB-0796, VX-745 and SB/GW-681323 (p38 MAPK inhibitors), PF-3644022 (MK2 inhibitor) and SB-747651A (MSK1 inhibitor) did not inhibit cell proliferation (Figure 7.2). However, drastic reduction in cell proliferation from 3μM was observed for cells treated

with MEK inhibitor, PD-184352 and statistical difference with and without PD-184352 at concentrations: 0 μM versus 5μM (p=0.0345) and 0 μM versus 10μM (p=0.0414). (Figure 7.2E).

When IL-10 secretion was evaluated, results showed dose dependent reduction in IL-10 across all inhibitors (Figure 7.3 B-G), except for SL-0101, where no effect on IL-10 secretion was observed (Figure 7.3A) which were not significantly different when compared with 0μM (untreated group). However, there was an overall statistical difference with and without BIRB-0796 (p=0.0317); and with and without PD-184352 (p=0.0115) but post hoc test revealed no specific point of significance**.**

Also, there was generally no effect on IFN-ɣ secretion between 0μM (untreated group) and treated groups across all donors (Figure 7.4), except for PD-184352 (Figure 7.4 E) treated group statistical difference with and without PD-184352 (p=0.0241) but post hoc test revealed no specific point of significance**.**

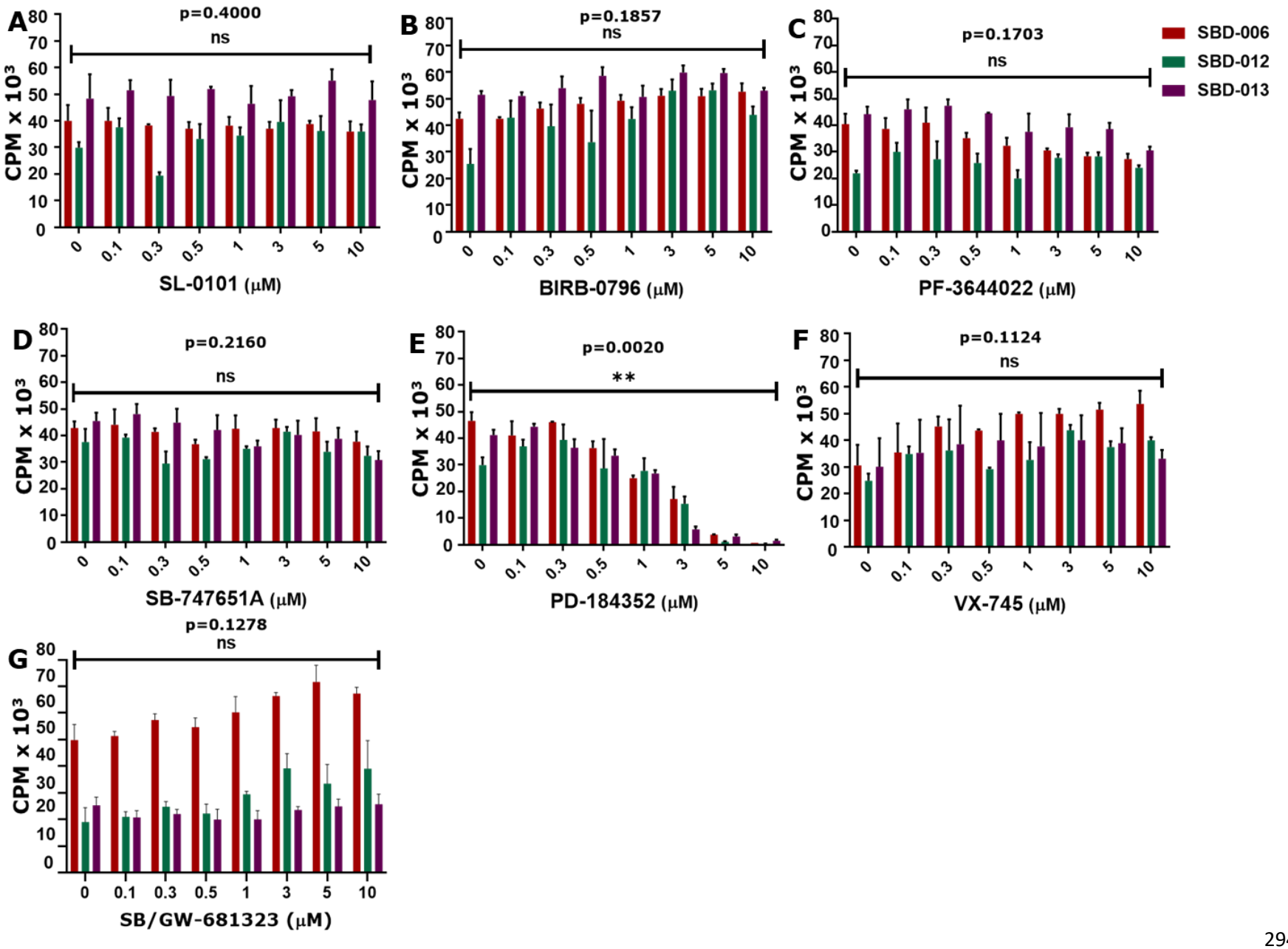

A
p=0.4000
ns
CPM x 10³
SL-0101 (μM)
B
p=0.1857
ns
BIRB-0796 (μM)
C
p=0.1703
ns
PF-3644022 (μM)
SBD-006
SBD-012
SBD-013
D
p=0.2160
ns
SB-747651A (μM)
E
p=0.0020
**
PD-184352 (μM)
F
p=0.1124
ns
VX-745 (μM)
G
p=0.1278
ns
SB/GW-681323 (μM)
0 0.1 0.3 0.5 1 3 5 10

Figure 7.2: Dose titration of select kinase inhibitors on proliferation of $CD4^+$ T cell following CD3/CD55 costimulation

Proliferation of total $CD4^+$ T cells in culture set up to contain 1.5 x 10^5 cells/well in 96 well plates, pre-treated with kinase inhibitors, SL-0101 (RSK1/2 inhibitor), BIRB-0796, VX-745 and SB/GW-681323 (p38 MAPK inhibitors), PF-3644022 (MK2 inhibitor), SB-747651A (MSK1 inhibitor) and PD-184352 (MEK inhibitor),in respective groups for 30 minutes before costimulation with plate-bound αCD3/αCD55 for 72 hours. Cell proliferation was assessed by incorporation of tritiated thymidine (0.5μCi[^{3}H]) in the last 15 hrs of culture measured in Counts Per Minute (CPM). **Red** bar shows proliferating cells for donor 1; **Green** bar for donor 2; and **Purple** bar for donor 3. Results showed mean ± SD from three donors in three independent experiments and statistical difference with and without PD-184352 at concentrations: 0 μM versus 5μM (P=0.0345) and 0 μM versus 10μM (P=0.0414). All other concentrations were not significant when compared with 0 μM. Statistical significance was determined by Repeated Measure One-Way ANOVA followed by Dunnett's Post hoc test, significant at P≤0.05. ns=not significant.

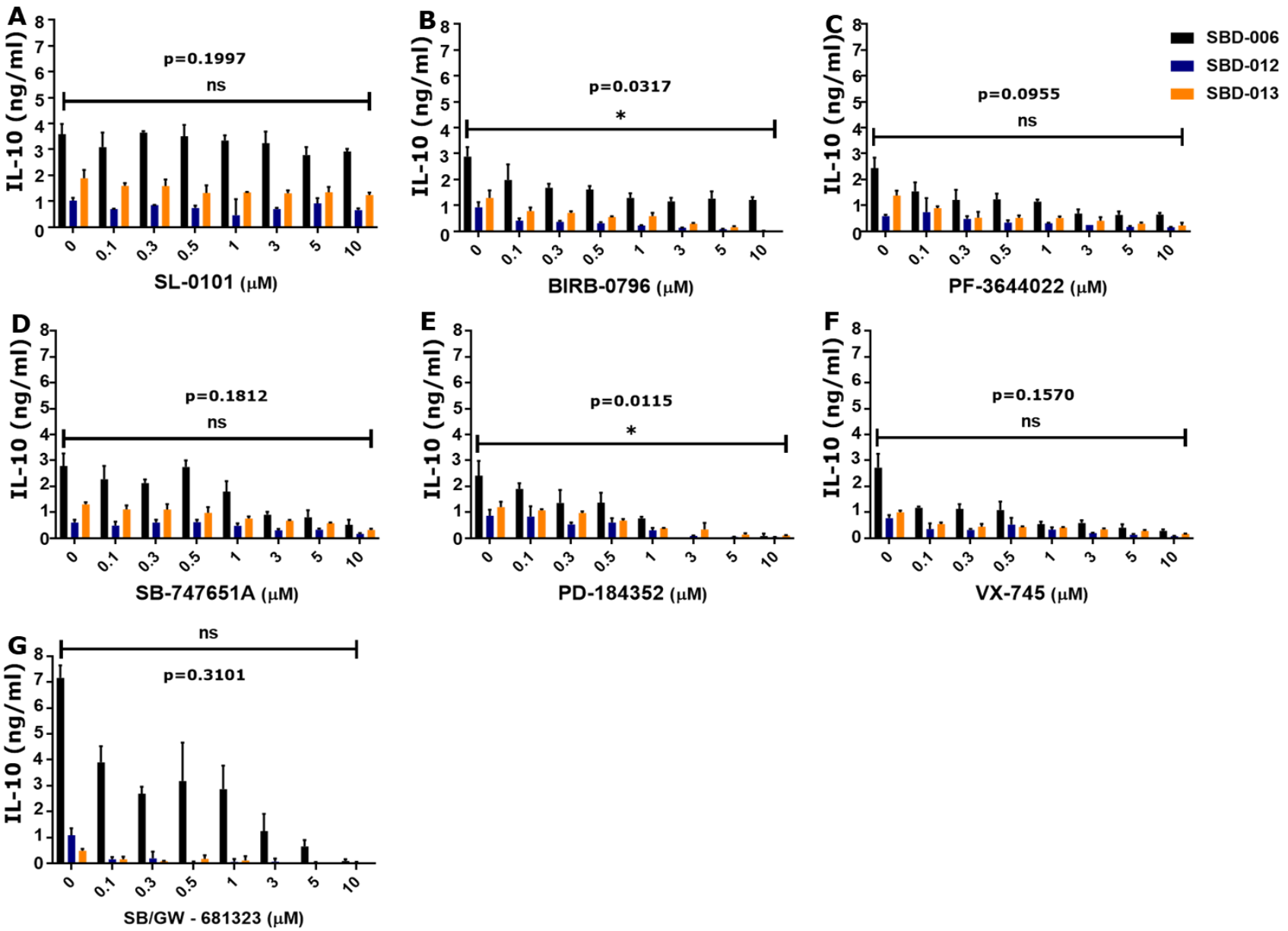

A
IL-10 (ng/ml)
p=0.1997
ns
SL-0101 (μM)
B
IL-10 (ng/ml)
p=0.0317
*
BIRB-0796 (μM)
C
IL-10 (ng/ml)
p=0.0955
ns
PF-3644022 (μM)
SBD-006
SBD-012
SBD-013
D
IL-10 (ng/ml)
p=0.1812
ns
SB-747651A (μM)
E
IL-10 (ng/ml)
p=0.0115
*
PD-184352 (μM)
F
IL-10 (ng/ml)
p=0.1570
ns
VX-745 (μM)
G
IL-10 (ng/ml)
ns
p=0.3101
SB/GW - 681323 (μM)
0
0.1
0.3
0.5
1
3
5
10

Figure 7.3: Dose dependent effect of kinase inhibitors on IL-10 secretion by Tr1-like cells following CD3/CD55 costimulation of $CD4^+$ T cells for 24 hours.

IL-10 secreted in culture by $CD4^+$ T cells after pre-treatment with kinase inhibitors, SL-0101 (RSK1/2 inhibitor), BIRB-0796, VX-745 and SB/GW-681323 (p38 MAPK inhibitors), PF-3644022 (MK2 inhibitor), SB-747651A (MSK1 inhibitor) and PD-184352 (MEK inhibitor), for 30 minutes and costimulation with plate-bound αCD3/αCD55 for 24 hours. Culture supernatant was collected at 24 hours and IL-10 levels quantified by Enzyme-linked Immunosorbent Assay (ELISA). Each bar represents amount (ng/ml) of IL-10 for respective concentrations (0, 0.1, 0.3, 0.5, 1, 3, 5, 10 μM) of kinase inhibitors. **Black** bar shows measured IL-10 for donor 1; **Blue** bar for donor 2; and **Orange** bar for donor 3. Results showed mean ± SD from three donors in three independent experiments. Overall statistical difference with and without BIRB-0796 ($p=0.0317$); and with and without PD-184352 ($p=0.0115$) but post hoc test revealed no specific point of significance**.** Statistical significance was determined by Repeated Measure One-Way ANOVA followed by Dunnett's Post hoc test, significant at $P \leq 0.05$. ns=not significant.

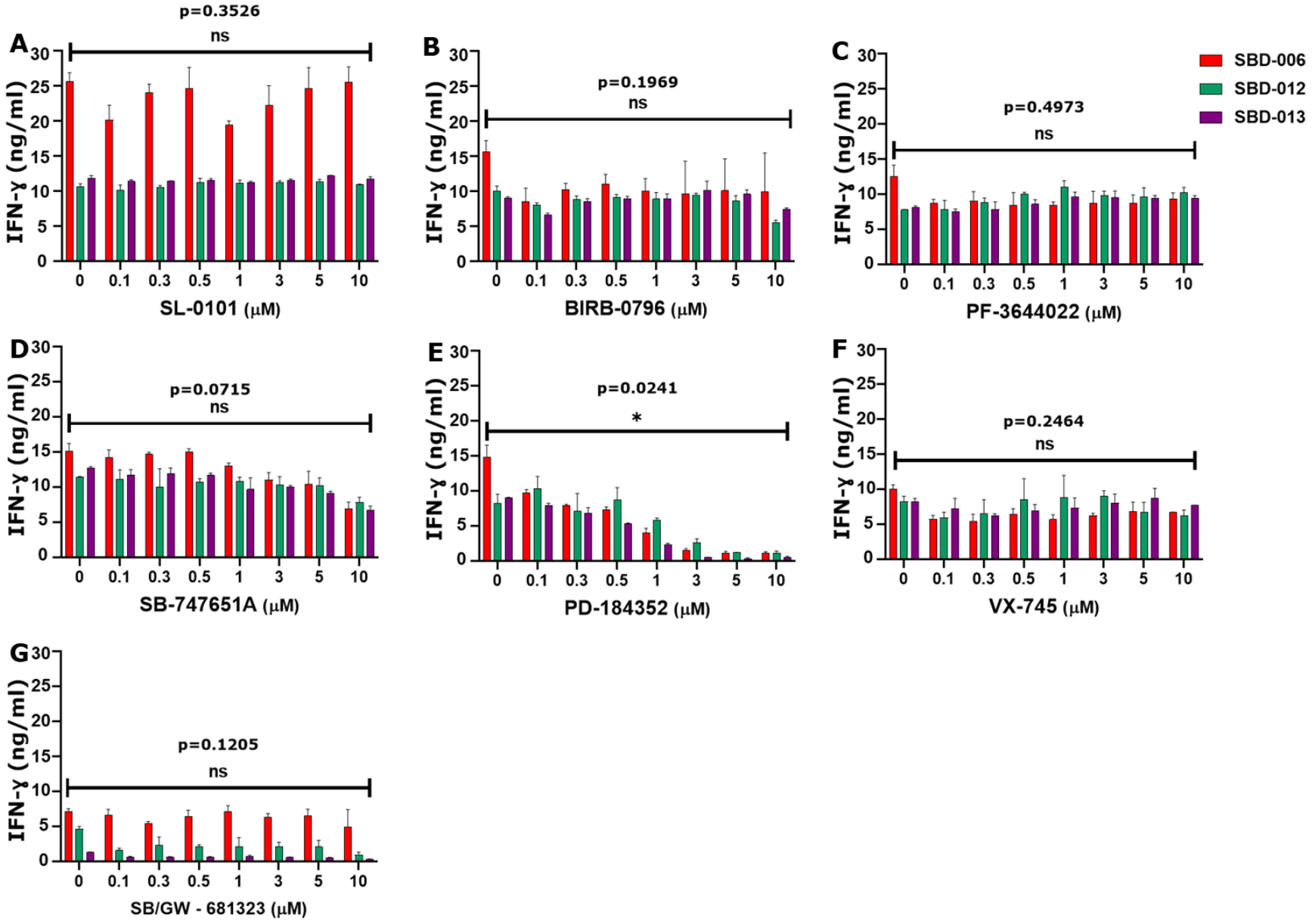

A
p=0.3526
ns
IFN-γ (ng/ml)
SL-0101 (μM)
B
p=0.1969
ns
BIRB-0796 (μM)
C
p=0.4973
ns
PF-3644022 (μM)
SBD-006
SBD-012
SBD-013
D
p=0.0715
ns
SB-747651A (μM)
E
p=0.0241
*
PD-184352 (μM)
F
p=0.2464
ns
VX-745 (μM)
G
p=0.1205
ns
SB/GW - 681323 (μM)
0 0.1 0.3 0.5 1 3 5 10

Figure 7.4: Dose dependent effect of kinase inhibitors on IFN-ɣ secretion by $CD4^+$ T cells after ` CD3/CD55 costimulation for 24 hours

IFN-ɣ secreted in culture by $CD4^+$ T cells after pre-treatment with kinase inhibitors SL-0101 (RSK1/2 inhibitor), BIRB-0796, VX-745 and SB/GW-681323 (p38 MAPK inhibitors), PF-3644022 (MK2 inhibitor), SB-747651A (MSK1 inhibitor) and PD-184352 (MEK inhibitor), for 30 minutes and costimulation with plate-bound αCD3/αCD55 for 24 hours. Culture supernatant was collected after 24 hours and IFN-ɣ levels quantified by Enzyme-linked Immunosorbent Assay (ELISA). Each bar represents amount (ng/ml) of IFN-ɣ for respective concentrations of kinase inhibitors. **Red** bar shows measured IFN-ɣ for donor 1; **Green** bar for donor 2; and **Purple** bar for donor 3. Results showed mean ± SD from three donors in three independent experiments with statistical difference with and without PD-184352 ($p=0.0241$) but post hoc test revealed no specific point of significance**.** Statistical significance was determined by Repeated Measure One-Way ANOVA followed by Dunnett's Post hoc test, significant at $P \leq 0.05$. ns=not significant.

Table 7.1: Overall effects of inhibitors on IL-10, IFN-ɣ secretion and proliferation following CD3/CD55 costimulation of $CD4^+$ T cells

Kinase inhibitors and working concentration used for further experiments following initial screening by dose titration

Drug	IL-10	IFN-ɣ	Proliferation	Inhibitor Concentration chosen (μM)
BIRB-0796	Reduced	No Effect	No Effect	1
PF-3644022	Reduced	No Effect	No Effect	1
SB-747651A	Reduced	No Effect	No Effect	5
VX-745	Reduced	No Effect	No Effect	1
SB/GW-681323	Reduced	No Effect	No Effect	1

Following from the observation that SL-0101 (RSK1/2 inhibitor) treatment had no effect on the measured parameters and PD-184352 (MEK1/2 inhibitors) treatment affected all the measured parameters, these inhibitors were excluded from future experiments. BIRB-0796 showed significant reduction in IL-10 in cultures and every other inhibitor marginally reduced IL-10 production without affecting IFN-ɣ secretion or cell proliferation. Since the hallmark of Tr1 cells is the production of high levels of IL-10, inhibitor concentrations [BIRB-0796 (1μM), VX-745 (1μM), SB/GW-681323 (1μM), PF-3644022 (1μM), and SB-747651A (5μM)] that showed about 50% reduction in IL-10 secretion was chosen for future experiment (Table 7.1). These working

concentrations have also been reported by other groups (T. Davis, Brook, Rokicki, Bagley, & Kipling, 2016; Franks et al., 2014; Singh, Diwan, Dastidar, & Najmi, 2018).

Single cell populations of $CD4^+$ T cells were assessed for both IL-10/ IFN-ɣ expression by dual cytokine secretion assay and percentage of cytokine positive cells measured after 24 hours of stimulation in the presence or absence of inhibitors, depicted by the gating strategy in Figure 7.5.

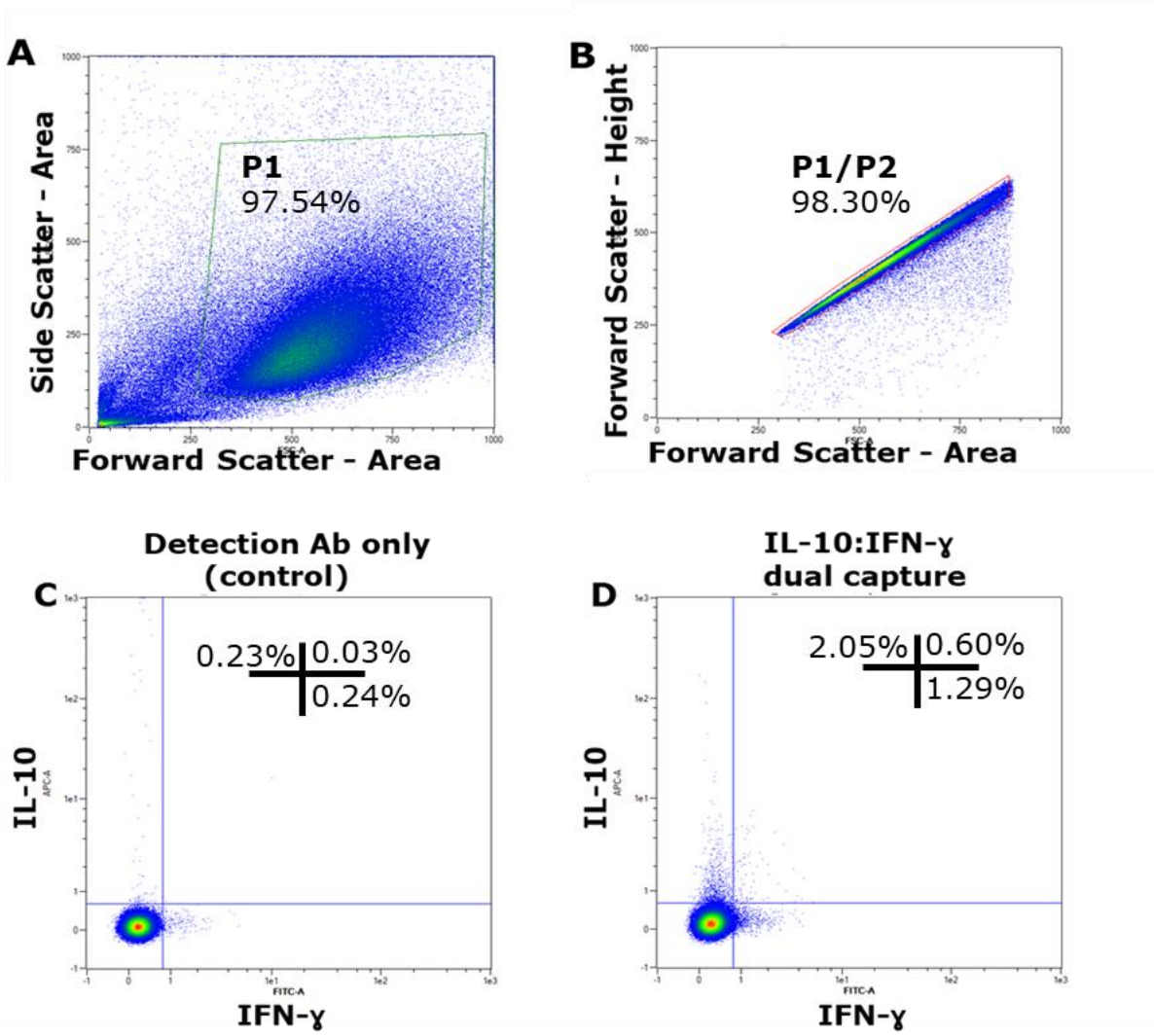


Figure 7.5: Determining the IL-10 and IFN-γ positive cells in CD4⁺ T cells following CD3/CD55 costimulation

Gating for IL-10 and IFN-γ on detection antibody (only) control and applied to dual cytokine capture to demarcate cytokine-negative and cytokine-positive cells. **A** Live cell inclusion on forward scatter-area, side scatter – area. **B** doublet discrimination and cell clumps exclusion by forward scatter- area, forward scatter – height. **C** IL-10/IFN-γ dual capture with detection antibody only – control. **D** Assessment of cytokine expression (IL-10/IFN-γ) following anti-CD3/anti-CD55 costimulation of CD4⁺ T cells for 24 hours.

7.2.2: p38 MAPK inhibitors (BIRB-0796, VX-745 and SB/GW-681323) potently inhibit IL-10 expression by Tr1-like cells

The p38 MAPK pathway plays a major role in cell proliferation, differentiation, cytokine production as well as apoptosis and this pathway has been a useful target in the treatment and management of many inflammatory diseases and cancers(Goldsmith et al., 2018; H. Y. Yong, Koh, & Moon, 2009). This study utilised three different p38 MAPK inhibitors (BIRB-0796, VX-745 and SB/GW-681323) in order to demonstrate the effect of p38 MAPK inhibition on IL-10 expression by Tr1-like cells.

$CD4^+$ T cells pre-treated with BIRB-0796, VX-745 or SB/GW-681323 were costimulated with CD3/CD55 for 24 hours. It was observed that treatment of cells with BIRB-0796, VX-745 or SB/GW-681323 resulted in reduction in percentage of IL-10 expressing cells; but no effect on IFN-ɣ expressing cells compared to non-treated group at 24 hours for all three donors assessed (Figure 7.6). IL-10 expression levels were significantly lower than untreated group across multiple donors (Figure 7.7).

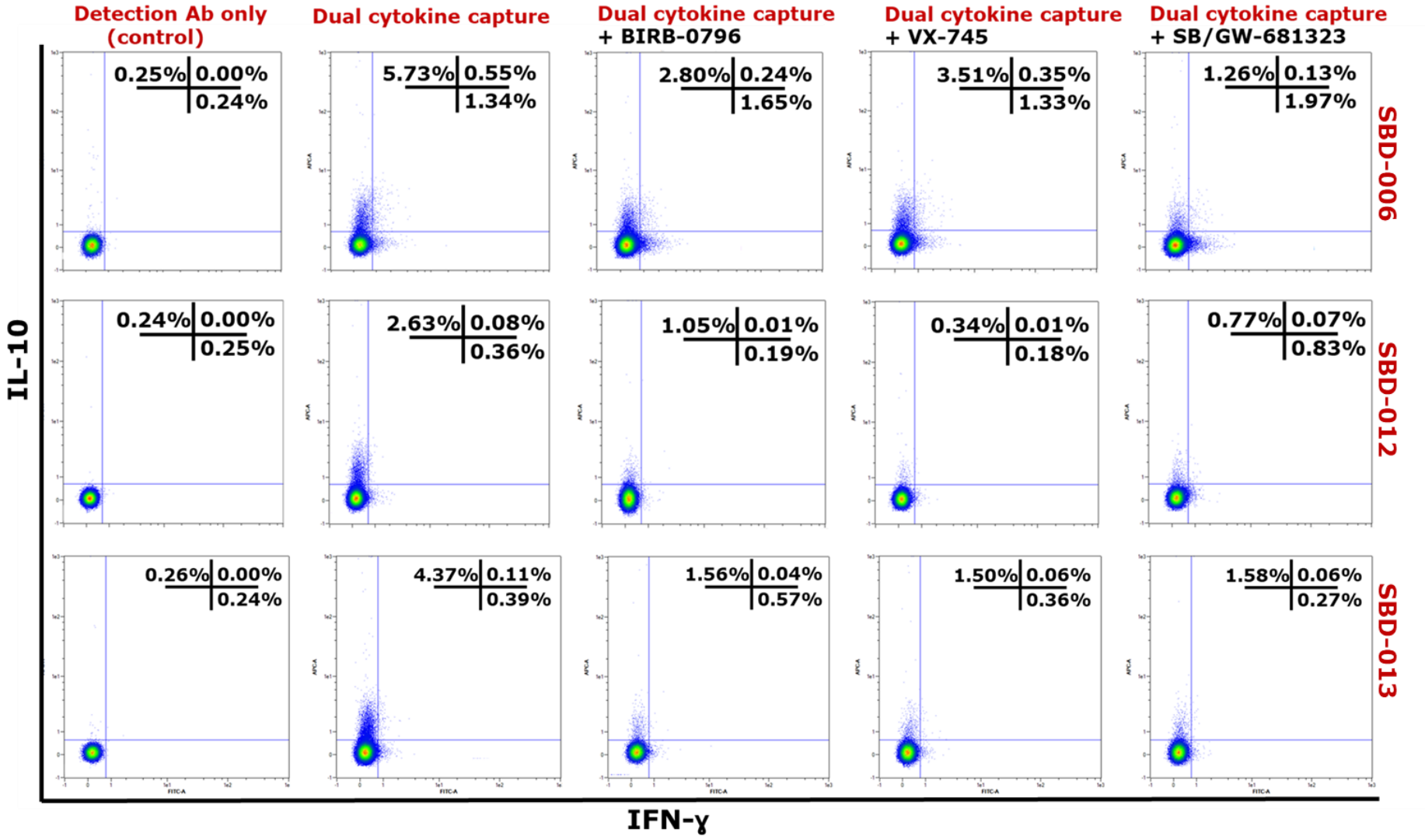

Detection Ab only (control)
Dual cytokine capture
Dual cytokine capture + BIRB-0796
Dual cytokine capture + VX-745
Dual cytokine capture + SB/GW-681323
SBD-006
SBD-012
SBD-013
0.25% 0.00% 0.24%
5.73% 0.55% 1.34%
2.80% 0.24% 1.65%
3.51% 0.35% 1.33%
1.26% 0.13% 1.97%
0.24% 0.00% 0.25%
2.63% 0.08% 0.36%
1.05% 0.01% 0.19%
0.34% 0.01% 0.18%
0.77% 0.07% 0.83%
0.26% 0.00% 0.24%
4.37% 0.11% 0.39%
1.56% 0.04% 0.57%
1.50% 0.06% 0.36%
1.58% 0.06% 0.27%
IL-10
IFN-γ

Figure 7.6: BIRB-0796, VX-745 or SB/GW-681323 treatment reduced the percentage of IL-10 expressing r1-like cells following CD3/CD55 costimulation of $CD4^+$ T cells for 24 hours.

$CD4^+$ T cells isolated from peripheral blood of healthy donors were pre-treated with BIRB-0796, VX-745 or SB/GW-681323 (p38 MAPK inhibitors) for 30 minutes and costimulated with CD3/CD55 for 24 hours. Cells were assessed for IL-10 and IFN-ɣ by cytokine secretion assay and data captured by flow cytometry. Secreted cytokines for both treated and untreated groups were evaluated against detection antibody (only) control. Flow cytometry plots are for three donors in three independent experiments, representative of six independent experiments.

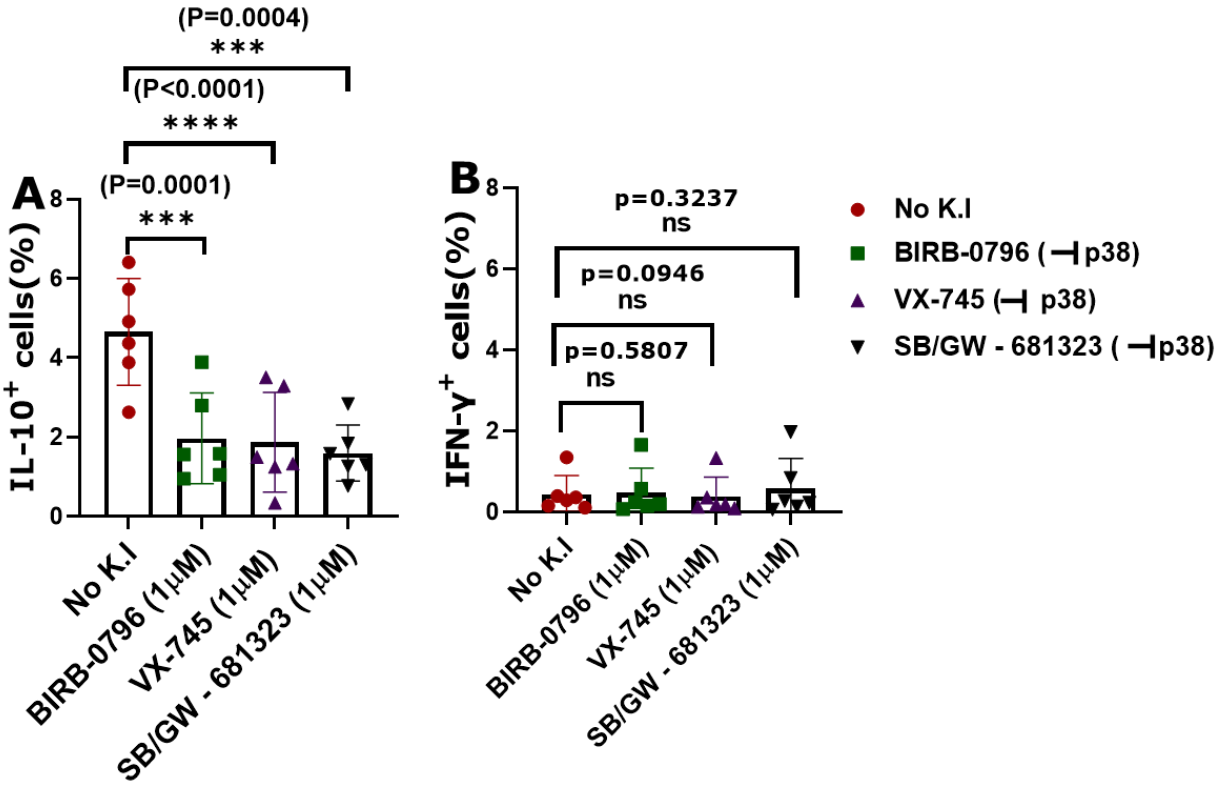


Figure 7.7: BIRB-0796, VX-745 or SB/GW-681323 treatment significantly reduced percentage of IL-10 expressing Tr1-like cells

Effect of BIRB-0796, VX-745 or SB/GW-681323 (p38 MAPK inhibitors) treatment on IL-10 expressing Tr1-like cells following CD3/CD55 costimulation of $CD4^+$ T cells for 24 hours compared to untreated group (No K.I). Data shown are percentage $IL\text{-}10^+$ cells (n=6) in six independent experiments. Statistical significance was determined by Paired Student's *t* test, significant at P≤0.05. ns= Not significant.

7.2.3: MK2 inhibitor (PF-3644022) reduced the percentage of IL-10 expressing Tr1-like cells following CD3/CD55 costimulation of $CD4^+$ T cells

PF-3644022 was the first MK2 inhibitor to be described with oral efficacy in both acute and chronic models of inflammation (Mourey et al., 2010; H. Song et al., 2015). MK2 acts downstream of the p38MAPK pathway and inhibition of MK2 has been reported as a preferred strategy in anti-inflammatory models (Singh et al., 2018). It is yet to be reported whether Tr1 cells mediate IL-10 signalling via MK2. Therefore, the effect of MK2 inhibition on IL-10expression by Tr1-like cells following anti-CD3/anti-CD55 costimulation of $CD4^+$ T cells was investigated.

$CD4^+$ T cells purified from human peripheral blood and pre-treated with PF-3644022, were costimulated with CD3/CD55 for 24 hours followed by measurement of IL-10 and IFN-ɣ expression. Results showed a reduction in percentage of cells expressing IL-10 in all three donors while no effects were observed for IFN-ɣ (Figures 7.8). This observation was consistent across multiple donors (Figure 7.9)

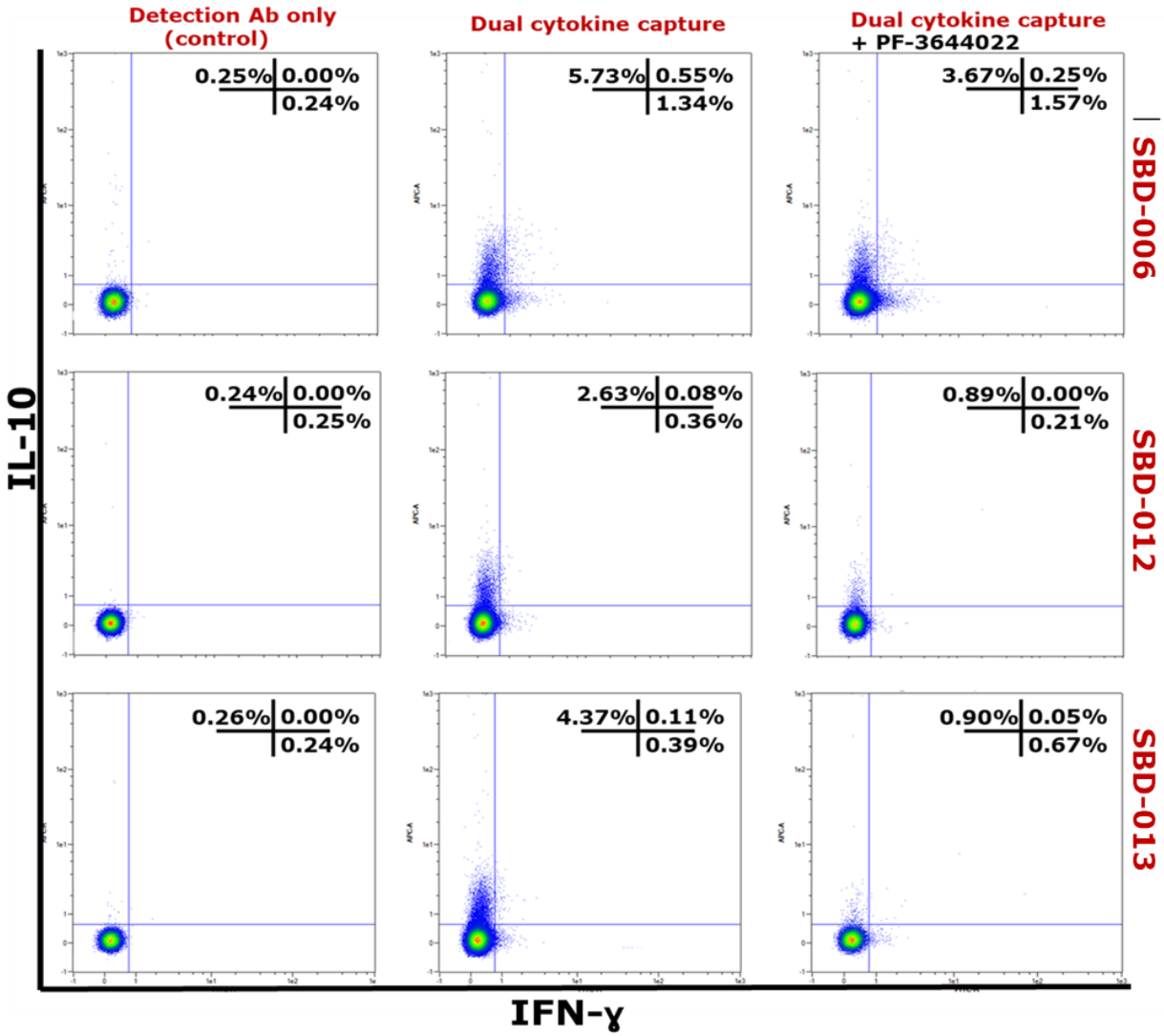


Figure 7.8: PF-3644022 treatment reduced percentage of IL-10 expressing Tr1-like cells following CD3/CD55 costimulation of $CD4^+$ T cells for 24 hours

$CD4^+$ T cells isolated from peripheral blood of healthy donors were pre-treated with PF-3644022 (MK2 inhibitor) for 30 minutes and costimulated with CD3/CD55 for 24 hours. Cells were assessed for IL-10 and IFN-ɣ by cytokine secretion assay and data captured by flow cytometry. Secreted cytokines for both treated and untreated groups were evaluated against detection antibody (only) control. Flow cytometry plots are for three donors in three independent experiments, representative of six independent experiments.

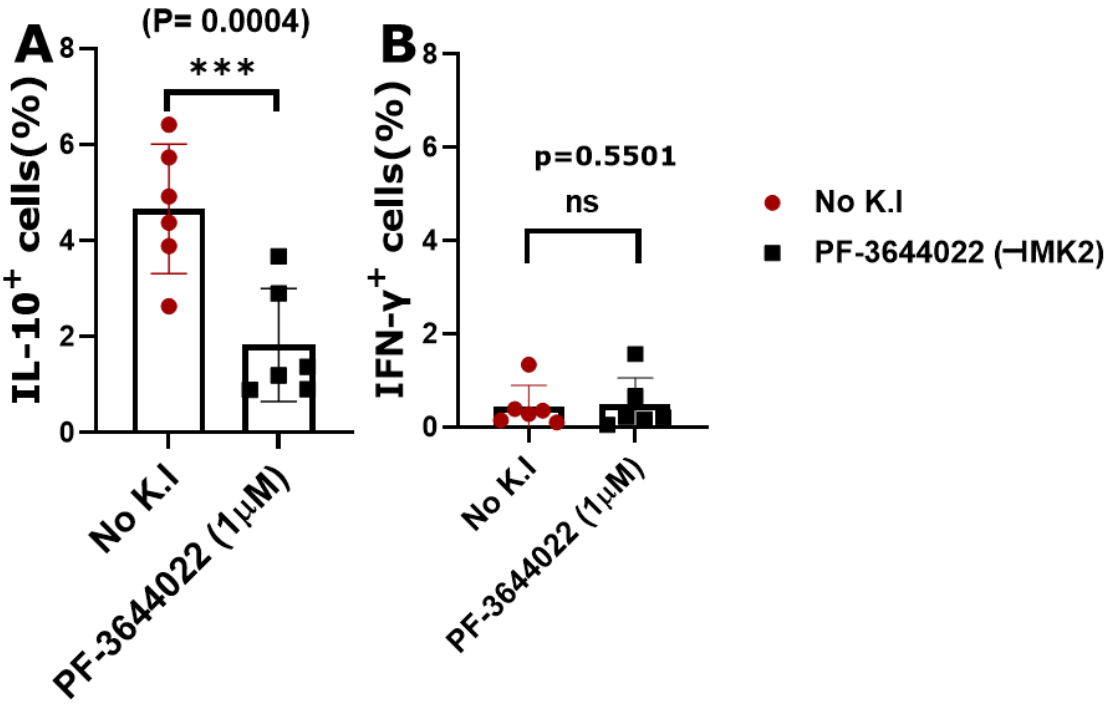


Figure 7.9: PF-3644022 treatment significantly reduced the percentage of IL-10 expressing Tr1-like cells

Effect of PF-3644022 (MK2 inhibitor) treatment on IL-10 expressing Tr1-like cells following CD3/CD55 costimulation of $CD4^+$ T cells for 24 hours compared to untreated group (No K.I). Data shown are percentage $IL\text{-}10^+$ cells (n=6) in six independent experiments. Statistical significance was determined by Paired Student's *t* test, significant at $P \leq 0.05$.

7.2.4: MSK1 inhibitor (SB-747651A) reduced the percentage IL-10 expressing Tr1-like cells following CD3/CD55 costimulation of $CD4^+$ T cells

SB-747651A is a MSK 1 inhibitor. MSK1/2 are nuclear protein kinases that regulate transcription downstream of the ERK 1/2(extracellular-signal-regulated kinase 1/2) and p38α MAPKs via the phosphorylation of CREB (c-AMP-response-element-binding protein) and histone H3. Therefore, this study evaluated the inhibitory effect of SB-747651A on MSK1 in relation to IL-10 expression by Tr1-like cells following CD3/CD55 costimulation.

$CD4^+$ T cells purified from human peripheral blood and pre-treated with SB 747651A, were costimulated with CD3/CD55 for 24 hours followed by measurement of IL-10 and IFN-ɣ expression. Results showed reduction in percentage IL-10 expressing cells in all donors assessed, with no effect on IFN-ɣ expressing cells (Figure 7.10) and this was consistent across multiple donors (Figure 7.11).

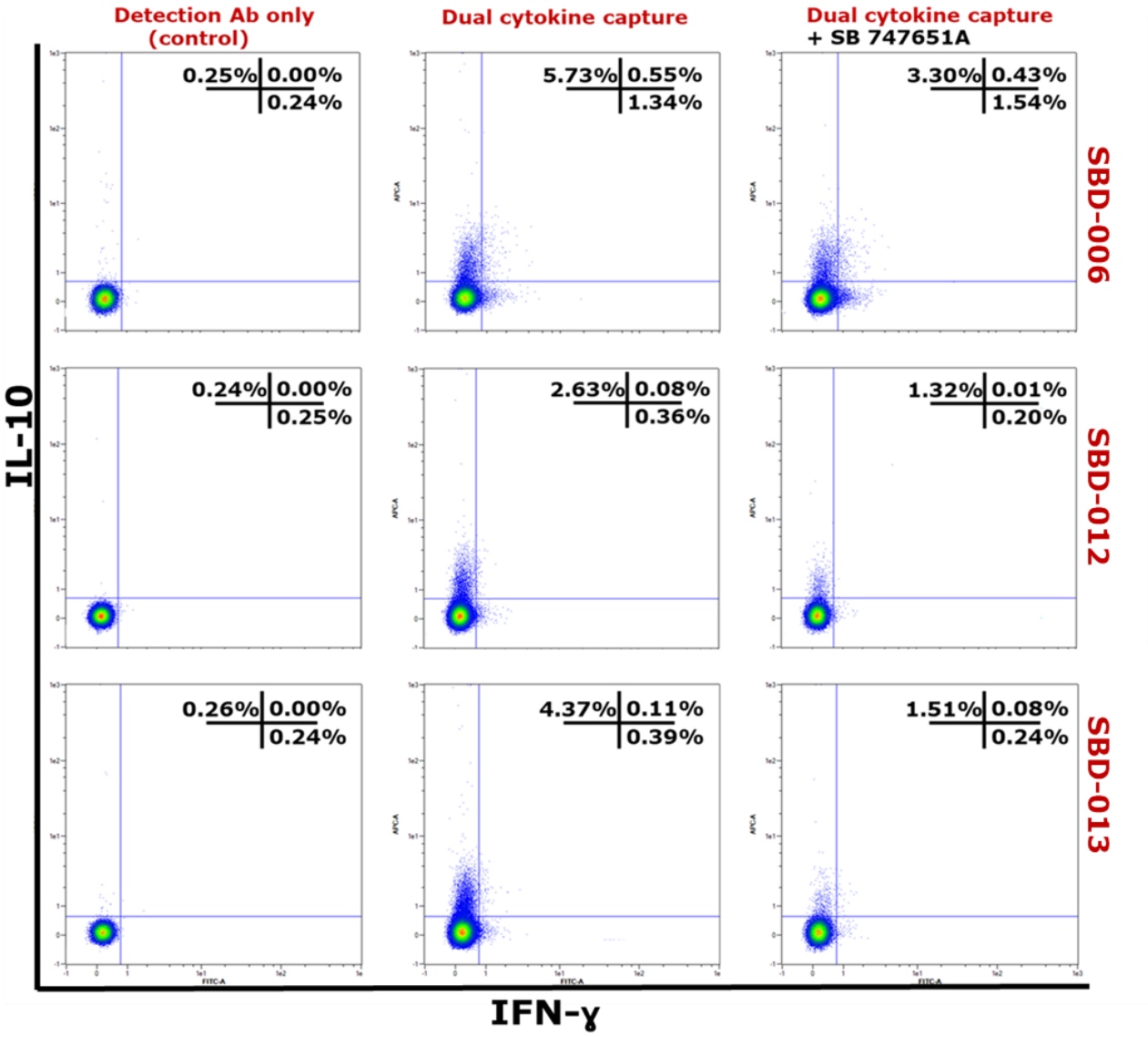


Figure 7.10: SB-747651A reduced the percentage of cells expressing IL-10 following CD3/CD55 costimulation of $CD4^+$ T cells for 24 hours

$CD4^+$ T cells isolated from peripheral blood of healthy donors were pre-treated with SB-747651A (MSK1 inhibitor) for 30 minutes and costimulated with CD3/CD55 for 24 hours. Cells were assessed for IL-10 and IFN-ɣ by cytokine secretion assay and data captured by flow cytometry. Secreted cytokines for both treated and untreated groups were evaluated against detection antibody (only) control. Flow cytometry plots are for three donors in three independent experiments, representative of six independent experiments.

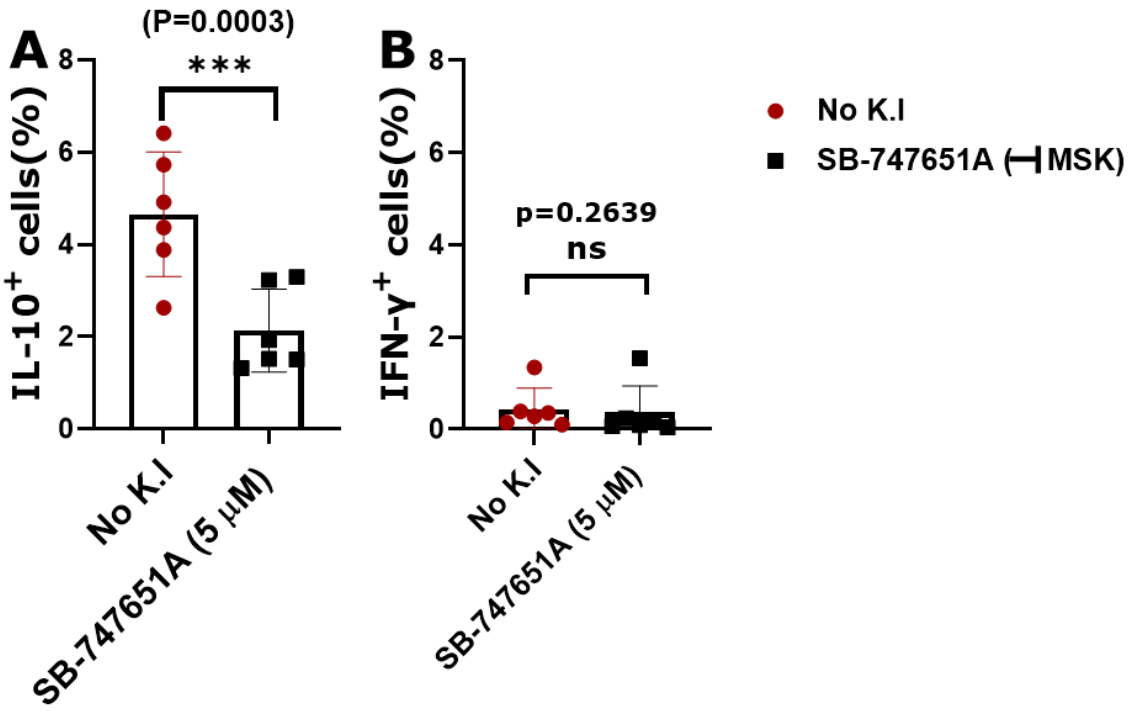


Figure 7.11: SB-747651A treatment significantly reduced the percentage IL-10 expressing Tr1-like cells following CD3/CD55 costimulation of $CD4^+$ T cells for 24 hours

Effect of SB 747651A (MSK1 inhibitor) treatment on IL-10 expression by Tr1-like cells following CD3/CD55 costimulation of $CD4^+$ T cells for 24 hours compared to untreated group (No K.I). Data shown are percentage IL-10^+ cells (n=6) in six independent experiments. Statistical significance was determined by Paired Student's *t* test, significant at P≤0.05.

7.2.5: Kinase inhibitors affected the percentage of IL-10 expressing cells; but not cycles of cell proliferation following CD3/CD55 costimulation of $CD4^+$ T cells

Treatment of $CD4^+$ T cells with inhibitors had no effect on overall proliferation of cells (Figure 7.3) as determined by tritiated thymidine incorporation. However, the impact of the inhibitor on proliferation and cytokine expression of the small Tr1-like (IL-10 producing) population was assessed by combining the Cell Trace Violet (CTV) dye dilution assay with the IL-10 cytokine secretion assay.

$CD4^+$ T cells were pre-treated with pre-determined concentrations of the respective inhibitors, stained with CTV and costimulated with CD3/CD55 for 72 hours followed by simultaneous IL-10 capture/cell proliferation cycles analysis as optimised in Figure 7.12. Results showed significant difference ($p=0.0030$) in $IL\text{-}10^+$ cells for SB-747651A treated group compared to the untreated (Figure 7.14) – which contrasted with the observations made when cells were treated with SB-747651A for 24 hours; but no difference in the proliferation cycles across all donors (Figure 7.13). However, treatment of cells with BIRB-0796, VX-745, SB/GW-681323 or PF-3644022 all marginally reduced the percentage of IL-10 expressing Tr1-like cells (Figure 7.16) with no effect on proliferation cycles of IL-10 positive or negative cells (Figure 7.15).

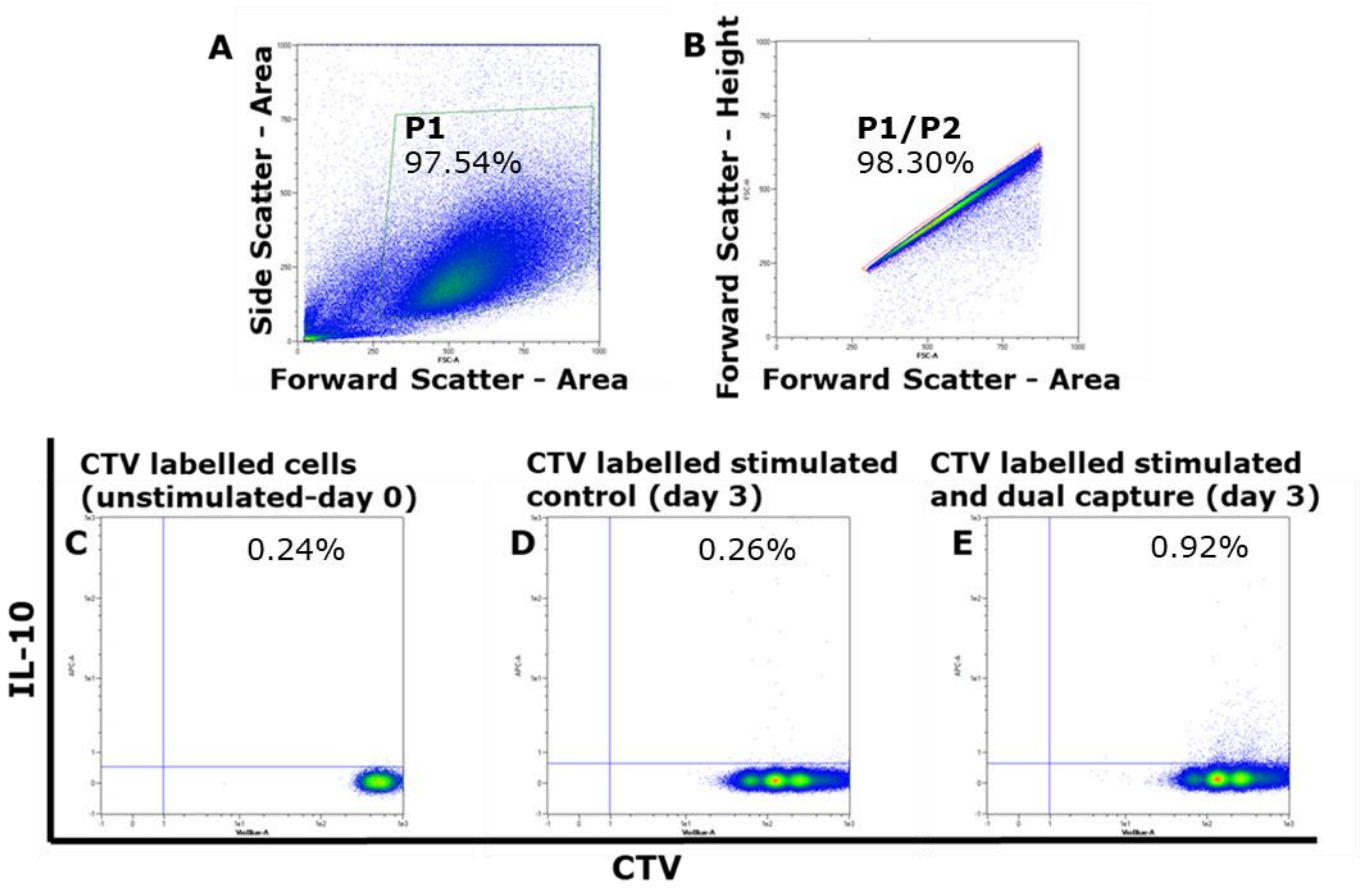


Figure 7.12: Determining the IL-10 positive cells following CTV labelling and CD3/CD55 costimulation of $CD4^+$ T cells

Gating for CTV labelled and IL-10 positive cells. **A** dead cell exclusion on forward scatter-area, side scatter – area. **B** doublet discrimination and cell clumps exclusion by forward scatter- area, forward scatter – height. **C** Uniformly labelled cells prior to costimulation (day 0). **D** Detection antibody only (control) of CTV labelled/stimulated cells (day 3). **E** Assessment of IL-10 and cycles of cell proliferation.

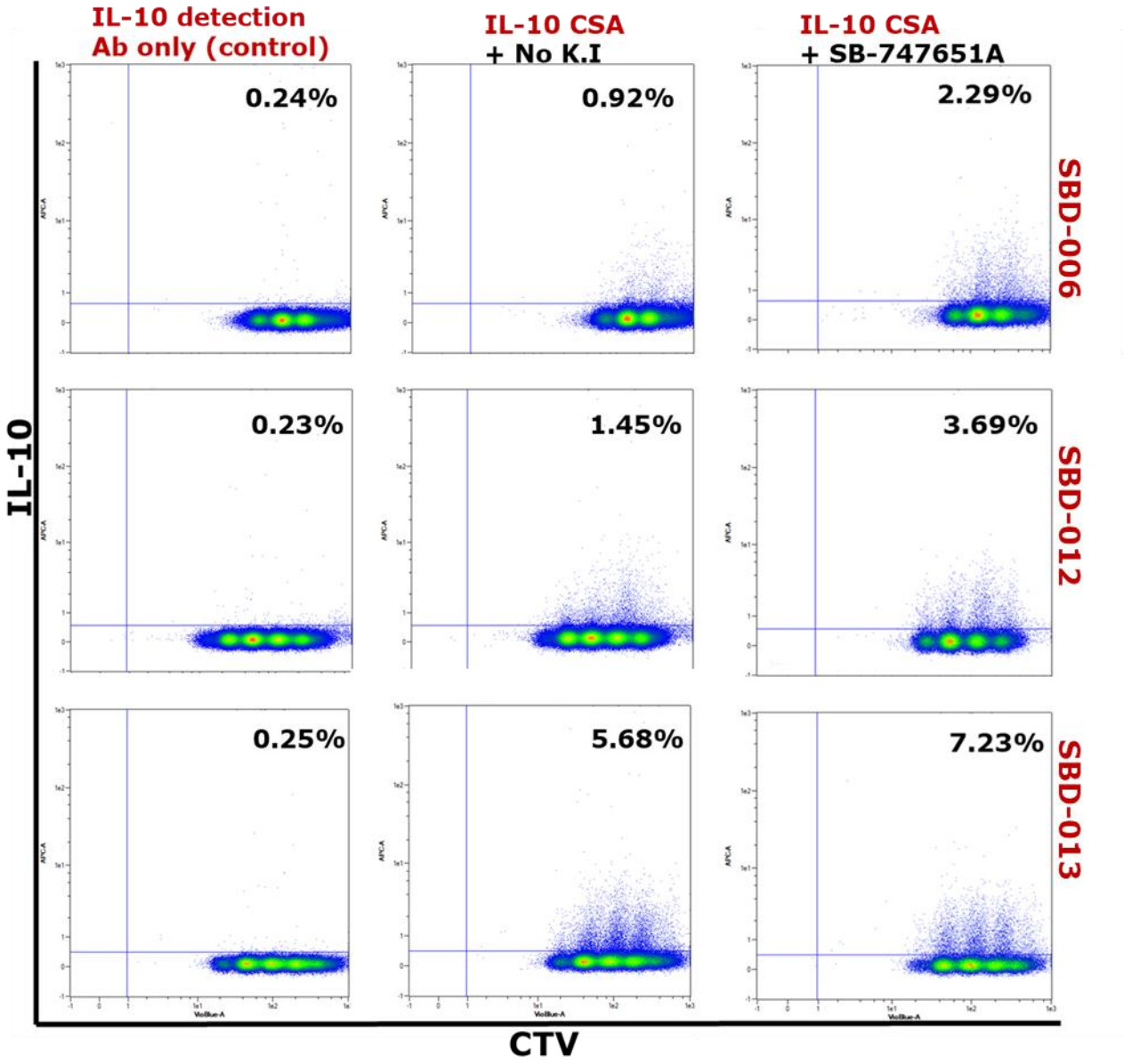


Figure 7.13: SB-747651A treatment enhanced percentage of IL-10 expressing Tr1-like cells without affecting cell proliferation cycles following CD3/CD55 costimulation of $CD4^+$ T cells for 72 hours

$CD4^+$ T cells isolated from peripheral blood of healthy donors were labelled with CTV dye, pre-treated with SB-747651A (MSK1 inhibitor) for 30 minutes and costimulated with CD3/CD55 for 72 hours. Cycles of cell proliferation were assessed by CTV dye dilution and IL-10 expression by cytokine secretion assay. Data capture was done by flow cytometry. IL-10 expression was evaluated against detection antibody (only) control. Data shown are for three donors, representative of six independent experiments.

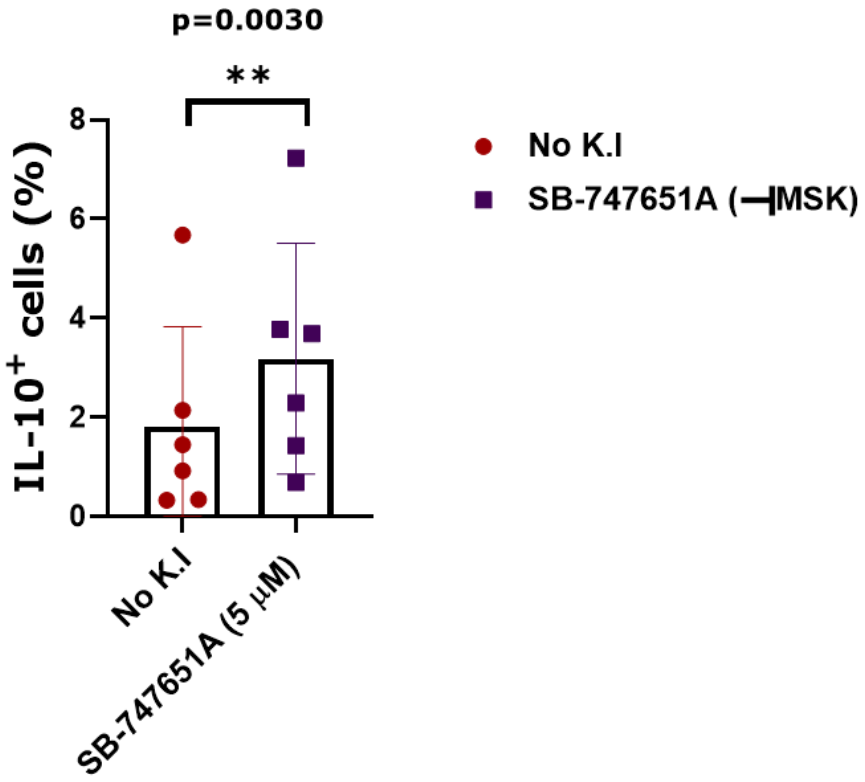


Figure 7.14 SB-747651A treatment marginally increased the percentage of IL-10 expressing cells following CD3/CD55 costimulation of CD4$^+$ T cells for 72 hours

Effect of SB 747651A (MSK1 inhibitor) treatment on percentage of IL-10 expressing Tr1-like cells following CD3/CD55 costimulation of CD4$^+$ T cells for 72 hours compared to untreated group (No K.I). Data shown are percentage IL-10$^+$ cells (n=6) in six independent experiments. Statistical significance was determined by Paired Student's *t* test, significant at P≤0.05.

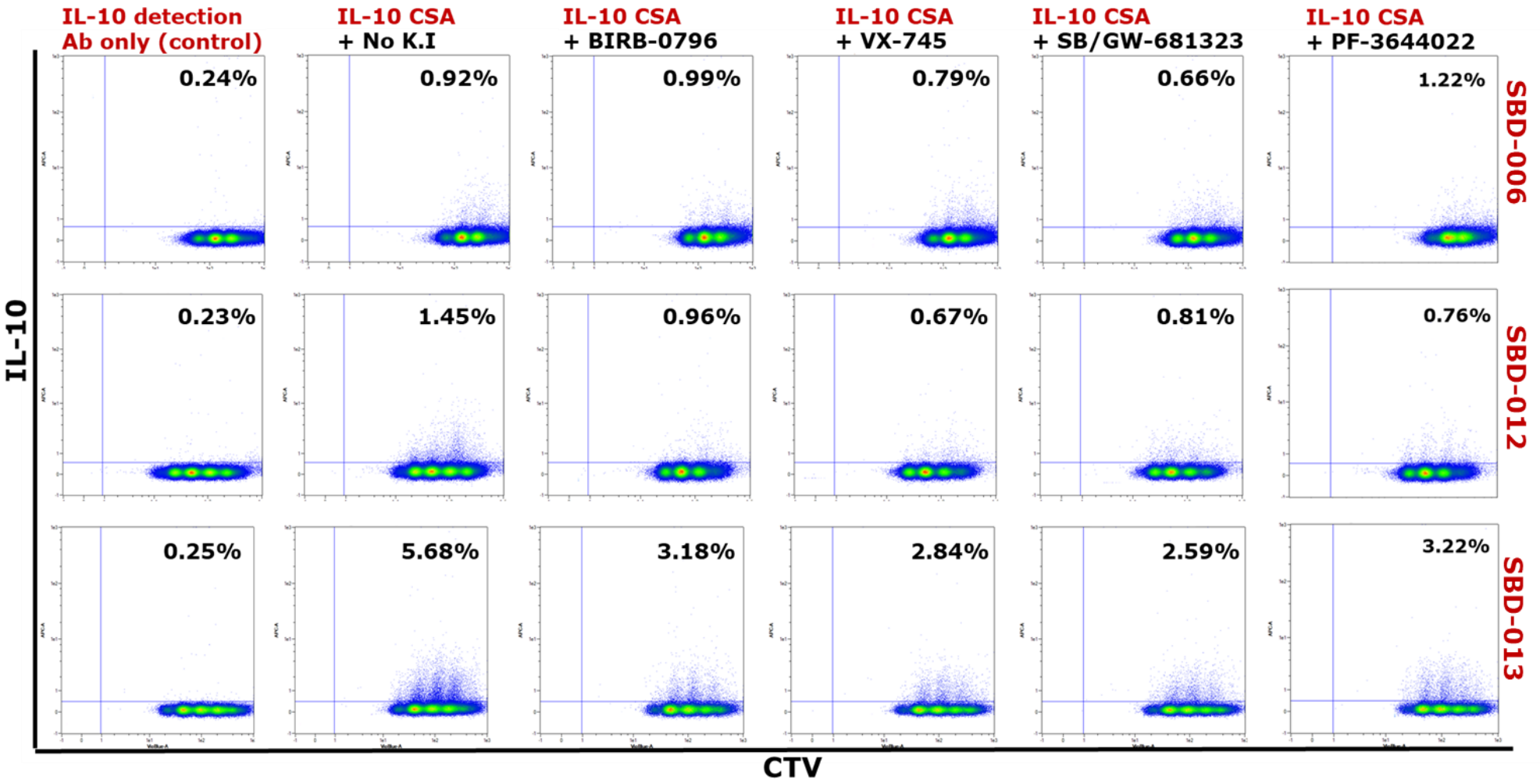

IL-10 detection
Ab only (control)
IL-10 CSA
+ No K.I
IL-10 CSA
+ BIRB-0796
IL-10 CSA
+ VX-745
IL-10 CSA
+ SB/GW-681323
IL-10 CSA
+ PF-3644022
0.24%
0.92%
0.99%
0.79%
0.66%
1.22%
SBD-006
0.23%
1.45%
0.96%
0.67%
0.81%
0.76%
SBD-012
0.25%
5.68%
3.18%
2.84%
2.59%
3.22%
SBD-013
IL-10
CTV

Figure 7.15: Treatment with BIRB-0796, VX-745, SB/GW-681323 or PF-3644022 reduced percentage of IL-10 expressing cells without altering overall cell proliferation cycles following CD3/CD55 costimulation of $CD4^+$ T cells for 72 hours

$CD4^+$ T cells isolated from peripheral blood of healthy donors were labelled with CTV dye, pre-treated with kinase inhibitors BIRB-0796, VX-745, SB/GW-681323 (p38 MAPK inhibitors) or PF-3644022 (MK2 inhibitor) for 30 minutes and costimulated with CD3/CD55 for 72 hours. Cycles of cell proliferation were assessed by CTV dye dilution and IL-10 expression by cytokine secretion assay. Data capture was done by flow cytometry. Secreted cytokines for both treated and untreated groups were evaluated against detection antibody (only) control. Flow cytometry plots are for three donors in three independent experiments, representative of six independent experiments.

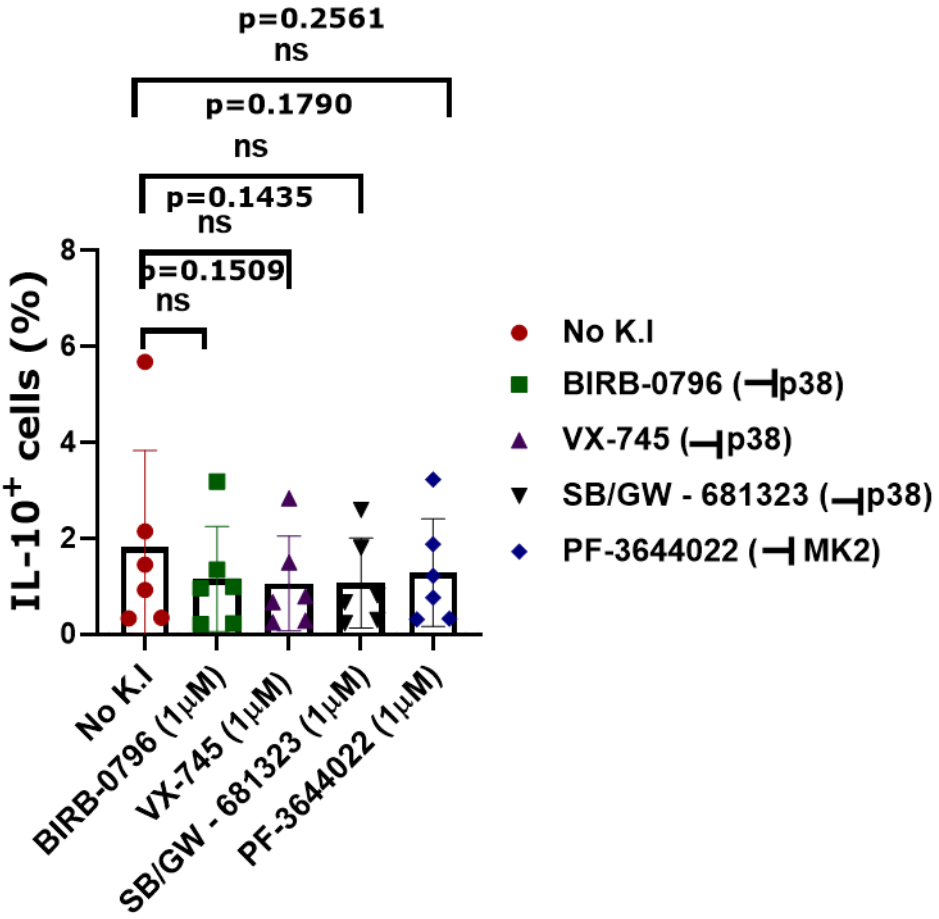


Figure 7.16 No significant difference in percentage of cells expressing IL-10 following treatment of CD4$^+$ T cells with BIRB-0796, VX-745 or SB/GW-681323 and CD3/CD55 costimulation for 72 hours

Effect of BIRB-0796, VX-745, SB/GW-681323 (p38 MAPK inhibitors) or PF-3644022 (MK2 inhibitor) treatment on percentage of cells expressing IL-10 following CD3/CD55 costimulation of CD4$^+$ T cells for 72 hours compared to untreated group (No K.I). Data shown are percentage IL-10$^+$ cells (n=6) in six independent experiments. Statistical significance was determined by Paired Student's *t* test, significant at P≤0.05.

7.3: Discussion

This study demonstrated that the MAPK pathway was a potential pathway for IL-10 production by Tr1-like cells following CD3/CD55 costimulation of $CD4^+$ T cells and targeting key enzymes (MSK, p38 and MK2) of this pathway could inhibit IL-10 production. The inhibitor against RSK 1/2 had no effect on either cytokine production or cell proliferation, while the inhibitor against MEK displayed profound reduction in cytokine production and cell proliferation. However, p38 MAPK, MK2 and MSK inhibitors displayed robust inhibition of IL-10 production with no apparent effect on IFN-γ production or cell proliferation.

Costimulation via CD55 has been reported to transduces signals into human T cells mediated by the interaction of the GPI-anchor with the protein tyrosine kinases $p56^{lck}$ and $p59^{fyn}$ (L. S. Davis et al., 1988a; Shenoy-Scaria et al., 1992b). Activation of T cells via CD55 leads to the recruitment of early components of the TCR-CD3 pathway, activation of ZAP-70 and downstream signals leading to IL-10 production (Dho et al., 2018; J. R. Hwang et al., 2020). Tr1 cells secrete high levels of IL-10 and are known to mediate immune tolerance through their strong immune-regulatory activity (Clemente-Casares et al., 2016; Gagliani et al., 2010). Although IL-10 is produced by several other cell types, Tr1 cells are specialized IL-10 producers in the T cell compartment and this makes them a major target in the treatment of autoimmune conditions

and cancers (Bacchetta et al., 2014; C. Pot, L. Apetoh, & V. K. Kuchroo, 2011; Roncarolo et al., 2018). Therefore, given its functions, tightly regulated mechanisms are required for IL-10 transcription to maintain the delicate balance between effective immunity and tissue protection. One such approach is to target kinases using small molecular weight compounds, which act to inhibit the phosphorylation of proteins, thus preventing their activation (Gaestel, Mengel, Bothe, & Asadullah, 2007). Thus protein kinases are now considered to be one of the most important groups of drug targets(Cohen, 2002).

In this study, kinase inhibitors were used to specifically target the MEK/ERK/RSK and the p38MAPK/MK2 pathways. Three major pathways have been described for MAPKs, including ERK, JNK and p38 kinases; but ERK and p38MAPK have been reported to play major roles in regulating cytokine production by cells in response to a wide range of stimulation. Studies have shown that MAPKs mediate signals in response to different stimuli (stress, LPS, cytokines and TLR ligands) in many cell types (Wortzel & Seger, 2011) including regulating the production of IL-10 and IL-12 in response to TLR ligands (Qian et al., 2006). Both ERK and p38MAPK pathways are reported to be equally involved in IL-10 induction in macrophages and dendritic cells following TLR ligand or LPS stimulation, as inhibition of either ERK or p38 MAPK activation leads to reduction in IL-10 expression (Bouhamdan et al., 2015; Saraiva & O'Garra, 2010). Similarly, all T helper cells produce IL-10 at one stage or another and this requires the activation of ERK,

suggesting a common molecular pathway for IL-10 production by Th cells (Saraiva et al., 2009). However, inhibition of p38 MAPK signalling did not lead to reduced IL-10 production, indicating that MEK/ERK/RSK pathway plays a dominant role over p38 MAPK in T_H1, T_H2 or T_H17 cells (Saraiva et al., 2009).

IL-27 differentiated Tr1 cells have been shown to require IL-10 receptor signalling to activate p38 MAPK, thereby sustaining IL-10 production, which ultimately mediated their suppressive activity (Brockmann et al., 2017). Additionally, both IL-21 and IL-27 are thought to induce the activation of ERK and STAT3, involved in IL-27 mediated upregulation by T cells(Fuqua, Akomeah, Price, & Adunyah, 2008; Stumhofer et al., 2007). In another study, the activity of the p38 MAPK and its downstream effectors MK2/3 are reported to be markedly enhanced in iTreg cells induced by tolerogenic DCs, and p38 inhibition resulted in complete loss of function (Adler et al., 2007). However, the pathway for IL-10 production by Tr1-like cells following CD3/CD55 costimulation of $CD4^+$ T cells has not been previously reported.

Findings from this study suggested that both MEK/ERK/RSK and p38MAPK/MK2 pathways were activated following CD3/CD55 costimulation. This is evidenced by the results showing that treatment of cells with SB-747651A (MSK1/2 inhibitor) targeting the ERK pathway, led to reduction in IL-10 expression by Tr1-like cells, in the short term 24 hours. However, the inhibitory effect of SB-747651A appeared to

have been transient (for treated group) as levels of IL-10^+ cells were observed to be significantly higher than untreated group at 72 hours. This was an intriguing observation and since this was a preliminary study, no further investigations were immediately done to address these observations, but possible explanation could be that the inhibitor had altered (delayed) the kinetics of cytokine expression and cells may only have started to express IL-10 later as the effect of the inhibitor weaned off. Conversely, it has been shown previously that the kinetics of IL-10 expression by $CD4^+$ T cells peaked around 24 to 30 hours, which might account for the low levels of IL-10^+ cells in the untreated group at 72 hours. Treatment of $CD4^+$ T cells with BIRB-0796, VX-745, SB/GW-681323 (p38 MAPK inhibitors) and PF-3644022 (MK2 inhibitor) targeted the p38MAPK/MK2 pathway and demonstrated significant and sustained reduction in IL-10 expression by Tr1-like cells. This finding suggested that p38MAPK together with its downstream substrate (MK2) played a major role in regulating IL-10 production by CD3/CD55 costimulated Tr1-like cells. It has been reported that p38MAPKs control multiple biological functions in the cell by phosphorylating numerous protein substrates, both in the cytoplasm and in the nucleus (Sanz-Ezquerro & Cuenda, 2021) and the more ubiquitously expressed isoforms (p38α and β)(S. Kumar et al., 1997) were specifically targeted in this study. Results also revealed that inhibiting of the ERK and p38MAPK pathways had no effect on IFN-ɣ production. This was consistent with the report

that IFN-ɣ production may be regulated by alternative pathways order than the ERK and p38 MAPK (de Souza et al., 2014).

Taken together, our data suggested that p38 MAPK/MK2 might be the dominant of the two pathways investigated in regulating IL-10 production by Tr1-like cells following CD3/CD55 costimulation. The IL-10 signalling pathways in Tr1 cells can be a good target for immunotherapies in certain cancers with immunosuppressive microenvironment or in autoimmune conditions (e.g. Multiple Sclerosis) where these pathways appear defective. Further studies would shed more light on the transcriptional events involved and whether there were some off target effects by inhibitors. Also, enrichment and sequencing of these IL-10 positive Tr1-like cells might give further insight on the transcriptome of gene expression following CD3/CD55 costimulation in the presence of kinase inhibitors. This would help identify other factors restricted to the Tr1 phenotype.

Chapter 8

Discussion and Future work

Tr1 cells have emerged as key players in the prevention of autoimmunity but also have a significant role in cancer where they suppress immune responses against tumour cells. This provides scope for huge therapeutic potential in the treatment of both conditions. However, understanding the underlying mechanisms that regulate the functions of Tr1 cells has been hampered by the difficulty in fully characterising these cells. Different groups have defined Tr1 cells differently and that has led to current vague positions about Tr1 cells. Given that iTreg / Tr1 cells all produce IL-10 as their inhibitory mediator, there are several possibilities that have emerged regarding the definition of these cells. These include

(A) That Tr1 cells are a differentiated population of cells producing IL-10 only in response to antigenic stimulation.

(B) That there are specific environmental cues that result in the induction of IL-10 from 'any' cell (T cell plasticity)(Abbas et al., 1996; John J. O'Shea & William E. Paul, 2010; Zhu, 2018).

(C) That there may be a point in the lifetime of any cell population where they start producing IL-10 (immune resolution phase, exhaustion)(Couper, Blount, & Riley, 2008; Ouyang & O'Garra, 2019).

The current study reported that IL-10 single positive cells existed in human peripheral blood, when stimulated by non-specific stimuli PMA/I or costimulation via CD28 or via CD55. Costimulation via CD55 appeared to favour a more Tr1-like (IL-10$^+$) cell phenotype compared to CD28 costimulation which favoured a more Th1-like (IFN-ɣ$^+$) phenotype. Also, the kinetics of IL-10 production is ahead of IFN-ɣ at 24-30 hours, compared to 30-40 hours for IFN-ɣ. Furthermore, this study reported that Tr1-like cells existed as effector, central and effector memory pools in similar ratios to Th1 cells. These IL-10 single positive cells expressed all the surface markers attributed to Tr1 cells and highly express c-MAF transcription factor associated with IL-10 production in Tr1. We have also shown that costimulation of CD4$^+$ T cells via CD55 triggered IL-10 production through the MAP kinase pathway, that can be inhibited by a range of MAPK inhibitors. Importantly these do not influence IFN-ɣ production in Th1 cells, suggesting this is a specific pathway that can be targeted.

CD55-CD97 interactions together with TCR engagement resulted in cell proliferation and IL-10 production and similar results were obtained when anti-CD55 antibody were used for CD4$^+$ T cells stimulation. Our study showed that stimulation of CD4$^+$ T cells with PMA/I, a potent polyclonal nonspecific agent, activated cells to secrete IL-10 and IFN-ɣ. The use of costimulatory molecules (CD3/CD28 or CD3/CD55) against the respective receptors also potently activated CD4$^+$ T cells to secrete cytokines such as IL-10 and IFN-ɣ. As expected, costimulation via CD28

resulted in a more Th1-like cell phenotype, driving high IFN-ɣ expression (Jonathan H. Esensten et al., 2016), while costimulation via CD55 favoured IL-10 single positive cell population, demonstrating that CD55 acts as the costimulatory pathway that drives Tr1-like cells to produce IL-10 (Capasso et al., 2006a; Sutavani et al., 2013). This suggested that TR1 cells existed in the peripheral blood as a small effector/memory pool, like the T helper populations and supports previous findings that costimulation through CD55 induced *in vitro* differentiation of an IL-10 single positive Tr1 cells population from naïve $CD4^+$ T cells. While CD28 costimulation failed to induce the differentiation of a Tr1 cell phenotype from naïve cells, it was capable of restimulating Tr1-like cells in the $CD4^+$ T cell pool of human peripheral blood. The kinetics of activation of these cells would also support the fact that they were effector or memory phenotype as they responded in 24 hours compared to 72 hours for naïve cells. These observations suggested that these were differentiated effector/memory population of cells that did not absolutely require costimulation from APCs for restimulation, but also raised a question about distribution of CD28 receptors (CD80/CD86) and CD55 (CD97) expression on naïve cells. Physiologically, it is possible that the APCs (immature or tolerogenic DCs) that costimulated naïve $CD4^+$ T cells through CD55 expressed CD97 but not CD80/ CD86 or had a higher ratio of CD97 to CD80/ CD86 expression. Hence, effector cells have lower ligand affinity threshold than naïve cells, low CD80/CD86 expression was enough to trigger the

restimulation of Tr1-like cells following CD28 costimulation (Guram et al., 2019; Kimachi, Sugie, & Grey, 2003).

The role of Tr1 cells in peripheral tolerance is widely acknowledged but the method of induction has been diverse (Fathman & Lineberry, 2007; Kalekar et al., 2016; Levings & Roncarolo, 2000; Macián et al., 2004). The many *in vitro* models for Tr1 generation potentially result in different Tr1-like phenotypes which make it even more difficult to characterise and begs the question whether these approaches are a true representation of *in vivo* processes for Tr1 induction. Stimulation of PBMCs with allogeneic monocytes in the presence of high dose IL-10 (Groux et al., 1997); costimulation of naïve $CD4^+$ T cells by immature DCs (Roncarolo, Levings, et al., 2001); tolerogenic DCs (Weiner, 2001a) or with DC-10 (DCs generated in the presence of IL-10) (S. Gregori et al., 2010); stimulation of T cells in the presence of immune modulators (vitamin D3), dexamethasone (Barrat et al., 2002) and rapamycin (Battaglia, Stabilini, et al., 2006; Bergmann et al., 2007); or costimulation with IL-27 (Apetoh et al., 2010), were some of the methods of *in vitro* Tr1 induction. It is possible that there are several *in vivo* means of Tr1 induction, and our group reported that costimulation via CD55 on T cells by CD97 on APCs was one of such, suggesting that alternate costimulators (CD97-CD55) may be a route to Tr1 induction.

This study demonstrated that once differentiated, Tr1-like cells maintained a distribution of effector/memory phenotypes like the Th1

cells. Our study showed that the classical costimulators, CD3/CD28 gave a predominantly IFN-ɣ positive cell population while CD3/CD55 resulted in IL-10 cell population that were predominantly effector memory T cells with similar T cell memory distribution compared to the well-established IFN-γ^+ Th1-like cells. Human memory Tr1 cells has not been previously described. Recent study in house dust mite model of allergic airway inflammation using 10BiT mice suggested that naturally arising Tr1 cells form immunological memory, but they, however, did not necessarily give rise to more Tr1 upon allergen re-challenge or contribute to tolerogenic memory(Yadava et al., 2019). On restimulation, we generally only saw a doubling of a population but not the clonal expansion often seen with Th1 or cytotoxic T lymphocytes (CTL) responses to an antigen (Arakawa et al., 2019; Bhattacharyya & Feng, 2020; Cukalac et al., 2014). This might imply a tighter homeostatic regulation of Tr1 cells, keeping numbers (%) relatively low by reduced expansion, shorter half-life or increased activation induced cell death (AICD). Immunological memory has been well characterised for T_H1, T_H2, T_H17 and T_{fh} cells (Golubovskaya & Wu, 2016; Kim et al., 2012) as well for FoxP3$^+$ nTreg cells (Brincks et al., 2013; Sanchez et al., 2012) and this study demonstrated that Tr1-like cells maintained a memory pool in human peripheral blood. Whether these memory Tr1-like cells were long-term antigen-specific tolerogenic memory is yet to be determined. The life cycle of memory Tr1 cells would be a great area to explore in future work. However, the Tr1-like memory described in

this study displayed some characteristics like the well described Th memory, which makes a case for them to be long lived. Moreover, memory T cells are antigen-specific cells that are long-lived after an infection and are equipped to rapidly respond with enhanced functionality upon re-challenge by specific antigen (Garcia et al., 1999; Gasper et al., 2014; McKinstry et al., 2008; Yamane & Paul, 2012). This supported the idea that Tr1-like cells were differentiated population producing IL-10 in response to a specific antigen.

The search for an exclusive set of surface markers and a master regulator for Tr1 cells has been elusive since the discovery of Tr1 cells. Several markers and transcription factors have been associated with Tr1 cells from other studies. The Tr1-like cells described in this study expressed all the major Tr1-associated cell surface makers including CD49b, CD226, LAG-3, PD-1, and CTLA-4. They also expressed high levels of c-MAF and granzyme B. As with other phenotypic markers of immune cells they are not exclusive to any one phenotype and combinations of these markers serves to refine the definition of these cells. This has been done most recently for nTreg cells, which started off as FoxP3 positive cells and have progressed to be best defined as CD4 positive, CD25 (hi), CD127 (low) and CD45RA/RO (Fontenot et al., 2003; Schmetterer, Neunkirchner, & Pickl, 2012; Valmori, Merlo, Souleimanian, Hesdorffer, & Ayyoub, 2005). Tr1 cells have been characterised using co-expression of CD49b and LAG-3 in both human and murine studies (Gagliani et al., 2013), although other studies

demonstrated that co-expression of CD49b and LAG3 was not restricted to the Foxp3$^-$ Tr1 cells. Foxp3$^+$ Treg cells and CD8$^+$ T cells that produced IL-10 also co-expressed these markers(W. Huang et al., 2018). CD49b is also expressed by epithelial cells, endothelial cells, fibroblasts, platelets and specific subsets of leukocytes(Cenni et al., 2001). Therefore, CD49b did not represent an exclusive marker for Tr1 cells, however the fact that interactions between CD49b and extracellular matrix ligands mediated important biological processes like inflammation and immunity, explains why it was put forward as a marker for Tr1 cells. LAP which binds to TGF-β1 to form a latent complex(Ali et al., 2008) is intimately involved in growth, differentiation, and immune modulation of several immune cell types(Boswell et al., 2011; Tran et al., 2009). CD4$^+$ T cells expressing LAP are thought to play an important role in the modulation of immune responses(Ochi et al., 2006; Tran et al., 2009). Scurr et al., reported that tumour infiltrating lymphocytes (TILs) that were CD4$^+$LAP$^+$ and co-expressed LAG-3 and CD25 were more potent supressing effector T cells compared with conventional FoxP3$^+$ Tregs(Scurr et al., 2014b). LAP has been shown to independently modulate immune cell function as it is expressed on immature DCs, involved in T cell differentiation, and induces epithelial cell migration(Gandhi, Anderson, & Weiner, 2007; Lu et al., 2002). CD226 competes with TIGIT and CD96 for binding to the ligands CD155 and CD112, which are abundantly expressed in tumors (Bi, 2021; Z. Huang, Qi, Miller, & Zheng, 2020). It has been reported

that expression of CD226 and TIGIT on human $CD4^{+}Foxp3^{+}$ Treg cells are related to their stability and function(Lozano et al., 2012). It is also involved in the killing of myeloid APCs by human Tr1 cells (Magnani et al., 2011) and CD226 deficiency has been reported to disrupt homeostasis and attenuate the suppressive capacity of Tr1 cells(N. Wang et al., 2021). The inhibitory receptors, CTLA-4, PD-1 and LAG-3 have all been reportedly expressed by Tr1 cells as potential checkpoint inhibitors(Roncarolo et al., 2018). CTLA-4 functions in inhibiting T cells responses by competing with CD28 for ligands, CD80/CD86 (Hosseini et al., 2020; Mitsuiki et al., 2019) while PD-1 binds to its ligands PD-L1/PD-L2 to control the induction and maintenance of peripheral T cell tolerance(Sharpe et al., 2007). However, the roles of CTLA-4 and PD-1 in mediating immune tolerance, including antitumor responses, are largely distinct. While CTLA-4 is thought to regulate T cell proliferation early in an immune response, primarily in lymph nodes, PD-1 suppresses T cells later in an immune response, primarily in peripheral tissues(Buchbinder & Desai, 2016; Fife & Bluestone, 2008; Francisco, Sage, & Sharpe, 2010). LAG-3, like PD-1 and CTLA-4 is not expressed on naïve T cells; its expression is induced upon stimulation and the levels of LAG-3 expression on cell surface directly correlates its inhibitory capacity(Maeda et al., 2019). The expression of CTLA-4, PD-1 and LAG-3 by Tr1-like cells in this study is thought to confer immune-suppressive properties on these cells. However, the suppressive

capacity of Tr1-cells was not explored in this study. Suppression assay with an enriched population would help demonstrate this in future work.

c-MAF is reported to transactivate IL-10 promoter(Xu et al., 2009) and interacts with aryl hydrocarbon receptor (AhR) to promote the secretion of IL-10 and IL-21(Apetoh et al., 2010), and is involved in Tr1 differentiation (Barroso et al., 2021; Imbratta et al., 2020). The expression Foxp3 identifies Treg cells in mice (Hori et al., 2003) and in humans(W. Liu et al., 2006; Miyara et al., 2009) and is reportedly crucial for Treg cells stability and function. However, FoxP3 expression alone is not sufficient to establish a Treg phenotype as non-Treg cells also transiently express FoxP3(Sugimoto et al., 2006). This therefore meant that transient expression of Foxp3 by Tr1-like cells was not a prerequisite for suppressive functions(W. Lin et al., 2007; J. Wang et al., 2007). Initially thought to be expressed exclusively by Th1 cells, T Bet is now reportedly expressed by both innate and adaptive immune cells during type 1 immune response (Kallies & Good-Jacobson, 2017) as well as by regulatory T cells (Levine et al., 2017). RoRγ(t), the master regulator for Th17 cells is reportedly expressed by a population of regulatory T cells in the colon (Hegazy & Powrie, 2015). Recent studies reported that Tr1 cells prevented the activation of antigen specific T cells by killing myeloid APCs through the expression of CD226 and the secretion of granzyme B (S. Gregori et al., 2012; Magnani et al., 2011). Killing assays in future experiments would further demonstrate the cytotoxic potential of Tr1-like cells.

Our data showed that IL-10$^+$ Tr1-like cells expressed T-Bet and RoRγ(t), transcription factors reported as master regulators for Th1 and Th17 cells, respectively. As these assays were performed 24 hours after stimulation, representing a snapshot of events, further studies would confirm whether the pattern of expression of these transcription factors by Tr1-like cells remained the same or changed over time. Although the distribution of T-Bet and RoRγ(t) expression on Tr1-like and non-Tr1 cells was similar, it nonetheless raised the possibility of plasticity. The revelation of plasticity in T cell populations/function has provided a huge increase in our understanding of how an immune response adapts to changing conditions (Soukou, Huber, & Krebs, 2021). It also meant that defining the cells involved in these responses and the mechanisms that control them has become increasingly difficult. Reports have shown that several cell types including Th1 cells (Meiron et al., 2008), Th2 cells (Heinemann et al., 2014; Pacciani et al., 2010; Pellerin et al., 2018), Th17 cells (Heinemann et al., 2014), memory CD4$^+$ T cell (Yao et al., 2015) as well as naïve CD4$^+$ T cells (Bacchetta et al., 2010; Sutavani et al., 2013) could be reprogrammed to Tr1-like cells. Recent study showed that induction of IL-10 by IL-27 differentiated Tr1 cells is mediated by STAT3 and Early Growth Response 2 (Egr-2) in a Blimp1-dependent manner but not c-Maf/Ahr, suggesting that IL-10 expression by Tr1-like cells may be dependent on environmental queues and molecular context (Vasanthakumar & Kallies, 2013), which suggests that there may be a point in the lifetime of any cell population where

they start producing IL-10 (Couper et al., 2008). However, the short kinetics (24 hours) of peak IL-10 production observed in this study demonstrated that Tr1-like cells secreted IL-10 only in response to costimulation. Furthermore, more than twenty transcription factors have been shown to directly activate IL-10 production, and it is largely unknown how c-Maf and other transcription factors interact with each other in the regulation of IL-10 (Gabryšová, Howes, Saraiva, & O'Garra, 2014; S. S. Iyer & G. Cheng, 2012; Rutz & Ouyang, 2016; Saraiva & O'Garra, 2010). This adds another layer of complexity to the current search for the master regulator for IL-10 induction by Tr1 cells. However, the high expression of c-MAF following CD3/CD55 costimulation may have been responsible for the IL-10 production by Tr1-like cells in this study. It may also be that costimulation via CD55 preferentially activated the molecules responsible for IL-10 signalling in these cells.

CD55-CD97 interactions together with TCR engagement via CD3 resulted in the phosphorylation of the protein tyrosine kinases $p56^{lck}$ and $p59^{fyn}$, ZAP-70 and the activation of other downstream signalling molecules, leading to the production of effector cytokines, IL-10 (Shenoy-Scaria et al., 1992a). However, the signalling mechanisms that led from CD3/CD55 to IL-10 production and c-MAF involvement are not fully understood. Preliminary findings from this study suggested that the p38 MAPK-MK2 pathway was the dominant pathway regulating IL-10 production by Tr1-like cells following CD55 costimulation. Future studies

are required to determine any off-target effects by the kinase inhibitors and viability stains would highlight potential toxic effect of the drugs on cell by discriminating live and dead cells.

There is need for the enrichment and transcriptome analyses (RNA-Seq) of Tr1-like cells, as sequencing of early Tr1 cells differentiated from naive $CD4^+$ T cells might identify the initiating 'master regulator' and analyses of Tr1 cells from total $CD4^+$ T cells would help confirm such transcription factor and identify the profile of memory Tr1 cells. These could be compared to the Th1 or other Th cells and to CD28 costimulated cells to show or confirm differences. Such approach could accurately quantify gene expression levels, assemble new transcripts regarding master regulator (s) of Tr1 functions as well as signalling events that regulate IL-10 production. This might help explain the lack of other cytokines or identify other factors restricted to the Tr1 phenotype.

In conclusion therefore, the distribution of memory T cells in this study supported the idea that Tr1 cells were differentiated population of cells that produced IL-10. CD3/CD55 costimulation of naïve $CD4^+$ T cells revealed Tr1 differentiation into a small population of cells in human peripheral blood that can be restimulated to secrete IL-10. Multicolour staining for multiple markers would provide more information on unique markers co-expressed by Tr1-like cells.

However, findings from this study supported the redefining of Tr1 cells as $CD4^{+}$IL-10^{hi}IFN-γ^{-}, IL-10^{hi}IL-4^{-}, IL-$10^{hi}$$CD226^{hi}$, IL-$10^{hi}$$PD\text{-}1^{hi}$, IL-$10^{hi}$$LAG\text{-}3^{hi}$, IL-$10^{hi}$c-$MAF^{hi}$, and IL-$10^{hi}$Granz-$B^{hi}$.

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