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Modulation of IL-10 production by CD3/CD55 induced Type 1 Regulatory (Tr1) T-cells

By Tajkia Musarrat, M.Sc.

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University of Nottingham
Faculty of Medicine and Health Sciences
School of Medicine
Academic Unit of Clinical Oncology

To Abu Rushd Husain (my father)
and
Latifa Chowdhury (my mother)

Abstract: A healthy immune system is maintained in a state of balance between pro-inflammatory and anti-inflammatory cells. The paradigm for T-cell activation requires CD80/86:CD28 engagement resulting in differentiation of CD4$^+$ T-helper cells. However, alternative costimulatory molecules may favour the induction of alternate T cell phenotypes such as Type 1 Regulatory (Tr1) T-cells. One such receptor-ligand pair is CD55-CD97. We have previously demonstrated that co-stimulation by CD3/CD55 results in the differentiation of naive CD4$^+$ T-cells into Tr1 phenotype, defined as IL-10$^+$, IFN-γ^- and IL-4$^-$. IL-10 is the predominant immune-suppressive cytokine produced by adaptive immune system and it is required for immune resolution, promoting tolerance and controlling autoimmunity. Considering the importance of IL-10 production in auto-immune diseases, we aimed to study the CD3/CD55 mediated IL-10 production in Multiple sclerosis (MS) patients. In our pilot study, CD3/CD55 stimulation of naïve CD4$^+$ T-cells resulted in significantly lower level IL-10 production as well as lower number of IL-10$^+$ Tr1 cells in MS patients compared to heathy donors. We further investigated the effect of MS associated immune-modulators on the CD3/CD55 mediated IL-10 production. Vitamin-D3 and Dexamethasone preferentially enhanced IL-10 secretion and increased the number of Tr1 cells following CD3/CD55 stimulation whereas IFN-β demonstrated similar effect with both CD55 and CD28 costimulation.

To validate the phenotype of these Tr1 cells, we characterised the CD3/CD55 induced Tr1 cells in terms of cell surface molecules and

transcription factors. CD3/CD55 induced IL-10$^+$IFN-γ^- Tr1 cells were LAG-3^{High}, TIM-3^{High}, CTLA-4^{High} and PD-1^{High}. These cells also expressed T-bet and c-MAF but did not express FoxP3, GATA-3 and HELIOS. The presence of immune-modulators that are used in MS treatment did not alter the transcription factor profile of the Tr1 cells. Importantly, c-MAF was only induced in IL-10$^+$ Tr1 cells in response to CD3/CD55 but not to CD3/CD28 stimulation. c-MAF expression was persistent upon restimulation with CD3/CD55 and it was not induced by non-specific stimulation with PMA/Ionomycin, indicating that c-MAF induction could be an integral part of signalling for CD3/CD55 mediated IL-10 production.

Thus, our study demonstrates for the first time that CD3/CD55 induced Tr1 cells are best defined as IL-10$^+$IFN-γ^-LAG-3^{High}PD-1^{High}c-MAF^{High}. These cells express c-MAF which is induced by CD55 costimulation. Furthermore, the presence of immune-modulators has a significant effect on the induction of Tr1 cells and may provide another mechanism to modulate Tr1 cells in MS patients.

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Abbreviation

APC	Antigen presenting cell
AhR	Aryl hydrocarbon receptor
BCL-6	B cell lymphoma 6
BSA	Bovine serum albumin
CD	Cluster of differentiation
CD55	Decay accelerating factor (DAF)
CD97-Fc	CD97 Fc fusion protein
Cells/mL	Cells per millilitre
CTV	Cell Trace Violet
CO_2	Carbon dioxide
CPM	Counts per minute
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte-associated protein 4
DC	Dendritic cell
EAE	Experimental autoimmune encephalomyelitis
ELISA	Enzyme linked immunosorbent assay
Foxp3	Forkhead box P3
GPI	Glycosylphosphatidylinositol
GvHD	Graft – versus – host disease
ICAM-1	Intercellular adhesion molecule – 1
IL	Interleukin
LAG-3	Lymphocyte activation gene – 3
LPS	Lipopolysaccharide
MFI	Median fluorescence intensity
MHC	Major histocompatibility complex
MS	Multiple Sclerosis
nTreg	Naturally occurring regulatory T cells
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
TCR	T cell receptor
TGF-β	Transforming growth factor beta
IU / ml	International Units per millilitre
μg / ml	Micrograms per millilitre
μm	Micrometre

1 Chapter 1: Introduction

1.1 Immune system

The immune system is defined as the complex and organized network consisting of cells, molecules, organs and processes – each attributed with specialized function, which ensures host defence against pathogens and controls development of any diseases [1, 2]. The immune system is comprised of two branches -I) Innate and II) Adaptive immunity, which responds in fundamentally different way to pathogens but cooperate with each other to mediate efficient elimination of the infection.

Innate immunity is the first line of defence against infection and cellular damage. This provides non-specific responses against pathogens and it does not alter or amplify over time upon repeated exposure to the same infection. Innate cellular responses are mediated by phagocytic cells (macrophage, monocyte, neutrophils), inflammation mediators (eosinophils, mast cells, basophils) and natural killer cells. Innate molecular mediators include the complement system, acute phase proteins and cytokines [2]. This immune response does not require prior encounter to particular pathogen and its components are consistently present facilitating disease-resistance mechanisms even before the onset of disease. This immune response is considered "Inbred" and has been coined "innate" [1, 3].

Adaptive immunity is the acquired ability to eliminate infection by proliferation, differentiation and coordinated function of antigen-specific

B-cells and T-cells that demonstrate unique specificity and memory in order to generate faster response upon reencounter to the same pathogen [1, 3]. This process entails recognition of antigen following processing and presentation by specialized antigen presenting cells. B-cells produce antibody and regulate the humoral response against the antigen. T-cells mediate cytotoxicity to eliminate virus infected cells, assist B-cell differentiation and maturation and support the activity of macrophages in eliminating intracellular pathogens [2].

1.2 Immune homeostasis and immune tolerance

Homeostasis was defined by Walter B. Cannon [4] as the maintenance of the steady state of physiological variables within a pre-defined range by established regulatory feedback mechanism in biological organism. In line with this definition, immunological homeostasis is described as the maintenance of the immune system in stable state where appropriate immune response is permitted while controlling both pro-inflammatory and anti-inflammatory to certain extent in order to prevent disruption of immune balance. Immune homeostasis is required to selectively eliminate immune responses against self-antigen while supporting sensitive mechanisms to detect and generate effective immune response to non-self pathogens. The tightly regulated and acquired unresponsiveness against self is denoted as immune tolerance. Tolerance

to self and reactivity against pathogens are situated on the opposite sides of the spectrum in the range of immune response. Compromised immune tolerance results in a chronic inflammatory response and auto-immune diseases. However, tolerance to self may also facilitate escape of anomalous self, such as mutated cancer antigens. On the other hand, reduced reactivity leads to persistent infection and immune-deficiency.
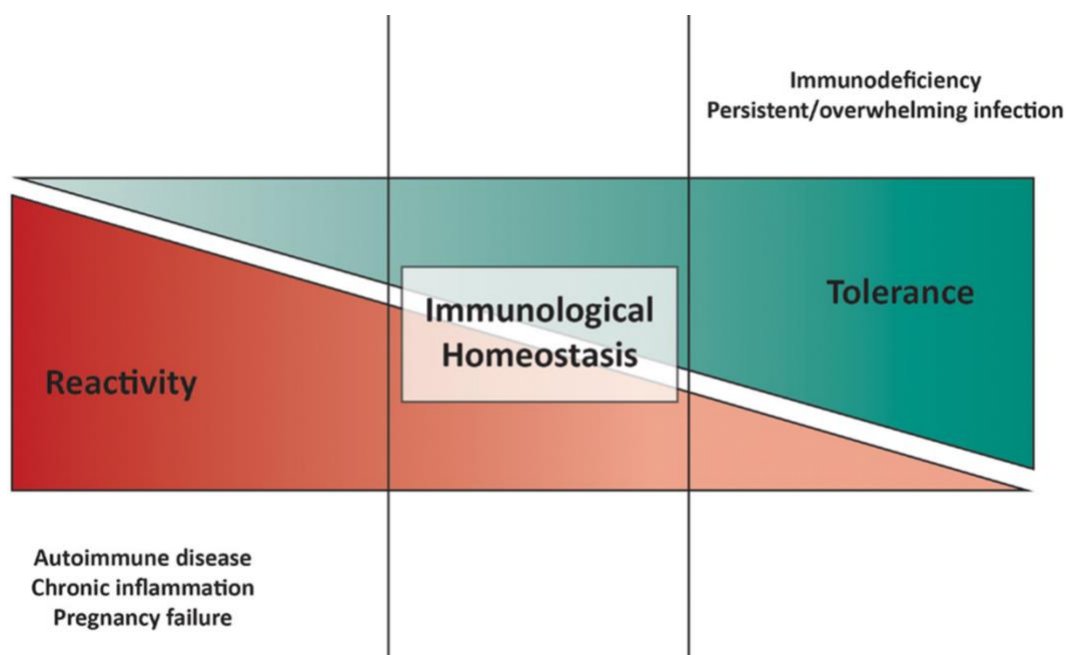
Figure 1.1: Model of immunological homeostasis. Dysregulated and excessive proinflammatory and anti-inflammatory immune response could lead to chronic inflammation and tolerance respectively. Autoimmunity and chronic inflammation prevail in absence of regulatory mechanisms whereas immunodeficiency and persistent infection manifest in absence of proinflammatory immune response. (Adapted from Povoleri, *Frontiers in immunology,*2013 [5])

Regulation of immune responses are salient for maintaining immune homeostasis. Immuno-suppressive regulatory T-cells such as Type 1 regulatory T-cells (Tr1) are important mediator of immune homeostasis as these cells regulate the pro-inflammatory immune responses and promote resolution at the end of immune response. Development and persistence of auto-immune disease has been attributed to impaired regulatory T-cell function [6] whereas prevalence of regulatory T-cells hinders anti-tumour immunity and promote progression of the disease [7].

1.3 T-cell

Thymus-derived lymphocytes (T-cells) were discovered in 1960s by Jacques Miller and Graham Mitchell as the cell population which could proliferate in response to antigen and even though they did not produce antibody, they enabled bone-marrow derived B-cells to develop into antibody forming cells [8, 9]. T-cells have been studied extensively in the last few decades leading to identification of multiple T-cell subsets with distinct functions.

1.3.1   Ontogeny of T-cells

T-cells originate from bone-marrow derived multipotent Haemopoietic Stem Cells (HSC) through a stringently regulated process [10]. The earliest T-cell specific precursor derived from HSC is known as the Early Thymic Progenitor or Early T-cell Precursor (ETP). This represents a small cell population in the thymus where the TCR genes were maintained in the germline state but differed in HSC as they expressed low level of CD-4 which was down-regulated in subsequent developmental stages [11]. Recent fate-mapping studies indicate that potentially there could be intermediate state between HSC and ETP, termed Thymic Seeding Progenitor (TSP) which migrate from bone marrow to thymus and serve as a precursor for ETP. Interestingly, ETP has the potential to differentiate into only T-cells in thymus but could give rise to both B-cells and T-cells if transferred intravenously into recipient mice [12]. However, further studies supported the observation that ETPs do not retain the potential to develop into myeloid lymphocytes under physiological condition [13-17]. With each gradual step in the generation of functional

T-cells from HSC, the precursor potential to develop into multiple progenitor becomes restricted. This is due to intra-thymic environment and establishes ETP as the earliest precursor committed to both $\alpha\beta$ and $\gamma\delta$ T-cell generation [12, 15, 18].

ETP progresses to Double Negative cells (DN, CD4$^-$ CD8$^-$) which can be further divided into multiple subgroups of progenitor cells. The DN subsets are determined on the basis of sequential expression CD44 and CD25 where CD44$^+$CD25$^-$, CD44$^+$CD25$^+$, CD44$^-$CD25$^+$ and CD44$^-$CD25$^-$ are termed as DN1, DN2, DN3 and DN4 respectively (Table 1). At the DN1 stage, Notch1 signalling ensures the lineage commitment to T-cell generation by preventing conversion to dendritic cells [19, 20]. The interaction between Notch1 receptor on DN1 with its ligand Delta-like 4 (DLL4) on Thymic Epithelial Cells (TEC) in the corticomedullary junction of the thymus [21, 22] serves as one of the checkpoints in T-cell development from ETP.

DN1 cells differentiate to DN2 cells following migration deeper into the cortex, where the cells prepare for the TCR gene rearrangement, mediated by RAG1 and RAG2 in DN3 cells [23, 24]. The extensive gene rearrangement in the β, γ and δ loci to determines the fate of the progenitor cells, emerging as either $\alpha\beta$, following β-selection or $\gamma\delta$ T-cells occur in the DN3 stage [25]. Signalling through the pre-TCR and Notch1 pathways are essential to progress from DN3 and DN4 to the Double Positive state (DP) [26]. Here the gene rearrangement in the α-loci leads to production of correctly assembled $\alpha\beta$ TCR [27].

Table 1.1: Cell surface markers during T-cell development. Stages of T cell development correlate with specific locations in the thymus, distinct cell-surface phenotypes, requirements for Notch signals, and TCR rearrangement. (Adapted from Koch, *Annual Review of Cell and Developmental Biology*, 2011 [16])

Table 1 Stages of T cell development correlate with specific locations in the thymus, distinct cell-surface phenotypes, requirements for Notch signals, and TCR rearrangement

Developmental stage	Cell surface phenotype	Location	Notch signal	TCRβ rearrangement	TCRα rearrangement
ETP DN1	$CD117^{hi}CD44^{hi}CD25^-$ $CD24^{-/lo}CD27^{hi}$	CMJ	+++	Germline	Germline
DN2a	$CD117^{hi}CD44^{hi}CD25^+$ $CD24^{hi}CD27^{hi}$	Cortex	+	Germline	Germline
DN2b	$CD117^{int}CD44^{hi}CD25^+$ $CD24^{hi}CD27^{int}$	Cortex	+	DJ_H	Germline
DN3a	$CD117^{-/lo}CD44^{-/lo}CD25^+$ $CD24^{hi}CD27^{-/lo}$	SCZ	+++	DJ_H, VDJ_H	Germline
DN3b	$CD117^{-/lo}CD44^{-/lo}CD25^{int}$ $CD24^{hi}CD27^{hi}$	SCZ	+++	VDJ^+	Germline
DN4	$CD117^{-/lo}CD44^{-/lo}CD25^{-/lo}$ $CD24^{hi}CD27^{hi}$	SCZ	+	VDJ^+	Germline
DP	$CD4^+CD8^+TCR\beta^{int}$	Cortex	–	VDJ^+	VJ

Abbreviations: CMJ, corticomedullary junction; D, diverse; DN, double negative; DP, double positive; ETP, early thymic progenitor; H, heavy chain; J, joining; SCZ, subcapsular zone; TCR, T cell receptor; V, variable. The single + for Notch signal indicates Notch1 receptor expression; however, a specific function or requirement has not been described. The triple +++ for Notch signal indicates a requirement for Notch signaling during T cell development.

The binding of both CD4 and CD8 on cells that bind to the invariant region of MHC class-II and MHC class-I respectively, ensures that useful MHC-restricted TCR expressing cells are rescued from death. This occurs as the co-receptors bring Lck into physical proximity with cytosolic domains of the engaged TCR to initiate signalling that potentially assists with RAG gene repression, long-term survival, migration into the medulla, and differentiation into mature T cells in a process called positive selection [28-30]. Cortical Thymic Epithelial Cells (cTECs) play critical role at the stage of positive selection as they express thymoproteasomes which are involved in generating unique peptide sequences for CD8 T-cell selection. cTECs also contribute to shaping the T-cell repertoire by expressing lysosomal proteases, cathepsin L and Prss16 (also known as 'thymus-specific serine protease'; Tssp) which are

essential for producing self-peptide required for the positive selection of CD4 T-cells. In the last stage- the negative selection process , the positively selected CCR7 receptor expressing $CD4^+$ or $CD8^+$ T-cells migrate to medulla in a CCR7 ligand - CCL19 and CCL21, dependent manner. The chemokines are highly expressed by Medullary Thymic Epithelial cells (mTECs) which are further divided into $mTEC^{hi}$ and $mTEC^{low}$ on the basis of MHC class- II and CD83 expression [31]. mTECs express various Tissue Restricted Antigen (TRA) which facilitates the elimination of T-cells specific for antigens usually expressed in the peripheral tissue. The medullary dendritic cells also contribute to the negative selection process as some of the mTEC derived TRAs are transferred to these cells. The expression of various TRA by mTEC is modulated by transcriptional regulator AIRE which is expressed by 30% $mTEC^{Hi}$ cells and a subset of thymic B-cells. Patients with mutation in AIRE develop disease called autoimmune polyendocrinopathy- candidiasis- ectodermal dystrophy (APECED) and demonstrates that lack of appropriate AIRE function could lead to abnormal T-cell selection of thymus which results in escape of self-antigen specific T-cells and disruption of T-cell mediated B-cell tolerance. However, TRA expression for the negative selection also depends on other transcription factor as it has been estimated that only ~40% of TRA expression is regulated by AIRE. Another key transcription factor Fezf2 (Fez family zinc finger 2, also called forebrain embryonic zinc finger-like protein 2) plays a critical role in TRA expression by mTEC in a manner independent of AIRE as it was reported that the deficiency of Fezf2 leads to manifestation of auto-immune disease with characteristics different from those noted in AIRE-

deficient mice [32]. These observations implicate that AIRE and Fezf2 regulate distinct sets of TRA expression in mTECs and co-ordinate to provide coverage for a wide range of TRA to eliminate self-reactive T-cells during negative selection process.

Cortex
cTEC
CD4$^+$CD8$^+$ $\alpha\beta$T cell   Positive selection

CD4$^+$ $\alpha\beta$T cell or CD8$^+$ $\alpha\beta$T cell

Medulla
TRA transfer
mTEC
Aire → TRA
Fezf2 → TRA
TRA-peptide   DC
MHC
TCR

Negative selection

Conventional T cell   Apoptosis
Regulatory T cell
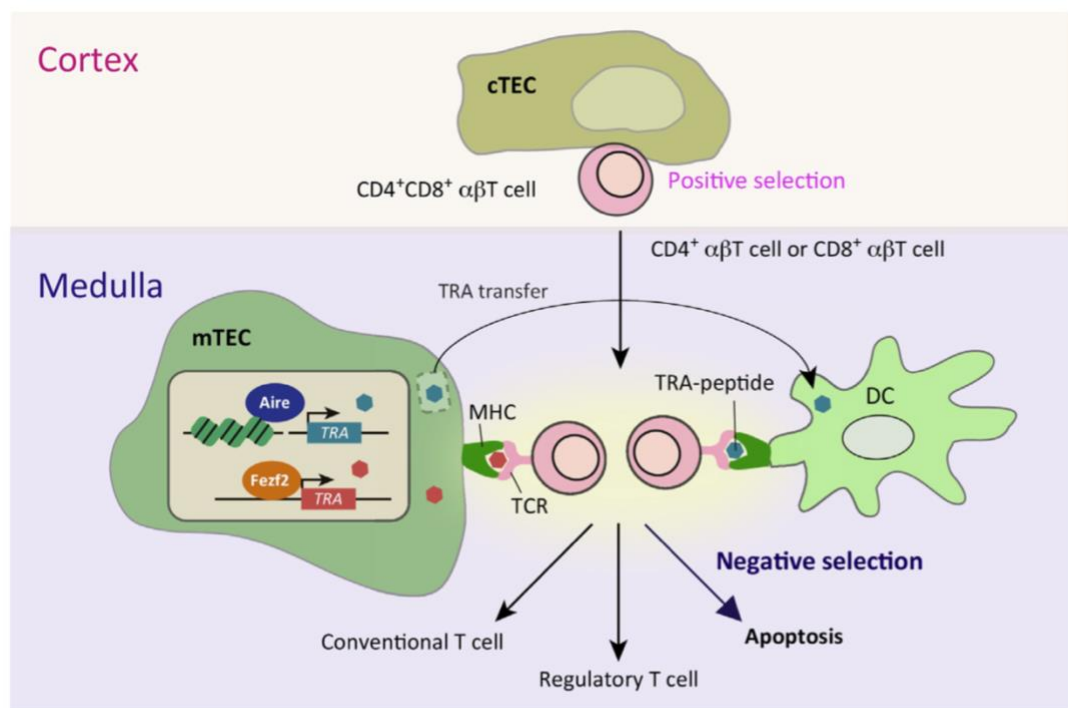
Trends in Immunology

Figure 1.2: Schematic Diagram of T Cell Selection and TRA Expression in the Thymus. Bone marrow derived early thymic progenitor (ETP) cells differentiate into $\alpha\beta$ TCR expressing CD4$^+$CD8$^+$ T-cells and interact with cortical thymic epithelial cells (cTEC) in context of peptide-MHC complex for positive selection. In the following stage, T-cells migrate to the medulla where medullary thymic epithelial cells (mTEC) present a wide range of tissue restricted antigen (TRA) to the T-

cells to eliminate the self-antigen specific T-cells by apoptosis in a process termed negative selection. mTEC derived TRAs could be transferred to thymic dendritic cells which further contribute to negative selection process. Different transcription regulators such as AIRE and Fezf2 mediate TRA expression by mTEC which determines the T-cells repertoire. (Adapted from Takaba, Trends in immunology, 2017 [33])

The binding of both CD4 and CD8 on cells that bind to the invariant region of MHC class-II and MHC class-I respectively, ensures that useful MHC-restricted TCR expressing cells are rescued from death. This occurs as the co-receptors bring Lck into physical proximity with cytosolic domains of the engaged TCR to initiate signalling that potentially assists with RAG gene repression, long-term survival, migration into the medulla, and differentiation into mature T cells in a process called positive selection [28-30]. In the last stage, cells go through negative selection process which results in the deletion of self-reactive T-cells before the single positive CD4$^+$ and CD8$^+$ T-cells emerge from the thymus and circulate in the periphery.
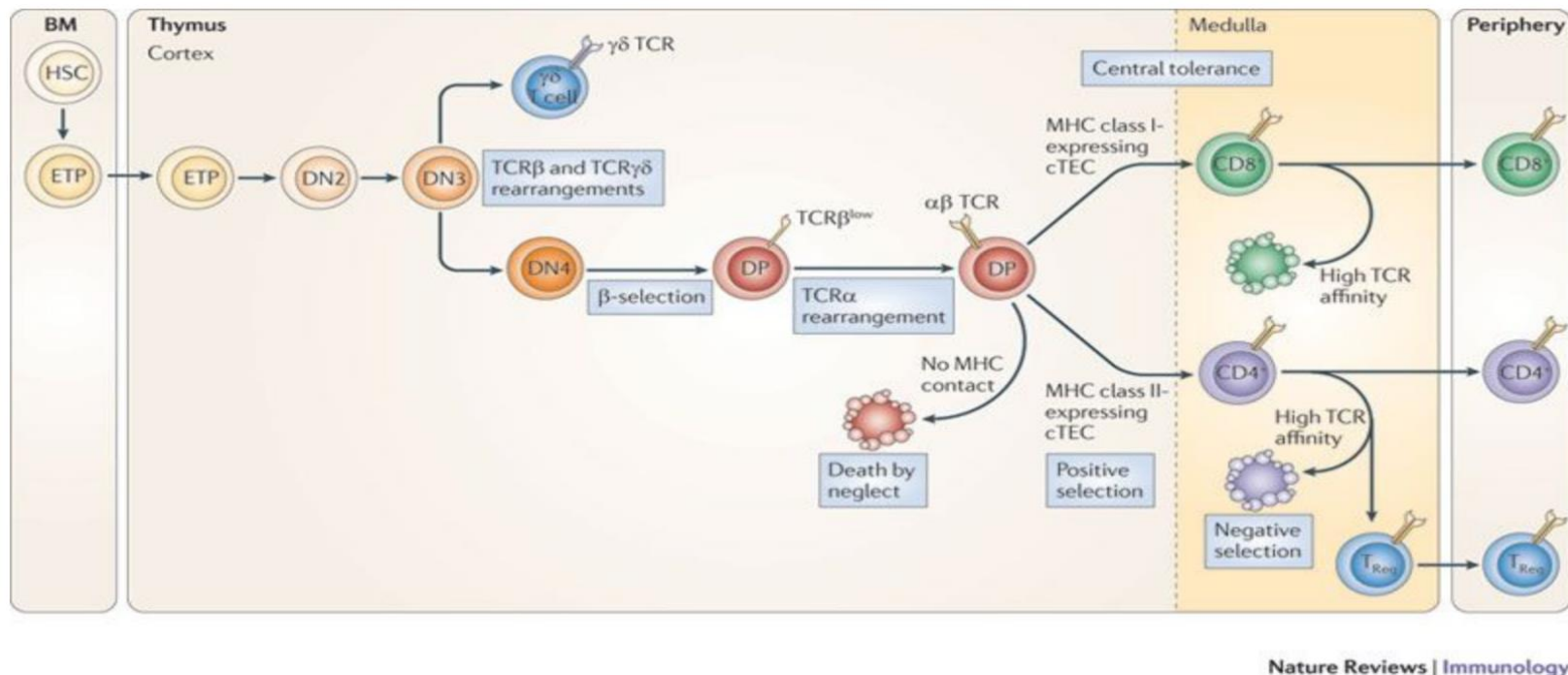
Figure 1.3: Development of T-cells in thymus. In the bone marrow, haematopoietic stem cells develop into early thymic progenitor (ETP) with some potential to   differentiate to not only T-cell but also B-cell and dendritic cells. ETP cells migrate to thymus and lose their potential to alternate fates by giving rise to double negative cells DN2 which do not express either T-cell receptor (TCR) or CD4 and CD8. In the following stages, crucial TCR gene rearrangement occurs which results in Double Positive (DP) cells. Then cells are selected on the basis of their ability to bind to the MHC molecules in a process term Positive selection and inability to contact MHC leads to death by neglect. If the avidity of the single positive (SP) cells for binding to MHC-peptide complex is higher than certain threshold, the cells are deleted in Negative selection process. However, a small fraction of high affinity TCR-bearing self-reactive cells develop into natural regulatory T-cells. The remaining cells egress as conventional T-cells in their naïve state. (Adapted from Miller, Nature Reviews Immunology, 2011 [10]

1.3.2   Different T-cells subsets depending on the activation state

Quiescent naïve T-cells migrate to the periphery and continuously recirculate between secondary lymphoid organs and blood via the lymphatic system until they encounter cognate antigen. Under the appropriate circumstances, with the help of costimulatory signals and supporting cytokine milieu, naïve T-cell become activated which results in proliferation and differentiation into various kind of effector and memory cells. T-cells, historically, were believed to terminally differentiate into different effector T-cell subsets. It is useful to maintain this idea of differentiated lineages as a starting point, although our ideas of the fixed nature of these has changed significantly in the past 10 years.

1.3.2.1   Naïve T-cells

Thymus derived antigen-inexperienced and inactive cells are usually considered naïve T-cells. Until recently, these were perceived to be part of a developmentally synchronized and homogeneous T-cell population. However, several studied have demonstrated heterogeneity, as they differ on the basis of phenotype, dynamics, location and function [34]. Human naïve T-cells have a considerably longer lifespan of 6-9 years compared to (6-11 weeks) in mice, where thymic output exclusively sustains the naïve T-cell pool throughout their life without any peripheral proliferation [35]. The human naïve T-cell pool is maintained by homeostatic peripheral proliferation of thymic emigrant T cells. This

maintains naïve T cell numbers later in life due to reduced thymic output and atrophy of thymus with age [35, 36]. Naïve Human T-cells can be defined by combinations of different markers. However, this usually includes; $CD45RA^+CD45RO^-CD62L^+CCR7^+$. Expression of homing receptor such CD62L and CCR7 assist the trafficking of naïve T-cells to secondary lymphoid organs after exiting the thymus. They also express intermediate level of CD28 and CD27 costimulatory markers [37, 38]. Human CD4 and CD8 naïve T-cells can also be divided into distinct subpopulations based on expression of CD31 and CD103 respectively [34].

1.3.2.2   Effector T-cells

Naïve cells can differentiate to effector cells, following antigen recognition and obtain the effector phenotype defined as $CD45RA^{+/-}CD45RO^+CCR7^-$ $CD62L^-$. These effector T-cells upregulate several activation marker such as CD69, CD71, CD25 and HLA-DR. Effector cells might also dynamically express proliferation markers, such as Ki67 [39]. Effector cells expand rapidly during immune response and at the end of the immune response, ~90% of effectors die during the contraction phase [40].

1.3.2.3   Central Memory T-cells (T_CM cells)

After activation, some effector T-cells develop into Effector memory or Central memory T-cells which are capable to responding to secondary antigenic challenge. Central memory (T_{CM}) are identified by expression of $CD45RA^-$ $CD45RO^+CCR7^+CD62L^+CD27^+$ $CD28^+CD127^+$. The re-expression of homing receptor provides them with potential to relocate to secondary

lymphoid organs. It has been suggested that T_{CM} might serve as a precursor for effector memory T-cells (T_{EM}) as T_{CM} have longer telomere and T_{CM} are able to generate T_{EM} *in vitro* [41, 42].

1.3.2.4   Effector memory T-cells

Effector T cells can also develop into effector memory (T_{EM}) T-cells which are defined as CD45RA$^-$CD45RO$^+$CCR7$^-$CD62L$^-$CD27$^{+/-}$CD28$^{+/-}$ CD127$^+$. The lack of homing receptor CCR7 and CD62L restricts these cells to the peripheral non-lymphoid tissue [43]. T_{EM} cells also express chemokine receptors such as CXCR3, CCR4 which allows them to be retained in the inflamed tissue [41, 44, 45] and have a more rapid response following antigenic restimulation [46].

Table 1.2: The differential cell surface markers expression associated with the sub-population of T-cells. (Adapted from Larbi, *Cytometry,*2013 [47]

	CD4+CD8+	CD4+	CD4+	CD8+	CD4+	CD8+	CD4+CD8+	CD4+	CD8+
CCR7		+	+	+	−	−	−	+	+
CD45RA	+	+	−	−	−	−	−	+	+
CD28	++	++	++++	+++	+	±	−	−	−
CD27	++	++	+++	++++	±	+	−	−	−
CD57	−	−	−	−	±	±	+	++	++
CD45R0	−	−	+	+	+	+	+	±	±
CD31	+	−	−	−	−	−	−	−	−
PD1	−	−	−	−	±	++	++	±	±
KLRG1	−	−	−	−	−	±	+	++	++
CD127	+	+	++	++	+	+	+	+	+

The expression of surface markers associated with differentiation is presented for CD4+ and CD8+ T cells. The sub-populations were categorized based on differential expression of the markers (arbitrary definition) but also for how this is related to the current consensus (classical definition). N: naïve; M: memory; -: no/very low expression; +: unit of expression. Expression levels of the markers have been compared between sub-populations (rather than within the same sub-population).

[N: naïve; M: memory; -: no/very low expression; +: unit of expression. Expression levels of the markers have been compared between sub-populations; EM: effector memory, CM: Central memory, TEMRA: Terminal Effector Memory RA$^+$]

1.3.2.5 Terminally differentiated effector memory cells re-expressing CD45RA (TEMRA) T-cells

In the recent years, another subset of activated T-cells has been identified which is characterised by the apparent re expression of the naïve marker CD45RA$^+$. They are CD45RA$^+$CD45RO$^{+/-}$CCR7$^-$CD27$^-$CD28$^-$PD-1$^+$KLRG1$^+$. TEMRA cells demonstrate relatively lower effector function and it has been suggested that TEMRA cells might derive from T$_{CM}$ cells in absence of antigen and in the presence of low Interleukin-2 and high interferon gamma secretion [48]. Although CD8$^+$ TEMRA cells have been studied more, CD4$^+$ TEMRA cells have also been identified in peripheral blood. These cells have been associated with protective immunity against Dengue virus and Cytomegalovirus (CMV) infection [49-52]. TEMRA cells can be further divided into two subset depending on the expression of CD57 along with GPR56$^+$ [53].

1.4 Different subsets of T-cells

1.4.1   CD4+ T-cells

T-cells were initially identified as supporting cells which were necessary to develop antibody responses by B-cells [54-58]. Later the it was discovered that a specific subset of T-cells, termed CD4 T-cells, produced soluble factor such as IL-4 and provide costimulation by CD40-CD40L which promoted antibody production against foreign antigens by B-cells [59-63]. These CD4 helper T-cells bind to the antigen in context of MHC class-II on antigen presenting cells [64, 65]. B cells, when acting as antigen presenting cells promote the activation of helper cells that in response produce cytokines that support B cell development. During activation of CD8 T cells by Dendritic cells the CD4 cells promote the licencing of DCs and support the development of memory CD8 responses [66-70]. In some animal studies, even in the absence of CD4 T-cells, primary CD8 T-cell responses were observed against viruses, but they failed to mount effective response upon secondary challenge [70-73]. CD4 T-cells constitutes a major part of PBMCs (25-30%) [74] and CD4 T-cell population consists of several distinct subpopulations. The characteristics and the role of various CD4 T-cell subsets are further discussed in 1.5.

1.4.2   CD8+ T-cells

The cytotoxic activity of thymus derived lymphocytes was noted in several early studies focusing on allogeneic, MHC-mismatched tissue,

tumour transplantation models and allogeneic mixed lymphocyte cultures [75-77]. However, it was the depletion of Ly-2 (CD8a) and Ly-3 (CD8b) bearing lymphocytes [78-80] as well as the studies with virus infected animal models which confirmed that specific T-cell receptor bearing CD8 T-cells recognize fragments of antigen in context of MHC class-I and mediated the killing of the target cells [76, 81-83]. Naïve CD8 T-cells (or CTLs) interact with antigen presenting DCs in the lymph node [84-86] and differentiate into short-lived effector cells (SLEC), which die after the infection is eliminated. Some become memory precursor effector cells (MPEC) which contribute to secondary immune response upon encounter with the same antigen [87-89]. CD8 T-cells not only develop robust immune response against intracellular pathogens [90, 91] but they are also capable of responding to exogenous antigenic targets by cross-presentation [92-95].

CD8 T-cells confer their cytotoxicity by two direct cell-cell contact dependent mechanism – i) interaction between Fas-Fas ligand , ii) granzyme and perforin mediated cytolysis; and indirectly affect target cells by producing pro-inflammatory cytokines including IFN-γ, TNF-α [96]. Fas-Fas ligand interaction between target cell and CD8 T-cells results in classical apoptosis of the target cell [97]. Granzyme and perforin mediated cytotoxicity to eliminate target cell without killing the bystander cells by caspase-dependent and -independent manner [98, 99]. It has also been reported that CD8 T-cells can kill 2-16 virus infected cells per day [100] and cooperate to increase this rate, while

demonstrating dual polarity in their ability to kill multiple targets simultaneously [100-102].

CD8 T-cells play important role in cancer and auto-immune diseases [103-106]. CD8 T-cells specific for neo-antigens expressed by tumour cells are critical for anti-tumour immunity. However, chronic antigenic exposure might impair activity rendering them ineffective in providing anti-tumour immunity [107-111]. On the other hand, self-antigen specific CD8 T-cells are also associated with the auto-immune disease initiation in diabetes [112, 113] and early-stage Multiple sclerosis (MS) [114, 115]. It was demonstrated that Myelin basic protein specific auto-reactive CD8 induce MS [116]. Depleting CD8 T-cells ameliorated the severity of disease by reducing the number of lesions and relapses [115, 117]. An altered gene prolife of CD8 T-cells were also found to correlate with poor prognosis in systemic lupus erythematosus (SLE) patients [118]. These findings suggest a multifaceted role of CD8 T-cells in immune system.

1.4.3 $\gamma\delta$ T-cells

$\gamma\delta$ T-cells represent an unconventional T-cells subset which constitute 1-5% of total population [119]. TCR of $\gamma\delta$ T-cells are composed of γ and δ TCR chain instead of α and β TCR chain like most of the CD3$^+$ T-cells. $\gamma\delta$ T-cells emerge earlier than $\alpha\beta$ T-cells in the thymus during T-cell development [120, 121]. $\gamma\delta$ T-cells distributed throughout the lymphoid

system and they are enriched as part of interepithelial lymphocytes (IEL) which are located within the epithelial layer of mucosal and barrier tissues [122-124].

$\gamma\delta$ T-cells recognizing antigens such as phosphoantigens, i.e., phosphorylated microbial non-peptide molecules that are metabolic intermediates of the isoprenoid biosynthesis [125-128]. Vδ1$^+$ $\gamma\delta$ T-cells also recognize glycolipid antigens as well as sulfatides in complex with CD1d [129, 130] that accumulate in lesions in MS patients [130]. Interestingly, $\gamma\delta$ T-cells were also termed the "innate adapter of immune system" [131] as they coordinate with neutrophil by releasing chemokines such as CXCL8 [132, 133], MCP-2 [134] and promote the differentiation into antigen presenting cells which in turn activate conventional CD4 and CD8 T-cells [133, 135]. They also secrete |IFN-g and IL-17 to provide a powerful proinflammatory environment thought to be important in the development of autoimmune disease [139-142]

1.4.4   Natural Killer T-cells (NKT cells)

Natural Killer T-cells (NKT) cells (only ~0.5% of total T-cell population) were first identified in 1987 as a distinct CD4$^-$CD8$^-$ (Double negative, DN) T-cell population which expressed intermediate level of $\alpha\beta$ TCR as well as NK cell marker NK1.1 [136-142]. NKT cells are defined by expression of CD1d-restricted αβ TCR, NK1.1 and recognition of hydrophobic ligand including various lipids and glycolipids (e.g. α-Galactosylceramide, glycosylphosphatidylinositol etc.) [143-147]. The TCR of NKT cells

predominantly express an invariant α-chain consisting of Vα14 Jα281 (now known as Jα18) [148, 149] required for the development of NKT cells [149].

NKT cells population consists of a heterogeneous population of cells with varied functions. CD4$^+$ NKT cells produce both Th1 (IFN-γ, TNF-α , IL-17) and Th2 (IL-4, IL-13) cytokines while the CD4$^-$, NKG2D$^+$ NKT cells secrete only Th1 cytokines upon activation [150-152]. It was also reported that CD8α^+ NKT cells produce higher amount of IFN-γ and have more cytotoxic potential in comparison to CD4$^+$ or CD4$^-$CD8$^-$ subsets. Moreover, all NKT subsets upregulate CD40L upon activation with antigen and promote IL-12 as well as IL-10 production by dendritic cells [152].

1.4.5   Mucosal associated invariant T-cells (MAIT)

Mucosal associated invariant T-cells (MAIT) are non-classical innate-like T-cell population which are restricted by highly conserved MHC class-I related molecule MR1 and is abundant in intestinal lamina propria (LP) of humans and mice [153, 154]. They are CD4$^-$CD8$^-$ (double negative, DN) T-cells with invariant α-chain - Vα7.2-Jα33/20/12 in humans and Vα19-Jα33 in mice, in conjunction with oligoclonal TCRβ chain [153, 155-157]. Further research revealed the existence of CD4$^+$ and CD8$^+$ MAIT cells [154, 158]. The selection and development of MAIT cells require B-cells as B-cells deficiency results in absence of MAIT [156, 159]. In human,

most of the MAIT cells are dependent on the transcription factor Promyelocytic Leukaemia Zinc Finger (PLZF) for development [154].

MAIT cells recognize various microbial Vitamin B2 metabolites (Riboflavin) and Vitamin B9 (folic acid) [160-166]. However, MAIT can also be activated in TCR-independent way by IL-18 in synergy with IL-12, IL-15 and/or interferon-α/β [164, 167]. Following activation, MAIT cells produce high level of IL-17 [168-170] and IFN-γ, IL-4, IL-10, GM-CSF etc [164]. MAIT produce granzyme-B (GzB) and perforin upon bacterial antigen recognition and mediate the cytotoxic killing of the infected cells [171] and have been associated with brain lesions in MS patients [172, 173].

1.4.6 Natural regulatory T-cells (nT$_{reg}$)

Thymus derived natural regulatory T-cells (nTreg), defined as $CD4^+CD25^{High}CD127^{Low}FoxP3^+$, are important regulator of immune responses and they are essential to maintain tolerance and prevent autoimmunity [174-176]. CD4 nTregs have been studied extensively in the last few decades and only recently several studies have also reported the important role $CD8^+CD25^+FoxP3^+$ nTreg in immune-regulation [177-183]. The development and function of nTreg is further discussed in 1.5.8.1.

1.5 Different CD4$^+$ T-cell subsets

CD4 T-cells play central role in adaptive immune system by supporting the development of humoral responses, maintaining CD8 T-cell memory responses and importantly, by influencing immune-regulation in order to maintain immune homeostasis. Although CD4 T-cells were originally divided into only two subsets (Th1 and Th2) by Mossman and Coffman [184], extensive research has revealed that CD4 T-cell population is more diverse and naïve T-cells can evolve into distinct phenotypes other than conventional Th1 and Th2 cells. Naïve T-cells recognize cognate antigens presented by APC in context of MHC class-II and depending on the costimulatory signal and cytokine milieu become activated, proliferate and differentiate down an effector pathway towards one of the following lineages.

1.5.1   T Helper 1 (Th1) cells

Th1 were originally identified as cells required for the cell-mediated immunity that help to control intracellular infections via the CD8 T-cell responses. They are defined by IFN-γ, TNF-α/β, IL-2 secretion and expression of the transcription factor T-bet [185-190]. Activation of naïve T-cells in the presence of IL-12 induces differentiation to Th1 cells [191-194]. Moreover, differentiation of naïve cells into Th1 cells is negatively regulated by IL-4 , IL-10 [195], IL-6 [196, 197], IL-23 and IL-17 [187].

Table 1.3: Different subsets of CD4 T-cells based on their ontogenic and functional requirements. The characteristic cytokine produced by each subset and their role in diseases are also summarized below. (Adapted from Tangye, *Nature reviews immunology*, 2013 [198])

CD4$^+$ T cell subset	Inducing cytokines	Activated STATs	Transcription factors	Suppressing cytokines	Canonical cytokines produced
T_H1 cells	• IL-12 • IFNγ	• STAT4 • STAT1	T-bet	IL-4 and IL-10	IFNγ
T_H2 cells	• IL-4	• STAT6	GATA3 and MAF	IFNγ	IL-4, IL-5 and IL-13
T_H17 cells	• IL-23 and IL-1β • IL-6 and IL-1β • TGFβ	• STAT3	RORγt and RORα	• IL-4, IFNγ, IL-27 and IL-2 • TGFβ (suppresses IL-22 expression)	IL-17A, IL-17F, IL-21, IL-22 and IL-26[*]
T_H9 cells	• TGFβ • IL-4	• STAT6	PU-1 and IRF4	IFNγ and IL-27[‡]	IL-9
T_H22 cells	• TNF • IL-6	• STAT1 • STAT3 • STAT5	RORγt and AHR	High doses of TGFβ	IL-22
T_{Reg} cells	• TGFβ and IL-2	• STAT5	FOXP3	IL-6	TGFβ and IL-10
T_{FH} cells	• IL-6, IL-21 and/or IL-27 • IL-12	• STAT3 • STAT4 • STAT1	BCL-6, IRF4, MAF and BATF	IL-2 and IL-10	IL-21, IL-4 and IL-10

1.5.2   T Helper 2 (Th2) cells

Th2 cells were identified as important mediators of humoral responses and are characterised by production of IL-4, IL-5, IL-6,IL-9, IL-10, and IL-13 [199-202]. These promotes B-cell proliferation and immunoglobulin class-switching [202]. IL-25, IL-33 and thymic stromal lymphopoietin (TSLP) derived from epithelial cells as well as IL-4 derived from innate lymphoid cells and basophils potentiates the differentiation to Th2 cells. The linage commitment to Th2 is determined by master regulator GATA-3 in both STAT6 dependent and independent manner [190, 203-205].

1.5.3   Th3 cells

Th3 are one of the regulatory T-cells which helps to maintain peripheral immune tolerance by secreting immunosuppressive cytokine TGF-β. The induction and the function of Th3 cells is described further in 1.2.4.

1.5.4   Th9 cells

Th9 cells are critical part of immunity against intestinal pathogens (e.g. helminth infection) but they are also mediator of respiratory allergic pathogenesis. Th9 cells produce high levels of IL-9 which contributes to – i) development of Th2 responses [206], ii) enhanced immunoglobulin production by B-cells, iii) differentiation and proliferation of mast cells; and iv) recruitment of eosinophils and lymphocytes at the site of

inflammation [207-209]. Th9 cells develop from naïve CD4 T-cells in the presence of TGF-β and IL-4, that prevents the generation of FoxP3$^+$ regulatory cells [206, 210, 211]. This is reportedly enhanced by IL-1 and IL-25 [212] while it is suppressed by IFN-γ and IL-23 [211]. Th9 cell differentiation is regulated by transcription factors- PU.1, STAT6, GATA3 and IRF-4 [213-215]. Their role in auto-immunity is not completely understood and conflicting reports indicate that Th9 cells promote tissue inflammation while others demonstrate a protective role [216, 217].

1.5.5   Th17 cells

In the recent years, Th17 cell has emerged as one of the main CD4$^+$ T-cell subsets which influences various immune responses by producing high mount of pro-inflammatory cytokine IL-17. They also produce IL-21, IL-22, IFN-γ and granulocyte macrophage colony-stimulating factor (GM-CSF) [218] and their differentiation is mediated by IL-23 and the transcription factor RORγt. The Th1 and Th2 cytokines- IFN-γ and IL-4 respectively, negatively regulate the generation of Th17 cells. The function of Th17 are specially noted in auto-immune diseases including multiple sclerosis (MS), psoriasis, rheumatoid arthritis (RA), inflammatory bowel disease (IBD) where they mediate the chronic inflammation [219, 220].

1.5.6   Th22 cells

Th22 cells play an important role in epidermal immunity and remodelling by producing proinflammatory cytokine IL-22, TNF-α and IL-13 [221-224]. Th22 cells were identified as a distinct memory T-cell population in skin which is regulated by transcription factor Aryl Hydrocarbon Receptor (AHR) and they also express chemokine receptor CCR6, skin-homing receptors CCR4 and CCR10 [222, 223]. It has also been demonstrated that the differentiation of Th22 is further promoted by T-bet but inhibited by TGF-β [225, 226]. The expression of homing receptors by Th22 cells allows them to be retained in the skin [249].

1.5.7   Follicular helper T-cells (T$_{FH}$)

Follicular helper T-cells (T$_{FH}$) represents a distinct T-cell subset localized within the germinal centre (GC) which helps to regulate B-cell fate and development of plasma cells [227]. T$_{FH}$ express CXCR5 which allows them to migrate in response to CXCL13 and relocate to follicles where they interact with follicular DC and B-cells [228, 229]. T$_{FH}$ differentiation is mediated by the master regulator transcription factor B-cell lymphoma 6 protein (Bcl6) and IL-6 as well IL-21 enhance the expression of Bcl6 but they are not limiting factor for T$_{FH}$ differentiation [230, 231]. T$_{FH}$ is a specialized T-cell subset as is specifically promotes development of humoral responses by producing cytokines- IL-21 and IL-4, chemokine CXCL13 and CD40-CD40L signalling. B-cells express Signalling lymphocytic activation molecule (SLAM) whereas T$_{FH}$ also expresses

Signalling lymphocytic activation molecule (SLAM)-associated protein (SAP). The interaction between T-cell : B-cell via SAP-SLAM is crucial for development of B-cell responses as it is required for IL-4 production by T_{FH} and to promote formation of germinal centres [232, 233].

1.5.8   Regulatory T-cells

The regulation and resolution of immune response is mediated by regulatory T-cells which confer immune-suppression through various contact-dependent / -independent mechanisms. In the adaptive immune system, regulatory T-cells are indispensable to prevent inappropriate immune response against self-antigen and maintain tolerance. Regulatory subsets, other than of CD4 origin, of different lymphocyte population has also been identified including CD8$^+$ Treg, regulatory B-cells [234, 235], regulatory $\gamma\delta$ T-cells [236, 237], regulatory NK cells [238-240], regulatory dendritic cells [241-243], regulatory macrophages [244]. Whether these cells are true regulatory cells or represent a regulatory phase in the life cycle of these cells is still to be clarified. The concerted regulatory activity of these cells ensures that the manifestation of autoimmune disease is averted, the proinflammatory responses are restrained after clearance of the pathogen and tissue damage is minimised in cases of chronic infection [245-247]. CD4 regulatory T-cells can be broadly divided into two groups depending on their ontogeny. One group of Treg consists of only natural regulatory T-cells (nTreg) which are selected and develop in the thymus. The other group, known as the inducible or peripheral regulatory T-cells (iTreg or pTreg), consists of

several distinct subset of T-cells with regulatory activity that develop in the periphery.
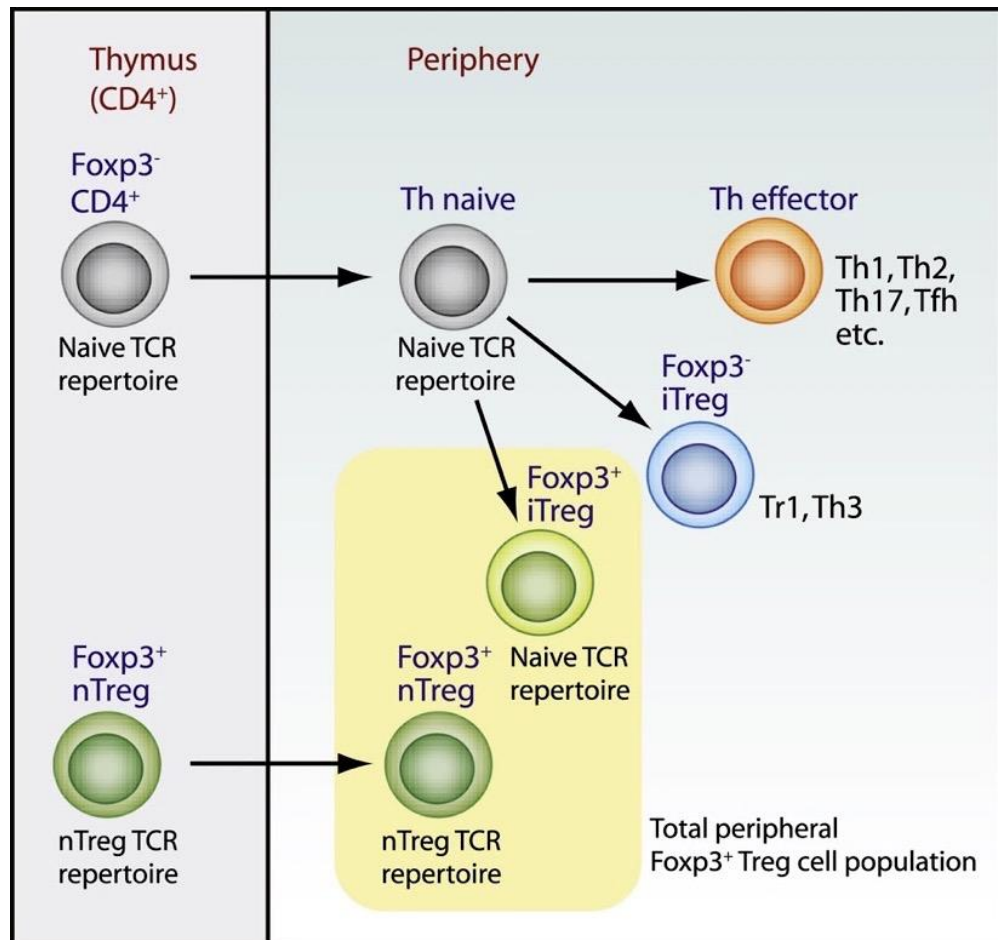
Figure 1.4: Thymic and peripheral generation of FoxP3+ Treg Cells. CD4 regulatory T-cell compartment is comprised of thymus derived natural Treg and inducible Treg (iTreg) developed in the peripheral immune system which develops from naïve CD4 T-cells. Depending on the differentiation stimuli, antigen repertoire and immunological cues, naïve T-cells could convert to Th3, Type 1 regulatory T-cells or FoxP3+ non-thymic iTreg. nTreg and iTreg might differ in their TCR repertoire and could potentially contribute to immune-suppression under variable immunological settings (e.g., mediation of autoimmunity, infection etc.). (Adapted from Lafaille, *Immunity*, 2009 [248])

1.5.8.1 Natural regulatory T-cells

The concept of regulatory cells, earlier known as "Suppressor cells", was introduced based on the observations from several studies conducted by Gershon and Kondo in early 1970's [249-251]. However, due to limitation of understanding and inability to identify these cells, the existence of these cells could not be validated for more than a decade. In 1995, the breakthrough study by Sakaguchi demonstrated a distinct CD25^{+} T-cell population which contributed to maintain immunological self-tolerance [252] which confirmed the role of suppressor cells and these cells are now known as natural regulatory T-cells (nTreg). nTreg constitutes upto 10% of peripheral CD4 T-cells [253, 254]. The seminal observations from the adoptive thymocyte transfer studies conducted by Gershon and Kondo paved the way for the discovery of nTreg [255, 256]. These studies demonstrated for the first time that T-cells not only elicited immune response to antigen challenge but a separate subset within the T-cell population also existed which suppressed the immune response. Extensive studies on these T-cells, initially known as suppressor cells, revealed the development of nTreg alongside conventional T-cells in the thymus as well as their characteristic phenotype and mechanisms of immune-suppression. Neonatal thymectomy experiments demonstrated that removal of thymus resulted in development of spontaneous autoimmune pathologies only if the thymus was removed on day 3 but not on day 7 [257]. Further studies reported that self-antigen specific TCR was required for the development of nTreg in thymus as selection of these cells were dependent on the expression of cognate antigen expression in the thymus. Moreover, it was also reported that higher

affinity for TCR is crucial for nTreg differentiation as cells with lower affinity for self-antigen fail to develop into nTreg [258]. Other determinants of nTreg development include intraclonal competition for antigen, interaction with intrathymic antigen presenting cells and overall strength of TCR signalling or avidity [259]. Several models of thymic development of nTreg have been proposed in the last few years. The instructive model suggests that the T-cells bearing TCR with intermediate self-reactivity would be selected for nTreg development. If the TCR self-reactivity was very high, the self-antigen specific T-cell would be deleted by negative selection whereas low self-reactivity would lead to the development of conventional T-cells [259-261].
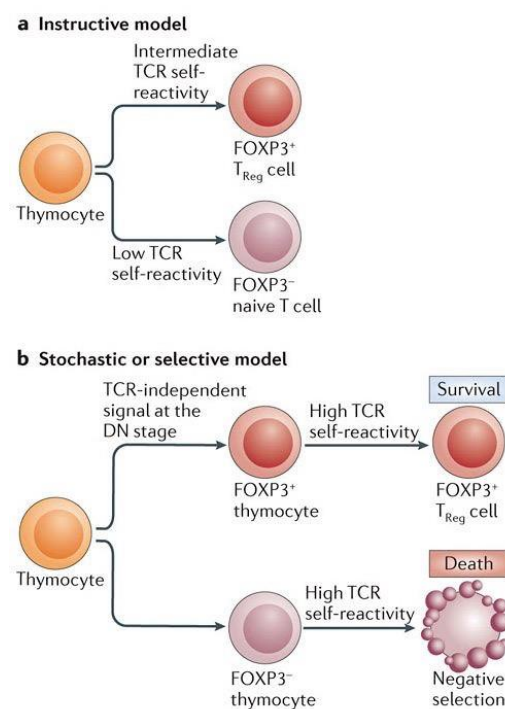
Figure 1.5: Models for thymic Treg cell development. The instructive model and stochastic model proposed that nTreg selection in thymus is dependent on intermediate self-reactivity and TCR-independent signal at double negative stage of thymocyte development respectively. Adapted from Hsieh, *Nature review immunology,* 2012 [259].

a **Gaussian distribution model**

Cell number

Positive selection

T_{Reg} cell selection

Negative selection

Relative avidity to self antigens

b **Poisson distribution model**

Cell number

Positive selection

T_{Reg} cell selection

Negative selection

Relative avidity to self antigens
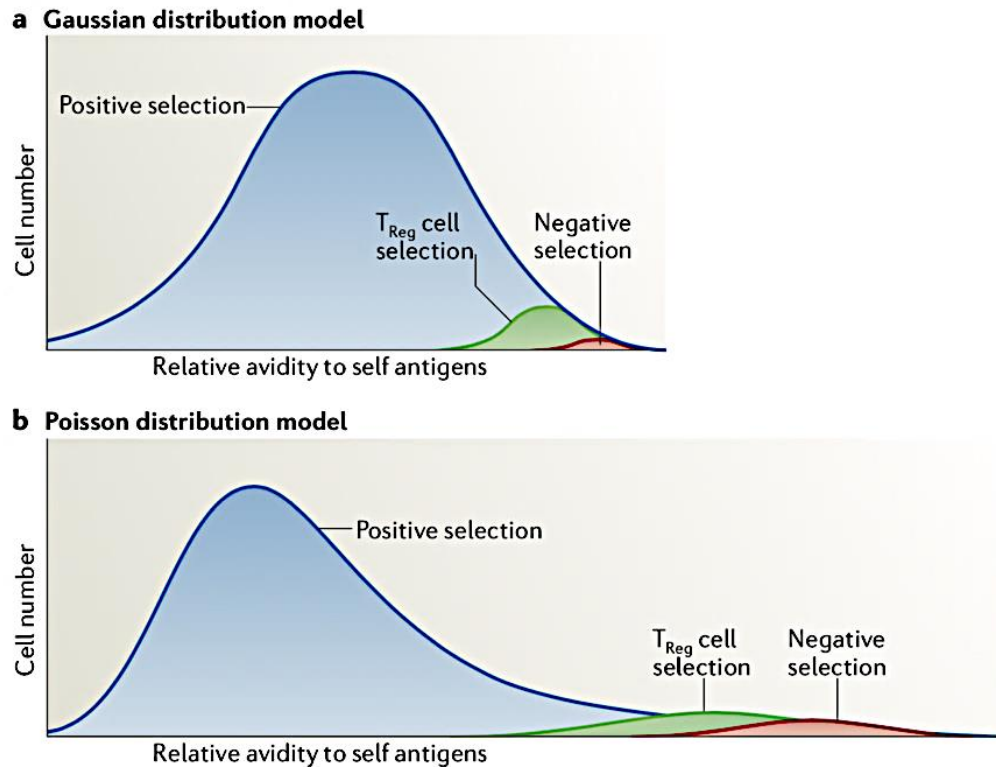
Figure 1.6: Models of self-reactivity and Treg cell generation. The Gaussian distribution model and Poisson distribution model suggest that nTreg selection in the thymus depends on higher self-reactivity than the level of self-reactivity permitted for conventional T-cell selection. These models also propose that the range of self-reactivity required for the development of nTreg resides closer to the threshold of high self-reactivity which leads to clonal deletion of cells by negative selection. Poisson distribution model differs from the Gaussian model in the larger predictive difference between the low self-reactivity necessary for positive selection of conventional T-cell and higher self-reactivity of nTreg. Adapted from Hsieh, *Nature review immunology,* 2012 [259].

In line with the instructive model, both Gaussian and Poisson distribution models predict that relatively higher TCR avidity results in nTreg selection and the range of TCR avidity required for nTreg selection lies closer to the threshold for negative selection than positive selection [259,

262]. However, Poisson distribution model proposed that the difference between the level of self-reactivity required for nTreg selection and positive selection is larger than speculated by the Gaussian distribution model. Contrary to the nTreg development models discussed above, the stochastic or selective model suggests that TCR-independent signals at the $CD4^-CD8^-$ stage of thymic development determines FoxP3 nTreg selection [263, 264]. Despite the conflicting theories about the nTreg selection model, it has been established that nTreg emanate from a distinct population of immune suppressive T-cells in thymus.

nTreg cells are currently defined as $CD4^+FoxP3^+CD25^{High}CD127^{Low}$ [252, 265-267]. nTreg cells also express LAG-3, 4-1BB, GITR, CTLA-4,OX-40, CD73, CD39 and neuropilin-1 [175, 268-273]. However, none of these markers are exclusively expressed by nTreg and a combination of markers are still required to identify these cells. Interestingly, nTreg cells could also be divided into two groups based on CD45RA and CD45RO expression [274-278]. The $CD45RA^+$ nTreg and $CD45RO^+$ nTreg cells are termed naïve and memory nTreg respectively. $CD45RA^+$ nTreg differ from $CD45RO^+$ nTreg in their reduced potential for proliferation and migration [274]. It was also reported that $CD45RA^+$ nTreg were preferentially located in the bone marrow and cord blood whereas $CD45RO^+$ nTreg were predominant in the skin and peripheral blood. Moreover, it was also demonstrated that naïve nTreg cells are less susceptible to CD95-CD95L (FAS-FASL) mediated apoptosis compared to memory nTreg [276]. Interestingly, $FoxP3^+CD25^+$ regulatory cells with similar suppressive potential as nTreg can also develop from naïve $CD4^+CD25^-$ T-cells in the

peripheral immune system. These non-thymic FoxP3$^+$CD25$^+$ cells are known as peripheral Treg (pTreg) and the induction of pTreg is dependent IL-2 as well as TGF-β [248, 279-281]. Comparative studies to determine their functional potential also demonstrated that pTreg cells were more immune-suppressive compared to nTreg with the same TCR specificity [282]. However, in that study, the suppressive activity of pTreg was attributed to IL-10 production and neutralizing antibody abrogated the effect of pTreg but not nTreg which indicates that these cells appear to have different or overlapping mechanism of action. These observations were further supported by another study which demonstrated that nTreg were essential to prevent auto-immune disease mediated lethality whereas pTreg cells were important for prevention of chronic inflammation [283]. It has also been suggested that neuropilin-1 (Nrp-1) could be used as a surrogate marker to distinguish nTreg, which express Nrp-1, from pTreg in order to further investigate the role of these immune cells in mediating tolerance [268, 284, 285].

nTreg cells exert their regulatory function cell-cell contact dependent suppression by reducing proliferation of the effector cells, modulation of maturation and function of dendritic cells, modulation of cytokine microenvironment, cytolysis and metabolic disruption of target cells [286-288]. nTreg mediated immune-suppression is of particular importance to impede the development of auto-immune diseases such as Rheumatoid arthritis (RA) and multiple sclerosis (MS). It has been reported that nTreg intercept the development of organ-specific auto-

immune pathogenesis by inhibiting the differentiation of auto-reactive effector cells [289]. It has also been reported that nTreg suppress Th1, Th17 mediated inflammation and their function is impaired in MS, RA as well other auto-immune diseases [272, 290-292]. While it has been reported that the frequency of nTreg might decrease in auto-immune diseases, elevated tumour infiltrating nTreg has been reported in several cancers including gastric and ovarian cancer [292-295]. The recruitment, accumulation and expansion of nTreg in tumour could potentially hamper the effector cell mediated anti-tumour immunity and targeting of nTreg is required to potentiate anti-tumour immune responses [286, 296]. Importantly, although controversial, plasticity of nTreg has also been noted in recent years and its implication on the nTreg mediated immune-suppression is yet to be deciphered [297-299].

1.5.8.2 Inducible regulatory T-cells

A) Th3 cells

Th3 cells were initially observed to be critical regulator of peripheral tolerance following oral administration of self-peptide myelin basic protein (MBP) and proteolipid protein (PLP) in Multiple Sclerosis patients [300]. Administration of the MBP and PLP resulted in induction of antigen specific TGF-β secreting Th3 cells and it was suggested that mucosal DC might promote the differentiation of naïve cells to Th3. Indeed, it was later determined that mucosal DC recognize commensal bacteria, present antigens and produce IL-10, TGF-β, and IL-6 which promotes differentiation of Th3 cell. The induction of Th3 cell in turn promotes IgA production by B-cells which is important to maintain tolerance against commensal bacteria [301-303]. Interestingly, Th3 cells can also express FoxP3 but they are distinct from the FoxP3^{+} nTreg. Moreover, It was demonstrated that upon repeated antigenic administration, Th3 cells induce FoxP3^{+} nTreg and suppress Experimental Autoimmune Encephalomyelitis (EAE) in mice [304]. Although Th3 cells express FoxP3, unlike nTreg which was affected in IL-2^{-/-} mice, the development of Th3 is not dependent on IL-2 [305]. Th3 exert their immune-suppressive activity by producing TGF-β which negatively regulates both Th1 and Th2 cells. Th3 cells also play crucial role in immune tolerance to non-self antigens as it was reported that Th3 mediate the antigen specific hypo-responsiveness in chronic human helminth infection and suppress T-cell proliferation by enhanced TGF-β and IL-10 production [306]. Th3 cells, like other Treg populations lack a single specific marker.

B)　Type 1 regulatory (Tr1) T-cells

Type 1 regulatory T-cells (Tr1) were first identified in patients with severe combined immunodeficiency (SCID) transplanted with HLA mismatched haematopoietic stem cells. The Tr1 cells were characterised as immune-suppressive cells which conferred tolerance for a graft by producing high-level of IL-10 [307]. Further studies reported that IL-10$^+$ Tr1 cells are of a different origin than nTreg and they develop from conventional T-cells in the peripheral immune system. It was demonstrated that Tr1 cell development is not impaired by FoxP3 mutation or deletion. The induction of these cells was observed in Immune Dysregulation, Polyendocrinopathy, Enteropathy, X-Linked (IPEX) Syndrome which is manifested due to mutation in *FoxP3* gene [308]. Importantly, immune-suppression mediated by Tr1 cells was determined to be comparable to that of nTreg cells *in vivo* and *in vitro* [309, 310]. In addition, it was reported that enforced expression of IL-10 confers Tr1 phenotype and function in human CD4 T-cells [311]. Several studies have explored the induction and function of Tr1 cells both in vivo and in vitro. However, due to differences in the experimental settings and method of generation in these studies, it still remains unclear if these cells constituted the same inducible immune-suppressive cell population or not. Several cell surface markers has also been associated with Tr1 cells but none of them are exclusively expressed by Tr1 cells [312].

I. Induction of Tr1 cells

There are several pathways which have been described for the generation of Tr1 cells in vitro and in vivo. These include: the differentiation of Tr1

cells from naïve CD4 T-cells by signalling via alternate costimulatory molecules (CD46, CD55, CD2) [313-315]; differentiation via CD28 costimulation in the presence of a range of cytokines (IL-27, IL-10, IFN-α, TGF-β, IL-6) [316-320]; immune-modulators (Dexamethasone, Rapamycin etc.) [321, 322]; differentiation mediated by regulatory dendritic cells [323] and switching to Tr1 cells from Th1 cells via CD46 signalling [324].

Dendritic cells play a critical role in the induction of all cells, including Tr1 cells. It has been reported that tolerogenic DC, known as DC-10, induces differentiation of Tr1 cells via IL-10 dependent ILT4/HLA-G pathway [323]. DC-10 have been characterised as $CD14^+CD16^+CD11c^+CD11b^+$ $HLA-DR^+CD83^+CD1a^-CD1c^-$ and ILT4 expressed on DC-10 interacts with HLA-G on T-cells to induce Tr1 cells. Importantly, HLA-G expression on T-cells is dependent on IL-10 produced by DC-10 and blocking the IL-10 signalling by using anti-IL10 receptor antibody reduced HLA-G expression by CD4 T-cells which in turn controlled induction of Tr1 [323]. The role of IL-10 receptor signalling in Tr1 was further supported in another study which demonstrated that IL-10 receptor (IL-10R) signalling was critical for Tr1 function *in vivo* as it helped to maintain sustained production of IL-10 via activating STAT3 and p38 MAP kinase [325]. Although IL-10R signalling was dispensable for the differentiation and function of Tr1 cells in the presence of IL-27, impaired IL-10R signalling resulted in lack of sustained immuno-suppressive function in the long term and failed to provide protection against colitis [325]. In addition, protein kinase θ was determined to be essential for the efficient induction of antigen specific

IL-10$^+$ CD4 T-cells regulated tolerance following antigen dose escalation studies [326]. Activation of naïve CD4 T-cells in the presence of immune-modulators such as Dexamethasone, Vitamin-D3, Rapamycin etc. have also been reported to induce IL-10$^+$ Tr1 cells [321, 322]. However, the characterisation of these induced cells was often limited to the induction of IL-10. Whereas the presence of other cytokines such as IFN-g could also be used to determine if the treatments resulted in induction of IL-10 in Th1 cells.

It has also been reported that repeated antigen stimulation with higher affinity peptide induced tolerance by development of antigen-specific IL-10$^+$ CD4 T-cells and provided protection against EAE whereas lower affinity peptides failed to do so to the same extent [327, 328]. Also, several studies reported that the dose of antigen and route of administration could potentially influence the induction of IL-10$^+$ cells *in vivo* and high antigen dose was required to induce tolerance by sequential transcriptional modification which suppressed the proinflammatory effector function and enhanced regulatory T-cell associated markers including c-MAF, NFIL3, LAG-3, TIM-3, PD-1 [329].

II. Function of Tr1 cells

Extensive studies revealed that Tr1 cells exert their regulatory effect not only by IL-10 production but these cells also utilize contact dependent mechanisms via CTLA-4/CD80 and PD-1/PDL-1 interactions [330], metabolic disruption of target cells by CD39,CD73 [331] and granzyme-B

mediated cytolysis [332-334]. However, IL-10 production is still

considered as the defining characteristic of Tr1 cells.
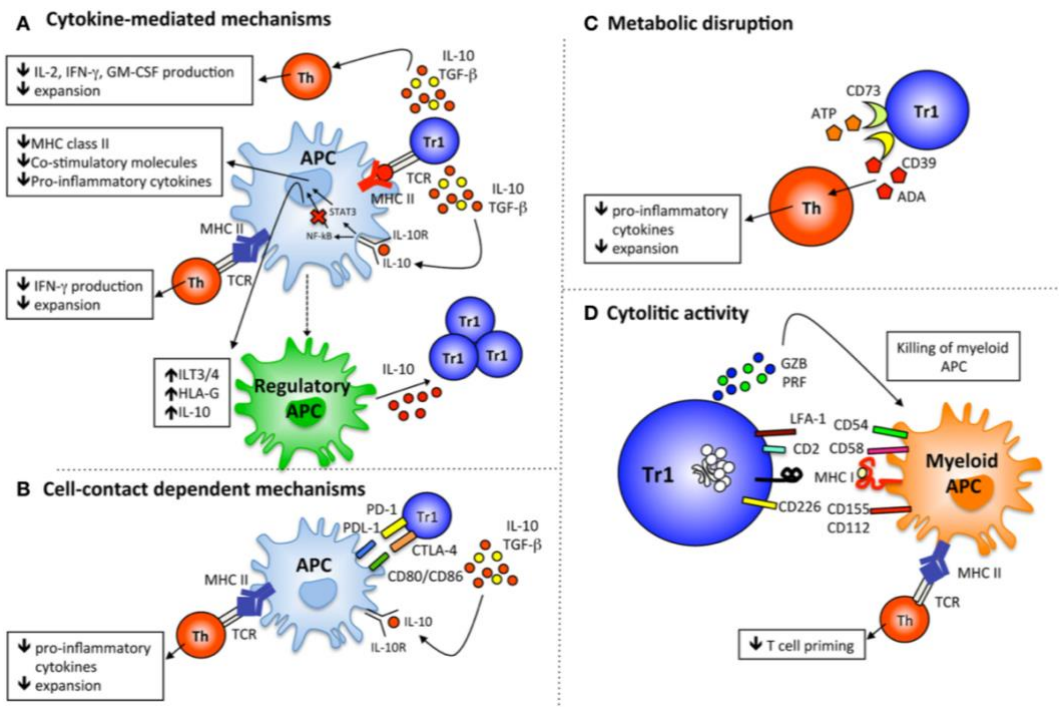
Figure 1.7: Function of Tr1 cells. Type 1 regulatory T-cells regulate immune responses by multiple mechanism which could be divided into four categories. A) Tr1 cells directly suppress target cells by secreting immune-suppressive cytokine such as IL-10 and TGF-β. B) Tr1 cells also suppress target cells by contact dependent mechanism CTLA-4 and PD-1 interaction. C) Tr1 cells instigates metabolic disruption of target cells by catalytic inactivation of extracellular ATP by using ectoenzyme CD39 and CD73. D) Tr1 cells disrupt priming of effector cells targeting and killing myeloid antigen presenting cell by granzyme-B and perforin mediated cytolysis which requires the interaction between CD226-CD1555/CD112. (Adapted from Gregori, *Frontiers in immunology,* 2012 [335])

IL-10 is one of the key immuno-suppressive cytokines which was initially

identified as a Th2 clone factor which suppressed the cytokine production

by Th1 cells and it was known as cytokine synthesis inhibitory factor

(CSIF) [336, 337]. Later it was discovered that many cells from both the

innate and adaptive immune system can produce inhibitory cytokine including; eosinophils, neutrophils, B cells, CD8^{+} T cells, and Th1, Th2, and Th17 CD4^{+} T cells. IL-10 exerts immune-suppression by negatively regulating maturation and function of dendritic cells and macrophages [338]. It also downregulated the MHC class-II and costimulatory molecule expression on APCs. Moreover, IL-10 modulates the production of IL-12 by APC which in turn affects the induction and function of Th1 cells [339, 340]. IL-10 also directly reduces IFN-γ production and proliferation of Th1 cells[341]. Importantly, the role of IL-10 in maintaining immune tolerance was noted in IL-10 deficient mice which demonstrated prolonged and exaggerated immune responses toward antigen, excessive inflammation, tissue damage and auto-immunity [342-345]. Interestingly, it was determined that IL-10 is a key mediator of homeostatic interactions with commensal microorganism as lack of IL-10 led to progression to pathological condition in response to gut microbiota [344, 346]. Tr1 cells also prevented the induction of inflammatory Bowel disease (IBD) in an IL-10 dependent manner when adoptively transferred into mice [4]. Moreover, it was also demonstrated that glycoantigen derived commensal bacteria induce potent Tr1 cell which express gut homing receptors [347]. IL-10 is important to control inflammation in autoimmune diseases such as multiple sclerosis, rheumatoid arthritis etc. [348, 349] and the level of IL-10 was determined to be inversely correlated with EDSS (define) score in progressive MS patients [350].

1.6 Differentiation of CD4 T-cells

1.6.1 The "Three Signal Model" of T-cell differentiation

Thymic development of T-cells from the haematopoietic stem cells results in quiescent but immune-competent naïve T-cells. Following the egress from the thymus, antigen-inexperienced naïve T-cells remain in the peripheral immune system until they recognize cognate antigen and differentiates into effector cells. However, the differentiation of naïve cells is a complex process that requires coordinated participation of multiple signals to ensure the initiation of immune response rather than T-cell unresponsiveness or anergy. The "Three signal model" of T-cell differentiation postulates that CD4 naïve T-cell could differentiate into effector cells when a TCR signal is triggered by recognition of antigen (signal 1) in conjunction with appropriate costimulatory signal provided by antigen presenting cells (signal 2) and in the presence of supporting cytokine milieu which supports the T-cell linage selection (signal 3) [351-354]. The recognition of antigen in absence of a costimulatory signal leads to the induction of anergy [355] or deletion of cells by apoptosis, which may play an important role in the maintenance of immune tolerance [356]. In contrast, the recognition of cognate antigen accompanied by costimulatory signal and cytokines results in cell differentiation which is characterised by IL-2 production, cell proliferation, upregulation of activation markers (e.g. CD69, CD25 etc.), acquisition of a specific phenotype and attaining the ability to respond to the same antigen upon restimulation [185, 357-360].

CD40L, CD40 ligand; DAMP, damage-associated molecular pattern; IL-12, interleukin-12; IL-12R, IL-12 receptor; PAMP, pathogen-associated molecular pattern; PRR, pattern recognition receptor.
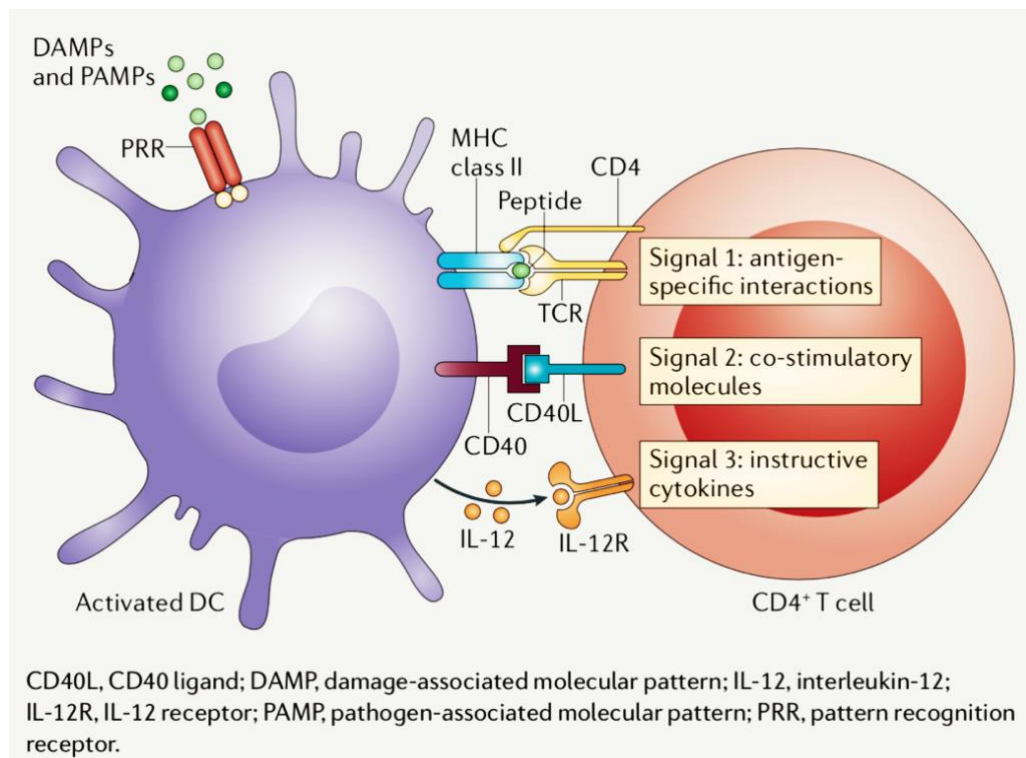
Figure 1.8: The three signal model of T-cell activation. The "three signal model" depicts the prerequisite signals essential for the differentiation of naïve T-cells. Signal 1 is provided by TCR signalling upon antigen recognition while signal 2 derives from the interaction of costimulatory moles on T-cells with their ligands on APC. Both signal 1 and signal 2 are absolute requirement for differentiation and cell survival whereas the signal 3 provided by the cytokine milieu is important for determining the phenotype and function of the cells. (Adapted from Kambayashi 2014 [361]).

Depending on the type of costimulatory signal (Signal 2) obtained from APC at the time of T-cell priming, naïve cells can differentiate into either proinflammatory or anti-inflammatory T-cells. The requisition of costimulatory signal to elicit T-cell responses derived from the seminal observations made in the organ transplantation studies by Lafferty and

Woolnough [355, 362]. Subsequent discovery of CD28 [363] and its ligand CD80 [364, 365] established the obligatory requirement of costimulation for the differentiation of naïve T-cells as the signal 2 [366].

The recognition of peptide-MHC complex initiates a signalling cascade where TCR complex is phosphorylated by the kinase Lck and phosphorylation of the signalling motif leads to recruitment of Zap70 which in turn phosphorylates adapter protein LAT and results in downstream signalling via different pathways [367]. Early studies differentiated CD28 costimulatory signal from CD3-TCR signalling based on the calcium dependency and sensitivity to cyclosporine A (CsA) of the signal 1. Later it was determined that intracellular signalling could be initiated by CD28 via direct phosphorylation of CD28 and binding to lipid kinase PIK3. In addition, ITK, and the signalling complex GRB-2–SOS can also bind to CD28. The Src homology domain 2 (SH2) of PI3K binds to the conserved pYMNM motif on CD28 and the kinase has been reported to be associated with various cellular function including growth factor–induced mitogenesis and apoptosis which have been attributed to CD28 signalling [368]. While CD28 still remains as one of classical costimulatory molecules, a myriad of costimulatory molecules has been discovered in the last few years along with the coinhibitory molecules which negatively modulate T-cell function. While the synergistic effect of various costimulatory signals promotes the differentiation and activation of T-cells, coinhibitory molecules modulate T-cell function at later stage and serve as response modifiers [369, 370]. Importantly, the cytokines produced by dendritic and innate immune cells act as signal 3 in

polarization of different phenotypes of T-cells. For instance, activation of naïve T-cells with antigen and costimulatory signal in the presence of sustained IL-12 produced by APC promotes differentiation of naïve cells into Th1 effector cells while presence of IL-4 leads to the development of Th2 effector cells [371-374].

1.6.2   Antigen presentation by Dendritic cells

The interaction between dendritic cells and T-cells is of utmost importance as all three components or signal required for T-cell differentiation and activation are controlled by dendritic cells. The phagocyte system is represented by a complex heterogeneous population encompassing Dendritic cells, macrophage and monocytes. After being discovered in 1973 [375], dendritic cells were referred as the "Sentinels of the Immune system" or "Immunogenic" due to their critical role in the induction of adaptive immunity against pathogens but it soon became evident that DCs are also the mediators of homeostasis and help to maintain tolerance. In the homeostatic immunological state, immature DCs circulate in blood or remain in the peripheral tissues and express lower amount of co-stimulatory molecules but demonstrate high endocytic activity. DCs can become activated upon encounter with common motifs expressed by pathogens that are recognised by a system of Pattern Recognition Receptors (PRRs) such as Toll-like receptors (TLRs), C-type lectin receptors (CLRs), Nucleotide Oligomerization Domain-like receptors (NOD-like receptors or NLRs), Retinoic acid–

Inducible Gene (RIG)-I-like receptors (RLRs) and Scavenger receptors [376-378]. After the capture of potential antigen by phagocytosis, endocytosis or micropinocytosis, the antigen is processed by degradation and presented to T-cells in context of Major Histocompatibility Complex (MHC) molecules. PRR activation leads to upregulation of those 'signal 2' costimulatory receptors and induction of cytokines 'signal 3' that can coordinate to prime the successful activation and differentiation of naïve T cells.

Different subpopulations of DC express specific chemokine receptors in their immature state and respond to local inflammatory stimuli induced CC and CXC chemokines (RANTES, MIP-1 alpha, MIP-1 beta, MIP-3 alpha, MIP-5 etc.) [379]. However, mature DCs lose the ability to respond to the above mentioned chemokines by down-regulation of the receptor or desensitization and instead upregulate trafficking receptor CCR7 to facilitate response to ELC/MIP-3 beta and SLC/6Ckine which allows mature DCs to migrate to T-cell rich areas of the lymphoid organs [379]. Thus, dendritic cells go through a complex maturation process which enables them to become interdigitating DC and modulate immune response to self and non-self antigens.

Cytokine secretion by dendritic cells is intricately associated with T-cell immunity. DCs produce both pro-inflammatory cytokine such as IL-12 which induces the differentiation of naive CD4$^+$ T-cells in IFN-γ$^+$ effector

T-cells and the production of IFN-γ$^+$ further elevates the IL-12 by DCs through a positive feedback loop [380]. However, IL-12 is not constitutively transcribed by DCs in the immature state and is upregulated upon activation through CD40 signalling in conjunction with IFN-α or TLR agonists [381]. On the other hand, DCs secret IL-10 in the immature state in order to prevent spontaneous maturation [382]. The production of IL-10 is further enhanced upon maturation of the DC and might have an impact on IL-12 production in an autocrine manner as blocking the IL-10 with neutralizing antibody resulted in augmented release of IL-12 that enhanced naïve T cell activation and augmented the activation off allogeneic T cell [382]. Thus, the balance between IL-10 and IL-12 is key to determine the direction and magnitude of immune responses.

1.6.3   The role of alternate costimulatory molecules in the induction of Tr1 cells

TCR signalling provides the primary signal required for T-cell activation but it is not sufficient to induce T-cell differentiation. Immune synapse formation, of the central, peripheral and distal supra-molecular activation complexes (cSMAC, pSMAC and dSMAC, respectively), is the primary reorganizing event which enables coordinated signalling through TCR and costimulatory molecules to initiate T-cell activation. The classical Th1 inducing costimulatory molecule CD28 has been studied extensively and

it has been demonstrated that CD28 is localized in the cSMAC along with TCR where it forms micro clusters in order to initiate downstream signalling via protein kinase θ [383]. However, there are other costimulatory molecules which have been reported to induce differentiation of not only pro-inflammatory but also anti-inflammatory T-helper cells. There are three costimulatory molecules which are associated with the induction of Tr1 cells from either naïve CD4 T-cell or effector CD4 T-cells.

CD2-CD58 was the first costimulatory receptor-ligand pair reported to induce differentiation of naïve T-cells into Tr1 cells [313]. The CD2 induced IL-2, no IL-4, some IFN-γ, and high levels of IL-10 production from T-cells. However, CD2 costimulation also induced anergy of the CD4 T-cell which was not dependent on IL-10. Later CD46 and CD55- two compliment regulatory proteins with costimulatory potential, were also reported to induce Tr1 cells [314, 315, 384-386]. Initially only
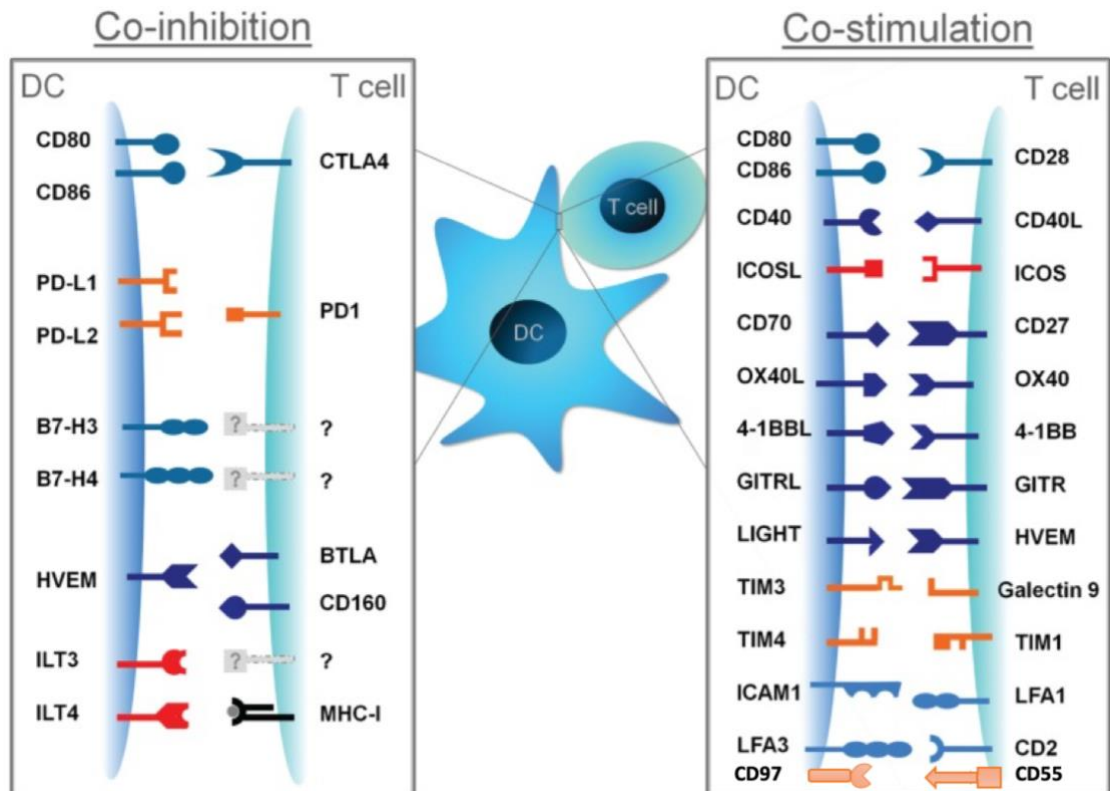
Figure 1.9: Co-stimulatory and co-inhibitory molecules and their cognate ligands. Depending on the maturation state, dendritic cells express many costimulatory and co-inhibitory molecules. The costimulatory molecules are required for naïve CD4 T-cell differentiation and activation of effector cell whereas co-inhibitory molecules attenuate T-cell responses at later stage by either competing with costimulatory signal or activating negative regulatory pathways. (Adapted from Bakdash, *Frontiers in immunology,* 2013 [369])

complement protein C3b was identified as the ligand for CD46 but later Notch signalling protein Jagged1 was also determined to be a ligand for CD46 which helped to elucidate underlying mechanism of CD46 mediated IL-10 production. It has been proposed that CD46 sequesters Jagged1 on the resting CD4 T-cells and prevents its interaction with the Notch1 which promotes Notch1-DLL1 signalling and serves as a break for T-cell

activation. Upon TCR signalling, C3b is generated locally which binds to CD46 and enables Jagged1-Notch1 signalling which results in IL-10 production. It has also been reported that α-E-catenin mediates the down-regulation of CD46 in activated T-cells which further promotes Jagged1-Notch1 interaction and the absence of α-E-catenin leads to impaired CD46 down-regulation which resulted in reduced IL-10 production [387]. Interestingly, CD46 regulated signalling cascade, leading to induction of Tr1, has been suggested as a mechanism for "switching" from Th1 phenotype to Tr1 phenotype [388]. According to this model of Tr1 induction, IFN-γ^+ Th1 cells transition to IL-10$^+$ cells at the end of an immune response via CD46 signalling and it challenges the notion that Tr1 cells represent a specific lineage of regulatory T-cells. In contrast, it was demonstrated that CD55 on T-cells interacts with its ligand CD97 and induced differentiation of naïve CD4 T-cells into a discrete IL-10$^+$ Tr1 population [315]. Similarly, it was demonstrated that both IL-27 and IL-6 induce differentiation of Tr1 cells from naïve T-cells when stimulated with CD3/CD28 [316, 317]. A key issue in the field of Tr1 cells is in providing a consensus in terms of a definition. The differentiation of Tr1 cells from naïve CD4 T-cells posits that these cells belong to a specific lineage. However, many of the studies mentioned here use IL-10 as a definition of Tr1, when IL-10 can be induced in a large range of cell lineages under different conditions. Whether these costimulatory molecules and cytokine-regulated induction of Tr1 cells share any common molecular pathway is yet to be determined.

1.6.4   T-cell plasticity

The discovery of Th1 and Th2 cells provided us a dualistic perception about the T-cell differentiation and commitment to specific lineage. However, this notion has been challenged in the recent years by the ever expanding classification of T-cell subsets which now also accommodates Th3, Th9, Th17, $T_{FH,}$ Th22, nTreg, pTreg and Tr1 cells. To further complicate our understanding of T-cell lineage as a differentiated phenotype, many studies have reported the emerging functional flexibility or plasticity of T-cells under health and disease conditions [389-393]. Plasticity has been defined as the ability of T-cells of a specific origin/lineage to attain the characteristics of one or more different lineages. This has been defined in terms of cytokines production and expression of master regulator transcription factors [393, 394]. For instance, it has been demonstrated that FoxP3 regulatory T-cells could be reprogrammed, under the appropriate environmental conditions, to produce IL-17 and express $ROR_{\gamma}t$ which is signature prolife of Th17 cells and vice versa [390, 395, 396]. In addition, induction of Th1/Treg cells was also reported where the Th1 cells attained FoxP3 expression while concomitantly expressing T-bet as well as IFN-γ and mediated immune-suppression by producing IL-10 in order to impede airway hyper-reactivity development [397].
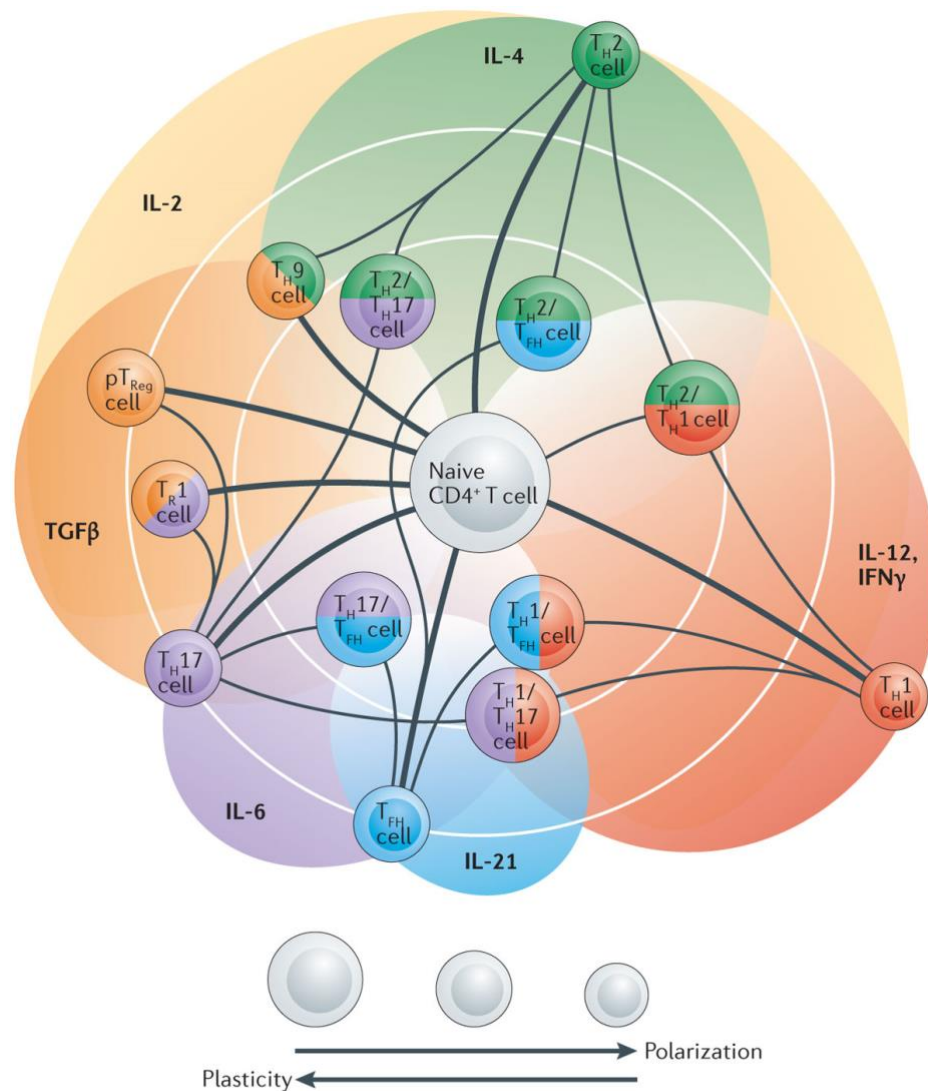
Figure 1.10: Cytokine-driven T cell plasticity. Inductive cytokine mediated plasticity of T-cells is depicted as transition of naïve T-cells to polarized and terminally differentiated T-cells via intermediate ephemeral state which is demonstrates the characteristics of more than one T-cell subset. Concerted effect of multiple cytokines or single cytokine might initiate reprogramming of a T-cell primed under different conditions. Repeated stimulation of T-cells under similar condition eventually leads to polarization which restricts the cellular function to specific subset and decreases susceptibility to plasticity. (Adapted from DuPage, *Nature reviews immunology*, 2016 [394])

The evolving paradigm shift in the concept of "plasticity" and "lineage stability" has been attributed to previously unfathomed influence of determinants such as cytokines, strength of TCR as well as costimulatory signal, microRNA, non-coding RNA, notch signalling and epigenetic modification on the polarization of differentiated effector cells [297, 393, 394, 398, 399]. It has now been demonstrated that the cytokines which influence the priming of T-cells during differentiation process could also serve as an inductive factor for plasticity of established T-cell subsets. For example, it was demonstrated that the concerted effect of type I and type II interferons along with interleukin-12 direct the reprogramming of LCMV-specific Th1 cells into T-bet$^+$GATA-3$^+$ Th1/Th2 cells which persisted for a long time in vivo [400]. Similarly, rapid *in vivo* conversion of IFN-γ producing Th1 into IL-4$^+$ Th2 cells was observed during helminth infection in a mouse model [401]. In addition, epigenetic modification has also been identified as one of the crucial factors which controls T-cell plasticity. Naïve T-cell differentiation represents the culmination of multiple integrated signals that determine T-cell fate. However, differentiation also facilitates the establishment of an epigenetically modified pattern of chromatin so that characteristic gene expression can be recapitulated in a heritable way by the progenitor cells upon restimulation. This process not only requires progressive changes in genes associated with specific lineage, but it also needs regulation of other genes which might suppress development of the selected lineage [402, 403]. However, differentiation does not completely abrogate the potential for flexibility, and it allows critical genes to remain in a "bivalent state" in order to respond to modified signals. It has been

confirmed by several studies that master regulator genes such as *Tbx21, Gata3, Rorc* etc. can remain in a bivalent state following differentiation which indicates a potential for obtaining characteristics of multiple subsets [297, 404]. Global Mapping of trimethylated lysine 4 on histone 3 (H3K4me3) and trimethylated lysine 27 on histone 3 (H3K27me3) associated with permissive and repressive chromatin modification respectively. This revealed that distinct epigenetic patterns are exhibited by different subsets of cells [297]. For example, repressive H3K27me3 modification of *Foxp3* gene was noted in Th1 and Th2 cell but not in Th17 cells which could potentially explain the existence of RORγ$^+$FoxP3$^+$ Th17 cells reported by several studies [297, 396, 405]. Similarly, *Tbx21, Runx3, RORc* genes were determined to be in bivalent state in iTreg cells which indicated that under appropriate stimuli, iTreg cells could obtain the expression pattern of multiple master regulator transcription factors. Moreover, it was also reported that Th2 cells retained H3K27me3 modification on *GATA-3* gene following differentiation from naive CD4 T-cells upon primary stimulation. Subsequent stimulation was required for polarization which was accompanied by removal of the H3K27me3 signature from *GATA-3* gene [297]. In line with these observations, the "Progressive T-helper cell differentiation" model suggests that naïve cells contain genes in a "poised" form which convert to a bivalent state in effector cells and permits flexibility of cellular function. Sustained polarizing stimulation would lead to either permissive (H3K4me3) or repressive (H3K27me3) chromatin modification which results in generation of terminally differentiated effector cells with restricted potential for plasticity [406]. This model was supported by study which

demonstrated that polarized long term Th1 cells acquired from repeated restimulation with IL-12 could not convert to Th2 cell whereas short term Th1 cells (1 week) obtained Th2 phenotype in the presence of IL-12 and IL-4 [407].
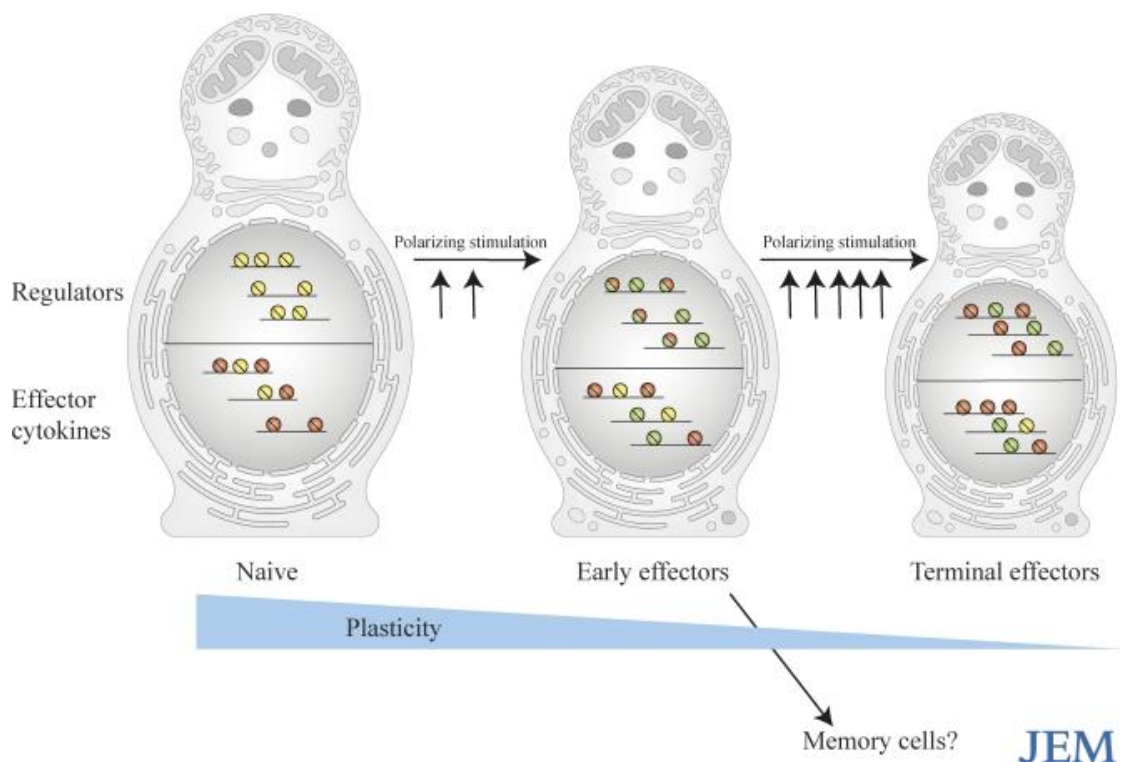
Figure 1.11: Epigenetic modifications accompany progressive T helper cell differentiation. (A) Naive T cells have master regulators (top) in receptive "poised" states (yellow balls) and effector cytokines (bottom) in epigenetically silenced chromatin (red balls), although some cytokine genes are poised for rapid expression after TCR stimulation. (B) After one or two stimulations under polarizing conditions, daughter cells express similar cytokines from activated loci (green), whereas other cytokines become silenced (red) or maintain a poised state awaiting stimulation. Some master regulators, however, remain bivalent (red and green), sustaining flexibility in the cells that allows redirection of cytokine

expression if the inflammatory milieu changes. (C) With continued polarizing stimulations, master regulators become silenced (red) or activated (green), leaving cells with an essentially fixed repertoire of cytokine effectors (bottom) caused by the "loss" of genetic space. Memory cells, which arise early after initial antigen stimulation, would be predicted to maintain more bivalent states at master regulators, thus leaving a more flexible cytokine repertoire in memory cells as compared with terminal effector cells. (Adapted from Locksley, *Journal of Experimental Medicine*, 2009 [406])

The observations related to T-cell plasticity are of particular importance in elucidating T-cell immune responses contributing to the disease pathogenesis and its implications raise concern for immunotherapy based on adoptive transfer of T-cells such as nTreg and CAR-T cell therapy for the treatment of autoimmune disease and cancer respectively [408]. This is also particularly relevant in the field of Tr1 cells, where multiple routes and vague criteria are used to define the Tr1 cells. Given the above points it is possible that many of the definitions of Tr1 cells are actually Th1 or other lineages that have been induced to express IL-10. Nonetheless, it has also been acknowledged that the plasticity of T-cells is required in order to adapt to the immunological cues and the reprogramming of established T-cell responses could also provide us with opportunity for therapeutic intervention where pro-inflammatory Th1 or Th17 cells driving auto-immune responses could be targeted to produce anti-inflammatory IL-10 instead.

1.7 The role of CD55 and CD97 in T-cell immunity

CD55, originally defined as Complement Decay Accelerating Factor (CDAF), is a glycoprotein encoded by a gene on Chromosome 1. It is a 70 kDa glycosylphosphatidylinositol (GPI)-anchored protein which disrupts the C3 convertases (C4b2b / C3bBb) and prevents C3b deposition on the cell surface and the subsequent formation of the membrane attack complex in both Classical and Alternative pathways of Complement system [409]. Initially only two isoforms of CD55 were identified- 1. GPI-anchored membrane bound protein (gCD55^{wt}), 2. A soluble form of CD55 (sCD55). While the gCD55 is expressed by all tissues, the soluble CD55 has also been detected in plasma, tears, saliva, urine, faeces, synovial and cerebrospinal fluids [410]. More recently, five more isoforms of CD55 were isolated from human lungs tumours, including three alternate soluble forms and two membrane bound forms containing intronic sequences. [411].

CD55 is expressed by all cells including erythrocytes, lymphocytes, dendritic cells, epithelial and endothelial cells etc. [412]. In normal epithelial and endothelial cells, CD55 expressed at low level. However, its expression is increased up to 40 times in tumours [413]. The dysregulation of CD55 expression in various cancers has also been reported by many studies [413-418]. Interestingly, it was reported by several studies that certain isoforms of CD55 were upregulated in neoplastic tissue. It has been suggested that the isoform gCD55^{int7+}, containing complete sequence of intron7, might serve as a marker for cancer diagnosis and could be a suitable targeted for immunotherapy as

gCD55[int7+] [419]. CD55 has already been used a target molecule for the treatment of Non-metastatic Gastric cancer [420] and Anti-CD55 IgM antibody (PAT-SC1, Patrys limited) has demonstrated promising results by increasing the survival rate of the patients in clinical trials. It was also reported that neutralizing antibody against CD55 increase the efficiency of anti-cancer drug Rituximab [421].

Although CD55 has been known as a complement regulatory protein, only recently its role as a mediator of T-cell immunity has emerged [422, 423]. To modulate the function of T-cells, CD55 interacts with its ligand CD97, which is an Epidermal Growth Factor seven-span Trans-membrane (EGF-TM7) receptor and known as an early leukocyte activation marker [424]. There are several isoforms of CD97, which arise due to alternate RNA splicing, and the smallest isoform (EGF1,2,5) binds to CD55 with highest affinity.

In MS, CD55 and CD97 were noted to be expressed by the infiltrating pre-active and active lesions. Also, the level of soluble CD97 in the serum of MS patients was significantly higher compared to the heathy individuals and it might influence the interaction of cellular CD97 interaction with membrane bound CD55 on T-cells [425]. Interestingly, biochemical and structural analysis revealed that the binding site for CD97 on CD55 is located in domains 1 and 2 and on the opposite face to the site that is involved in the complement regulation [426]. It has also been demonstrated that CD55-CD97 interaction does not impair its ability to inhibit complement. So, CD55 can simultaneously regulate T-cell function and the complement system.
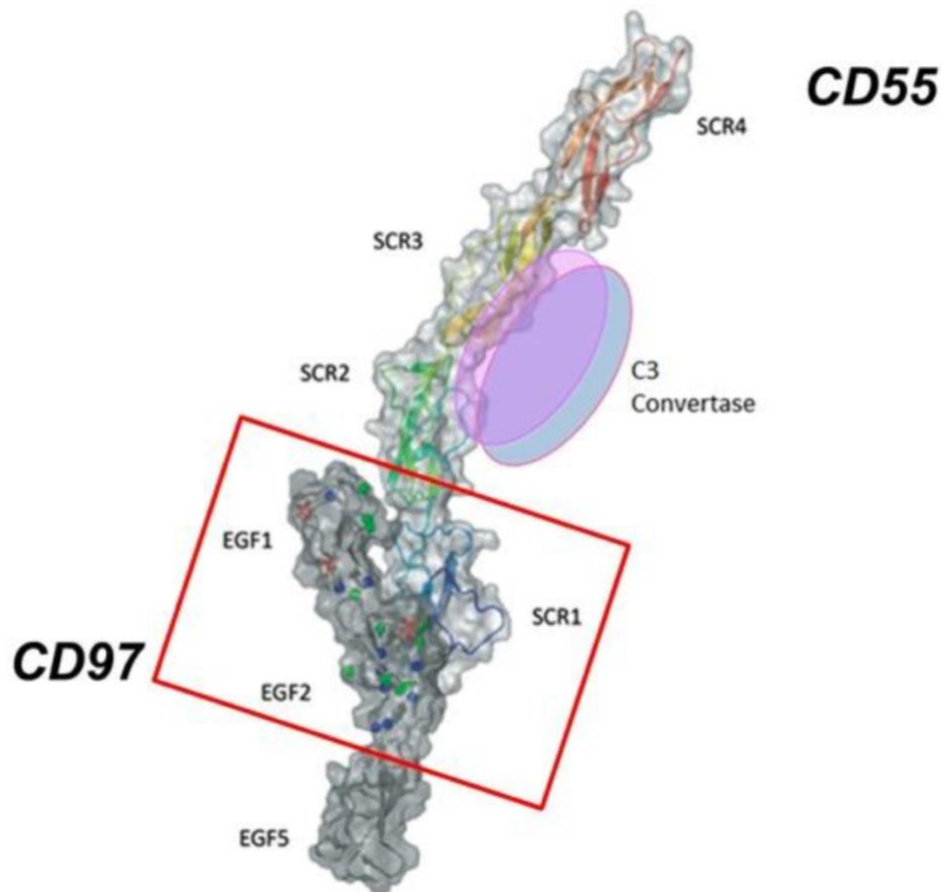
Figure 1.12: Intermolecular interaction between CD55 and CD97.
CD55 has the ability to interacts with its cellular ligand CD97 which expressed by antigen presenting cells and C3 as well as C5 convertases of the complement system. The N-terminal SCR1 domain of CD55 interacts with the N- terminal EGF1 and EGF2 domains of CD97, while a hydrophobic patch cantered on the linker between the SCR2 and SCR3 domains of CD55 binds to the convertases. (Adapted from Abbott et al., *J Biol Chem.*, 2007 [426])

CD55 co-stimulation, via CD97 or by crosslinking Antibody (Ab) in conjunction with CD3 (CD55/CD3) co-engagement on CD4^{+} T-cells leads to enhanced proliferation, expression of activation markers such as CD69, CD25 as well as cytokine production [386]. The CD55 co-stimulation of CD4^{+} T-cell is independent of its role in complement inhibition and comparable to CD28 and CD3 (CD28/CD3) co-engagement.

IL-10 is produced upon CD55 costimulation in the presence or absence of IL-2. The addition of IL-2 increases proliferation, activation, IL-10 and IFN-γ in a mixed population [386]. As elevated IL-10 production by CD4$^+$CD25$^+$ T-cells is one of characteristics of Regulatory T-cells, further investigation revealed that CD55 indeed is associated with induction of discrete Type1 Regulatory T-cell (Tr1) which represent only $\sim$ 0.5-4.5% of the cell population [315].

The potential of CD55 costimulation to induce activation, IL-2 production and proliferation of naïve CD4+ T-cells is comparable to CD28 costimulation even though they lead cells to differentiate into Tr1 (IL-10^{High,} IFN-γ^{Low}, IL-4$^-$) and Th1 (IL-10$^-$,IFN-γ^{High},IL-4$^-$) phenotype, respectively. CD55 induced CD4$^+$ T-cells maintain their IL-10$^+$ phenotype over multiple rounds of restimulation by CD55. Although no specific unique surface marker has been established to define the Tr1 phenotype, many studies reported similar cytokine profile to identify these regulatory T-cells [427-430] and the cell population obtained after CD55 induction demonstrated similar properties (IL-10^{High} IFN-γ^{Low} IL-4$^-$). CD55 co-stimulation also induces the expression of some of the markers that have been associated regulatory phenotype of T-cells such as CD49b, LAG-3 and CD226 [431].

The primary stimulation of T-cells with CD55 gives rise to two distinct populations - a small IL-10$^+$ population which produces high level of the suppressive cytokine and a larger IFN-γ^+ population. The restimulation of these cells with CD55 results in expansion of both populations. Notably

CD55 was capable of maintaining IL-10 production in the Tr1 cells and also restimulated the Th1 cells. However, CD28 was only capable of restimulating the Th1 cells and the Tr1 cells failed to produce IL-10, suggesting that CD55 is the driving force of IL-10 production by Tr1 and once committed the Tr1 cells are unresponsive to CD28.

The CD55 induced Tr1 cells also demonstrated the ability suppress proliferation of CD28 co-stimulated cells via IL-10, as neutralizing the cytokine abrogated the inhibitory effect [315]. The differentiation of naïve CD4 T-cells into Tr1 by CD55 demonstrated a novel receptor mediated route to Tr1 induction.

1.8 Role of regulatory T-cells in Multiple Sclerosis (MS)

Multiple sclerosis (MS) is an autoimmune, chronic inflammatory disease of the central nervous system characterised by demyelination and axonal loss. The aetiology of MS is still unknown but several genetic factors (both MHC and non-MHC genes) as well as environmental factors (vitamin D, lowered UV radiation exposure, cigarette smoking, obesity, and EBV exposure) have been associated with the development of MS. The clinical prognosis of MS can be divided into three stages: 1) a pre-clinical stage when both genetic and environmental factors initiate the disease and it is detectable only by MRI; 2) a relapsing-remitting (RRMS) stage characterized by episodes of neurologic dysfunction followed by resolution; and 3) a progressive stage, which usually evolves from the relapsing stage [432, 433]. The neurodegeneration leads to irreversible

disabilities in MS patients and it was reported that decreased IL-10 associated with higher disability in secondary progressive MS patients [434]. Immune cells, including lymphocytes and monocytes contribute to the inflammation which results in compromised blood-brain barrier and lesion areas become oedematous [433]. There are ten FDA approved immunotherapeutic or disease modifying treatments (DMT) for MS which target T cells, regulatory cells, B cells, and cell trafficking into the nervous system. Interestingly, emerging data suggests that there is not only predominance of T-cell mediated inflammation, the regulatory T-cells function is also impaired in MS patients which might contribute to the disease pathogenesis [435-442]. Importantly, high frequency of FoxP3$^+$ nTreg cells obtained Th1-type characteristics with secretion of high IFN-γ was determined in MS patients and these cells demonstrated reduced suppressive activity in vitro [443, 444]. In addition, it was also reported that CD46 mediated differentiation of Tr1 cell was altered in MS and IL-10 production in response to CD46 costimulation was significantly reduced in the MS patients in comparison to heathy individuals [6]. Interestingly, Vitamin-D3, which has immuno-modulatory potential, was able to rescue the IL-10 production by CD46 induced Tr1 cells in MS patients [445, 446]. IL-10 serves a protective role in MS as it has been demonstrated that decreased IL-10 is associated with the disease progression and dysregulation of both IFN-γ as well as IL-10 has been implicated to contribute to immunopathogenesis in MS patients [442, 447-449].

1.9 The effect of immune-modulators on pro-inflammatory and anti-inflammatory cytokine production by T-cells

Immune-modulators are therapeutic agents which have the potential to reinstate the immune balance by enhancing or curtailing pro-inflammatory and anti-inflammatory immune responses. In the recent years, immune-modulator or disease modifying treatments (DMTs) have been used for the treatment of many auto-immune diseases including MS. Reports from several studies indicate that the frequency of self-reactive effector cells might be higher in auto-immune disease patients while the immunosuppressive function of regulatory T-cells is compromised [450, 451]. These immune-modulators affect the T-cells immunity- particularly regulatory T-cell responses via direct and indirect mechanisms. Immune-modulators suppress the pro-inflammatory auto-reactive immune responses by promoting the anti-inflammatory function of regulatory T-cells. For example, it was demonstrated that dexamethasone treatment resulted in expansion of nTreg *in vivo* which led to prolonged survival of the transplanted graft in the GVHD mouse model [452]. It was also demonstrated that $CD4^+CD25^+$ cells expressed higher levels of glucocorticoid receptors and those cells more resistant to dexamethasone mediated apoptosis compared to $CD4^+CD25^-$ cells [453]. Similarly, it was reported that IFN-β treatment significantly enhance IL-10 production in relapsing-remitting multiple sclerosis

(RRMS) patients compared to healthy donors while no sustained effect was observed in the level of IFN-γ [454, 455]. Although the level of IL-10 secreting cells were lower in untreated patients, longitudinal studies demonstrated that the IL-10 producing cells increased over the period of treatment whereas the IFN-γ secreting cells did not alter significantly [348, 456]. It was also reported that blocking IL-10 by using antibody abrogates the IFN-β mediated suppressor cell function [457].

Vitamin D3 is considered as a candidate for immunotherapy because of its immuno-modulatory potential, its deficiency being associated with development of MS as well as its protective role in the established disease by reducing clinical activity [458-461]. Interestingly, CD97 is an early responding Vitamin-D3 target gene which contains one prominent Vitamin D3 receptor (VDR) binding site (P1$_{CD97}$) 56 kb upstream and a minor VDR-binding site (P2$_{CD97}$) 250 kb upstream of its transcription start site (TSS) and in monocytic cells, the gene was determined to be in accessible chromatin structure [462]. As the both Vitamin D3 and Tr1 cells have been implicated to be involved in the dysregulated immune responses in MS, it would be interesting to study if vitamin D3 affects the induction of CD3/CD55 induced Tr1 cells in MS patients.

1.10 Hypothesis and aim

The emerging role of alternative costimulatory molecules, such as CD46, CD55 etc. on the induction of Tr1 cells demonstrated the differential effect of costimulatory signal on the initiation of diverse T-helper cell responses [6, 315]. It also emphasizes the role of Tr1 cells in the dysregulated immune responses in autoimmune diseases including Multiple sclerosis [6]. Moreover, the effect of immune-modulators on altering the cytokine profile to promote immuno-suppressive IL-10 production by both CD28 induced Th1 cells and CD46 induced Tr1 cells have been reported [321, 445]. In the light of these observations and following on from our previous work on the induction of Tr1 cells by CD3/CD55 costimulation, we hypothesized that:

1) CD55 induced Tr1 cells in MS patients would be altered compared to normal donors.

2) Immune-modulators such as Vitamin D3, Dexamethasone and IFN-β would alter the regulatory function of CD55 induced Tr1 cells by affecting IL-10 and IFN-γ production

3) Competition between CD55 and CD28 would alter the induction of Tr1 cells.

4) Dendritic cells expressing CD97 could induce Tr1 differentiation *in vitro*.

2 Chapter 2: Materials and Methods

2.1 Ethics and Donors

Blood was collected from each donor after all donors provided written consent according to the Ethical rules of Nottingham City Hospital and University of Nottingham. Blood taken from healthy donors was covered by Nottingham Research ethics approval: 161-1711. Blood obtained from MS patients was covered by Nottingham Research Ethics approval to Dr. Bruno Gran (Project: Role of immune system in Multiple sclerosis).

2.2 Isolation of T-cells

2.2.1 Isolation of Peripheral Blood Mononuclear Cells (PBMCs)

Blood was collected in heparin coated tubes (for volumes upto 40 mL) or syringes (for volume over 40 mL) from the peripheral circulation of healthy donors and Multiple Sclerosis (MS) patients on the day of the experiment. Peripheral Blood Mononuclear Cells (PBMC) were isolated from blood by using density gradient centrifugation following an optimised protocol. Briefly, blood was diluted with sterile Dulbecco's Phosphate Buffer Saline (DPBS; Product code: D8537- Sigma Aldrich) in 1:1 ratio and 35mL of the diluted blood was layer over 15mL of Histopaque®-1077 (Product code: 10771, Sigma Aldrich) in a 50mL conical tube. This was immediately centrifuged at 800g for 25 minutes

with deceleration set to one to ensure clear separation of PBMC layer. After centrifugation, the PBMC layer (the distinct white interface between Histopaque® and plasma was harvested, and cells were washed twice with additional amount of DPBS (1:1 dilution) at 300g for 10 minutes. After the second wash, the supernatant was discarded and the cell pellet was disrupted. Cells were resuspended in DPBS and counted using a haemocytometer. Dead cells were excluded from the count by inclusion of trypan blue prior to counting (2.2.2). PBMCs were either used for isolation of Naïve CD4$^+$T-cells (described in 2.2.3) / 2.2.4(what about total CD4s) or labelled with cell proliferation dyes (described in 2.6) for various experiments.

2.2.2   Determining the number of isolated cells

The number of cells were determined using a Haemocytometer using Trypan Blue dye exclusion to exclude any dead cells from the cell count. 10μL of cell suspension was sampled and mixed with 10μL of Trypan blue and transferred onto the Haemocytometer chamber for counting on a light microscope. Four quadrants were counted, dead cells excluded, and total cell count determined according to the equation below.

Total Cells= $\frac{\text{Total number of cells counted}}{4}$ X Dilution factor for Trypan Blue X 10^4 X Volume of cell suspension

2.2.3   Isolation of Naïve CD4$^+$ T-cells

Naïve Human CD4$^+$ T-cells were isolated from PBMCs using Naive CD4$^+$ T Cell Isolation Kit II (Product code: 130-094-131, Miltenyi Biotec, Germany) using a negative selection process. After isolating the PBMCs and washing them with DPBS, the supernatant was discarded, and cell pellet was disrupted before resuspending in 40μL of MACS buffer per 10^7 cells. Then 10μL of biotin-conjugated monoclonal antibody cocktail, constituted of antibodies against CD8, CD14, CD15, CD16, CD19, CD25, CD34, CD36, CD45RO, CD56, CD123, TCRγ/δ, HLA-DR, and CD235a (Glycophorin A) which would bind to the non-target cells, were added to the cell suspension and cells were incubated at 4°C for 5 minutes. Following the incubation period, 30μL of buffer was added per 10^7 cells and 20μL of microbead-conjugated antibody cocktail containing Anti-CD61 and Anti-biotin antibody were added to the cells for 10 minutes at 4°C before the cell suspension was put through a column attached to a magnetic field. The flow through from the column was collected and it contained the unlabelled, enriched Naïve CD4$^+$ T-cells (CD45RA$^+$, CD45RO$^-$). The cells were washed with DPBS at 300g for 10 minutes and resuspended in T-cell culture media before they were used for other experiments. The purity of the isolated cells was assessed by Flow Cytometry (cells were only used for experiment if the purity was more than 97%), based on the expression of CD45 RA and CD45RO.

2.2.4   Isolation of total CD4$^+$ T-cells

Human CD4$^+$ T-cells were isolated from PMBCs by positive selection using microbead-conjugated Anti-CD4 antibody (CD4 Microbeads, Human; Product code- Order no. 130-045-101; Miltenyi Biotec, Germany). PBMCs were counted and washed with DPBS at 300g for 10 minutes. Then the supernatant was discarded, and cell pellet was disrupted before resuspending cells in 80μL of buffer per 10^7 cells. After preparing the cell suspension, 20μL of CD4 microbeads were added per 10^7 cells and mixed gently. Then the cells were incubated at 4°C for 15 minutes and 20 mL of buffer was added before the cells were centrifuged at 300g for 10 minutes. Following the centrifugation, the supernatant was discarded, and cells were resuspended in 500μL of buffer. Cell isolation column (MS or LS column depending on the number of expected yields of isolated cells) was placed into a magnetic field and was prepared by flushing with buffer before the cell suspension was applied onto the column. The microbead-conjugated antibody bound CD4$^+$ cells would remain inside the column and the unbound cells would flow though the column representing the non-target cells. Once the column was empty, it was washed with appropriate amount of buffer three times and the column was removed from the magnetic field in order to recover the positively selected CD4$^+$ T-cells. These were washed with DPBS for 10 minutes at 300g and were resuspended in T-cell media. The purity of the isolated cells were evaluated by Flow cytometry (based on CD3 and CD4 expression) and cells were only used for the experiments if the purity of the enriched cells were more that 97%.

2.3 In vitro culture of isolated Naïve CD4$^+$ T-cells

2.3.1   Cell culture media

In all experiments (except antigen specific stimulation experiments), cells were cultured in T-cell Medium which consisted of RPMI-1640 supplemented with 10% Heat inactivated Foetal Bovine Serum (FBS), 1% Non-essential Amino acid, 1% 2nM L- Glutamine, 1% Penicillin-Streptomycin, 1% Sodium pyruvate and 2% HEPES buffer (all the components were purchased from Sigma-Aldrich). For the experiments where T-cell responses to antigen was studied, autologous plasma derived from donor's blood was used in T-cell media instead of FBS.

2.3.2   Preparing antibody coated plate for cell stimulation

High binding 96-well plate (Product code:3361, Corning® Costart®) and 48-well plate (Product code:3548, Corning® Costart®) were pre-coated with antibodies overnight at 4°C. Antibody solution was removed by aspiration prior to addition of cells for culture. 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) along Anti-CD3 antibody (Clone–OKT3, Mouse IgG2a,in-house) were diluted in sterile PBS to prepare the antibody solution. All antibodies were titrated to determine optimal concentrations. Both Anti-CD55 and Anti-CD28 antibody was used at 5μg/mL whereas Anti-CD3 antibody was used at the sup-optimal concentration of 1μg/mL. Once Anti-CD3/Anti-CD55 and Anti-CD3/Anti-CD28 antibody solutions were

prepared, 200μL and 1mL was added per well of 96-well and 48-well plate respectively.

2.3.3 Cell culture conditions

Isolated Naïve CD4$^+$ T-cells were suspended at 0.5X10^6 cells/ mL in T-cell media (TCM) which was supplemented with rhIL-2 (50IU/mL). For all the experiments, 0.1X10^6 cells (200μL of cell suspension) were used for culture per well of 96-well plate whereas 0.5X10^6 cells (1000μL of cell suspension) were used per well of 48-well plate.

2.3.4 Primary and secondary cell stimulation

Purified Cells were stimulated with combinations of antibodies (anti-CD3, anti-CD55, anti CD28). Primary stimulation was for three days under standard culture conditions. Secondary stimulation of naïve cells took place after 7 days rest, usually day 10. The restimulation conditions were the same as for primary stimulation and analysis was carried out between 24 and 72 hours, depending on the assay.

Naïve CD4$^+$ T-cells were stimulated with plate bound antibodies for the primary stimulation or secondary stimulation (preparation of plates described in 2.3.2). Usually for primary stimulation, Anti-CD3 along with either Anti-CD55 or Anti-CD28 was used to stimulate the isolated naïve CD4$^+$ T-cells in presence or absence of immune-modulators (described in the section 2.4) on Day 0 (the day cells were isolated) and the activated cells were cultured for 72 hours (till Day3) at 37°C, 5% CO_2 in the

incubator. On Day 3, cells were used to evaluate their cytokine production and to identify their characteristic phenotype. If the experiment required secondary stimulation to study the cells upon restimulation, they were harvested on Day 3 following primary stimulation and transferred to either 48-well plate or 24-well plate depending on the number of the cells. Then the cells were rested for another 7 days. During the resting period, cells were monitored every day and the half of the cell culture media was replaced if needed. On Day 10, the resting cells were harvested and washed with warm TCM at 300g for 10 minutes and resuspended in fresh TCM supplemented with rhIL-2 (50IU/mL) before they were restimulated under various conditions.

2.4 Immune-modulators

Cells were treated with various immune-modulators including; Dexamethasone (Aspen, 3.8 mg per vial) 10^{-3} M stock in DMSO, Vitamin-D3 (Product code: D1530-1mg) 10^{-2} M stock in ethanol and IFN-β (Betaferon, 250 microgram/mL equivalent to 8×10^6 IU/mL, Bayer Global Pharma). Dilutions of these stocks of immune-modulators were prepared on the day of experiment to study their effect on cytokine production by the cells following Anti-CD3/CD55 and Anti-CD3/CD28 stimulation. All stocks were stored at -20°C and diluted in TCM prior to use.

2.5 Cell proliferation assays and Cell cycle analysis

2.5.1　　Cell labelling with CellTrace™ Violet (CTV)

CellTrace™ Violet (CTV) is a cell proliferation dye which was used to monitor the proliferative responses of CD4$^+$ T-cells to various stimuli such as co-stimulatory signal and antigen-specific stimulation. Briefly, stock solution of Cell Trace Violet (Product code- C34557, Invitrogen, ThermoFisher Scientific) was prepared by adding 20μL of sterile Dimethyl Sulfoxide (DMSO; Product code- ,Sigma Aldrich) per vial of CTV and gently mixing it by using a vortex to obtain a 5mM stock. A working concentration of 5uM, 1μL of stock solution per 999μL PBS, was used to label cells. CTV solutions were protected from exposure to light by covering it with foil. Isolated Naïve CD4$^+$ T-cells or PBMCs were washed at 300g for 10 minutes with excess (>2ml) amount of sterile DPBS per 1X10^6 of cells to remove any FBS / autologous plasma present in cell isolation buffer. After centrifugation, the supernatant was discarded and the cell pellet was disrupted. Cells were resuspended with 1mL CTV per 1X10^6 cells, mixed immediately and incubated at room temperature in the dark for 10 minutes (determined based on optimisation experiment). FBS or autologous plasma containing TCM was added to quench the remaining unbound dye and incubated for another 5 minutes. Cells were then washed at 300g for 10 minutes and resuspended in appropriate TCM for other experiments.

2.5.2   Thymidine incorporation assay

The effect of various stimuli on the proliferation of CD4$^+$ T-cells was determined by thymidine incorporation assay. Naïve CD4+ T-cells (1×10^5/well) were stimulated with various combination of plate bound antibodies in 96-well plate for 72 hours and later pulsed with 0.5µCi [3H] Thymidine (Amersham Bioscience) for 16 Hours at 37°C, 5% CO$_2$ in the incubator. After the incubation period following addition of thymidine, cells were harvested on a 96-well harvester filter (Filtermate196, Packard/PerkinElmer) and incorporated radioactivity was measured using a TopCount Scintillation Counter (Packard/PerkinElmer) to determine T-cell proliferation.

2.5.3   Cell cycle analysis by Propidium staining

The cell cycle analysis was performed by Propidium Iodide (PI) DNA staining. Cells were harvested after 72 hours of cell stimulation and washed with PBS. Then supernatant was discarded and single cell suspension was prepared before cells were fixed with cold 70% ethanol for 45 minutes at 4°C. Then cells were washed with PBS at 500g for 10 minutes before cells were treated with ribonuclease by adding 50 µl of a 100 µg/ml sock of RNase (Sigma Aldrich). Finally, 10µL of PI (stock solution 1mg/mL, Sigma Aldrich) was added to the cells and these samples were used to obtain data by flow cytometry to determine the cell cycle.

2.6 Isolation of Dendritic cell (DC) and DC : T-cell co-culture experiment

2.6.1 Isolation of CD14$^+$ monocytes and generation of Monocyte Derived Dendritic Cells (moDC)

The monocyte derived Dendritic cells were generated from the either fresh blood collected from healthy donors. The CD14$^+$ monocytes were labelled with Anti-CD14 antibody conjugated with microbeads (Human, Product code: 130-050-201, Miltenyi Biotec) and separated using magnetic columns following the manufacturer's protocol. The isolated CD14$^+$ monocyte cells were checked for viability and purity (more than 97% CD14+ pure cells were used for experiments) using flow cytometry. Purified cells were cultured for 5 days with Dendritic cell media (DCM; RPMI-1640 media with 10% FBS or autologous plasma and 1% sodium pyruvate) which was supplemented with GM-CSF (1000U/mL) and IL-4 (1000U/mL) to differentiate the CD14$^+$ monocytes into moDCs. On Day 3, half the amount of cell culture media was added along with GM-CSF and IL-4 (1000IU/mL each) to the cells. Immature Monocyte derived Dendritic Cells (moDC) were harvested on Day 5 for further experiments.

2.6.2 Isolation of CD1c$^+$ Dendritic cells

PBMC were isolated from healthy donors. Peripheral blood lymphocytes (PBL) were obtained by depleting PBMC of CD14$^+$ cells. PBL were used for isolation of CD1c (BDCA-1)$^+$ Dendritic Cells (Product no: 130-090-506, Miltenyi Biotec). PBLs were first depleted of CD19$^+$ cells to remove any

CD1c+ B-cells. The CD19 depleted PBLs were used to positively isolate CD1c$^+$ cells, using CD1c microbeads following the manufacturers recommendation. The isolated CD1c$^+$ cells were washed with PBS at 300g for 10 minutes and resuspended in DC culture media supplemented with GM-CSF and rested for an hour at 37°C and 5% CO_2 in the incubator before they were used for other experiments. Also, the CD1c$^-$ cells were collected, washed and resuspended in T-cell media supplemented with IL-2 before they were rested overnight. The CD1c$^-$ PBLs were later used for MLR experiments.

2.6.3   DC : T-cell co-culture experiments

DC: T-cell co-culture experiments were performed to study the effect of blocking CD97-CD55 interaction between DC and CD4$^+$ T-cells by using Anti-CD55 antibody (Clone: 791T/36, Type- Mouse IgG2b). moDC and CD1c$^+$ DCs as well as PBLs from the same healthy donor was used to set up the co-culture experiments. The CD1c$^+$ DCs were used for these experiments on the day of isolation whereas the moDCs were harvested and used for co-culture with T-cells on Day 5 following the initial CD14$^+$ Monocyte isolation (on Day 0). Briefly, $1X10^4$ CD1c$^+$ DCs or moDCs were used with $50X10^4$ PBLs (1:50 of DC: PBL) for each condition. In all conditions except the immature DCs (iDC), CD1c$^+$ DCs or moDCs were stimulated with PolyI:C (TLR3 agonist, Invitrogen) and R848 (TLR7 and TLR8 agonist, Invitrogen) and incubated for 24 hours to generate mature DCs. After the maturation of DCs, PBLs were added and incubated for another 24 hours before Cytokine Secretion Assay was performed to

evaluate the induction of a T-cell response. In experimental conditions where the effect of blocking of CD97-CD55 interaction between DC: T-cells were observed, the PBLs were pre-coated with either Anti-CD55 antibody (1μg/mL for 100X10^4 PBL/mL) or Isotype Mouse IgG2B antibody (1μg/mL for 100X10^4 PBL/mL) for 10 minutes at room temperature before they were added to the DCs.

2.7 Evaluation of cytokine production

2.7.1   Cytokine Secretion Assay (CSA)

Cytokine production by activated cells were evaluated by flow cytometry using Cytokine Secretion Assay (CSA). For the Dual CSA to determine IL-10 and IFN-γ production, IL-10 Secretion Assay – Detection Kit (APC), (Human, Product code: 130-090-761, Miltenyi Biotec) and IFN-γ Secretion Assay – Detection Kit (FITC) (Human, Product code: 130-090-433, Miltenyi Biotec) were combined. CSA was performed after 72 hours of the primary stimulation and 36 hours after secondary stimulation according to the optimised protocol.

To perform CSA, cells were harvested after the incubation period in a 15mL tube and washed with 5mL cold buffer (4°C) at 1500 rpm for 7 minutes. Then the supernatant was discarded to remove as much buffer as possible without disturbing the cell pellet. Then single-cell suspension was prepared and 40μL of cold T-cell media (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. The catch reagent (primary antibody) is a bi-specific antibody which recognizes CD45 as well as the cytokine (IL-10 for IL-10 CSA and IFN-γ for IFN-γ CSA). The samples were gently mixed before they were immediately transferred to ice, in order to prevent cytokine secretion, for 10 minutes. Following the incubation period, 5mL of warm TCM was added to each sample and placed in a rotating platform at 37°C for 45 minutes so that the cytokines could be secreted from the cells and bind to the catch antibody. Then 12mL of cold buffer was added to each tube and cells were washed at 300g for 10 minutes. After the wash, the supernatant was discarded and cell pellet was disrupted before the 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). The cells were mixed gently with the detection ab and incubated at 4°C for another 15 minutes. Lastly, cells were washed with buffer before the supernatant was discarded and cells were resuspended in fixing solution (PBS with 4% formaldehyde) for data acquisition using a flow cytometer.

Also, matched cells from the same experiment were used to prepare negative controls for the CSA experiment and they were processed in a similar way as describes in the previous section. However, the control samples were not labelled with the catch reagent and were only stained with the detection ab (instead of using isotype control) so that background level of non-specific binding by the fluorescent detection antibody could be determined.

2.7.2 Enzyme-linked Immunosorbent Assay (ELISA)

Culture supernatants were harvested after 72 hours of cells stimulation and they were used to determine IL-10 and IFN-γ production by cells using the Human IL-10 DuoSet ELISA (Product code: DY217B, R&D Systems) and Human IFN-γ ELISA Set (Product code: 555142, BD Biosciences) respectively, according the manufacturer's recommended protocol. Briefly, for IL-10 ELISA, high binding flat-bottom 96-well plates were coated with Anti-IL-10 (Human) capture antibody overnight at 4°C. Then the plate was washed three times with washing buffer (PBS with 0.05% Tween®20, pH 7.2-7.4) and blocked with the reagent diluent (1% BSA containing DPBS) for 1 hour at room temperature. Then plate was washed three times with the washing buffer and samples (cell culture supernatant) were diluted with reagent diluent prior to adding them to the plate. After incubating for another two hours, the unbound components were removed by washing with wash buffer before adding the detection antibody (dilution) and incubating for a minimum of two hours. Later, Streptavidin-HRP (dilution) was added for 1 hour, washed three times and substrate was added before reading the plates with a Microplate Reader at 450nm. The data collected from ELISA were analysed to determine the amount of IL-10 produced by the cells. This was achieved by reading off the sample data against a duplicate set of standards and using the equation of the line to determine sample concentrations. Similarly, the supernatants were also assessed for IFN-γ secretion by ELISA following the manufacturer's protocol.

2.8 Flow cytometry to study the expression of various markers

2.8.1 Extra-cellular staining for cell surface markers

Flow cytometry was used to determine the expression of various cell surface markers. In order to determine the level of expression of any marker by IL-10$^+$ Tr1 cells, extracellular staining was combined with CSA. The cells were harvested after 72 hours of cell stimulation (described in 2.3.4) and CSA was performed following the standard protocol (described in 2.9.1) until the step where the detection antibody is added to cells to determine cytokine production. At this stage, the antibodies specific for various cell surface markers (listed in Table 2.2) were added along with the detection antibodies for CSA. Then the cells are incubated at 4°C for 15 minutes and washed before the cells were resuspended in fixing solution (PBS with 4% formaldehyde) to obtain the data using a flow cytometer.

Table 2.1: Antibodies for cell surface markers

Marker	Clone	Fluorochrome	Manufacturer	Volume/ test (dilution)
LAG-3	17B4	FITC	Miltenyi Biotec	1:50
CD226	DX11	PE	Miltenyi Biotec	1:50
CD49b	AK7	FITC	Miltenyi Biotec	1:50
LAP	CH6-17E5.1	Vio-Bright FITC	Miltenyi Biotec	1:50
TIM-3	REA384	Vio-Bright FITC	Miltenyi Biotec	1:50
CTLA-4	BN13	PE	Biolegend	1:50
PD-1	PD1.3.1.3	PECy-7.0	Miltenyi Biotec	1:50
Isotype Control	REA Control(I)	Vio-Bright FITC	Miltenyi Biotec	1:50
Isotype Control	Mouse IgG1 (IS5-21F5)	PE	Miltenyi Biotec	1:50
Isotype Control	S43.10	Vio-Bright FITC	Miltenyi Biotec	1:50

2.8.2 Intra-cellular staining for Transcription factors

Cytokine Secretion Assay (CSA) was combined with Intracellular staining for Transcription Factor (TF) to determine the characteristics phenotype of the IL-10$^+$ Tr1-like cells. CSA was performed following the standard protocol (described in 2.9.1). After the final wash following CSA, a single-cell suspension was prepared by discarding the supernatant and disrupting the cell pellet. Usually cells were suspended in ~ 100µL of residual buffer and an Intracellular Staining kit (FoxP3 Staining Buffer Set, P130-093-142, Miltenyi Biotec) was used to proceed with the rest of the experiment. Then 1 mL of Foxp3 Fixation/Permeabilization buffer was slowly added to each tube while gently mixing buffer with the cell suspension by using the pulse vortex. After adding the buffer, the cells were incubated for 60 minutes at 4°C in the fridge and protected from light. Following the incubation period, 2 mL of 1X Permeabilization buffer was added to each tube and the samples were centrifuged at 500g for 10 minutes at room temperature. The supernatant was discarded, and the cell pellet was disrupted to make cell-suspension which was stained for various TF with fluorochrome-conjugated antibodies (listed in the Table 2.3) and incubated 45 minutes at room temperature in the dark. Then 2 mL of 1X Permeabilization buffer was added to each tube and the samples were centrifuged at 500g for 10 minutes. After the final wash, the supernatant was discarded, and the cells were fixed with the fixing solution (PBS with 4% Formaldehyde) and the samples were used for data acquisition on a flow cytometer.

Table 2.2: Antibodies for Transcription Factors

Marker	Clone	Fluoro-chrome	Manufacturer	Volume/ test (dilution)
FoxP3	3G3	PE	Miltenyi Biotec	1:50
T-bet	REA102	PE	Miltenyi Biotec	1:50
GATA-3	REA174	FITC	Miltenyi Biotec	1:50
ROR-γ_c	REA278	PE	Miltenyi Biotec	1:50
Helios	REA829	PE	Miltenyi Biotec	1:50
c-MAF	sym0F1	PE	eBioscience™	1:25
c-MAF	sym0F1	PerCp-eFluoro710	eBioscience™	1:25
Isotype Control	REA Control(I)	PE	Miltenyi Biotec	1:50
Isotype Control	Mouse IgG1 (IS5-21F5)	PE	Miltenyi Biotec	1:50
Isotype Control	REA Control(I)	FITC	Miltenyi Biotec	1:50
Isotype Control	Mouse IgG2b (eBMG2b)	PerCp-eFluoro710	eBioscience™	1:50

2.9 Data analysis

2.9.1   ELISA data analysis

The data obtained from the ELISA experiments were analysed with Microsoft Excel. Then the results were transferred to GraphPad Prism (Version 6.0) to prepare graphs and do statistical analysis.

2.9.2   Statistical Analysis

All statistical analysis for the data collected from various experiments were analysed using GraphPad Prism software (version 6). We thank Dr. Andrea Venn (School of medicine, University of Nottingham) for her kind assistance to review all the statistical analysis.

2.9.3   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. The data for the cell cycle analysis (PI staining) was also analysed with FlowJo (Version X).

2.10 Gating strategy for analysing Flow Cytometry data

2.10.1   Analysis of Cytokine Secretion Assay

Flow cytometry data analysis was required to interpret the data acquired from CSA assays which evaluate the cytokine production by cells.

Standard gating strategy was prepared at the beginning of the study and it was followed rigorously to analyse all the CSA data. To analyse the data, the dead cells and debris were excluded at the first step by looking at the Side Scatter (SSC-A) vs. Forward Scatter (FSC-A) and only the live cells were selected (called the Live cells population or gate). Then the doublets were excluded from the "Live cells" in order to avoid false positive signal by looking at Forward Scatter Height (FSC-H) vs. Forward Scatter Area (FSC-A). The cells selected (by placing a gate) at this stage is termed "Single cells". In the following steps, only the gated single cells will be included in the analysis. Then the background fluorescence level was determined by selecting appropriate gate on the "Detection Antibody Only" sample which is the negative control for CSA experiments. The gate from the negative control was applied to the test samples to evaluate the percentage of cells which produced the cytokine of interest.

A)

B)

C)

IL-10 APC

IFN-γ FIT

Detection
(Secondary)
Antibody only
Control
(Negative
control)

D)

IL-10 APC

IFN-γ FITC

Dual IL-10 and
IFN-γ Cytokine
Secretion Assay
(CSA)
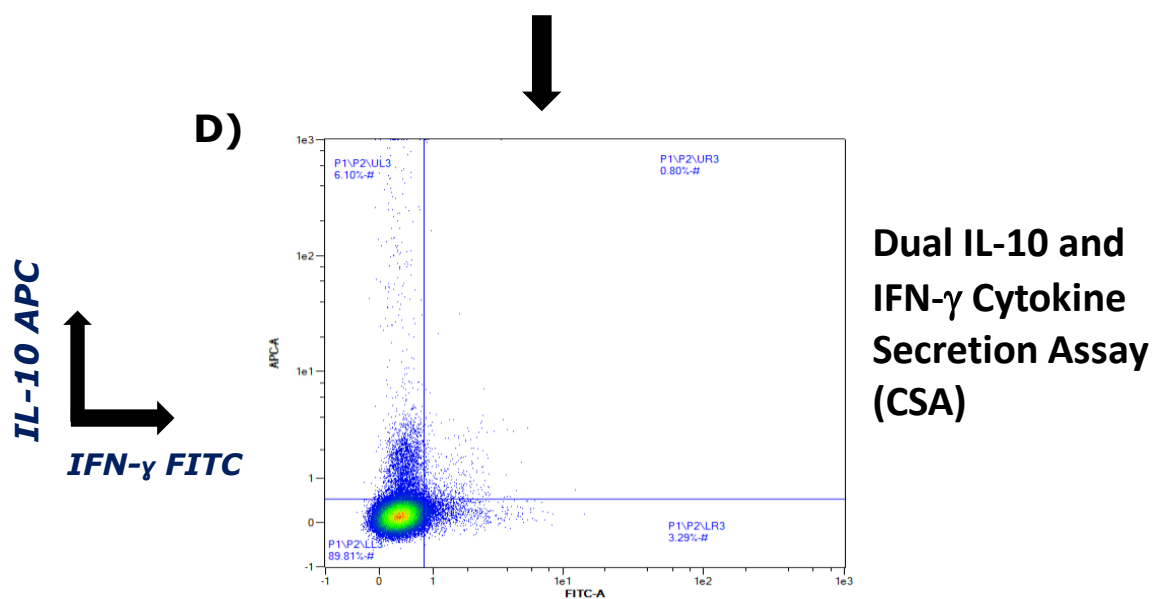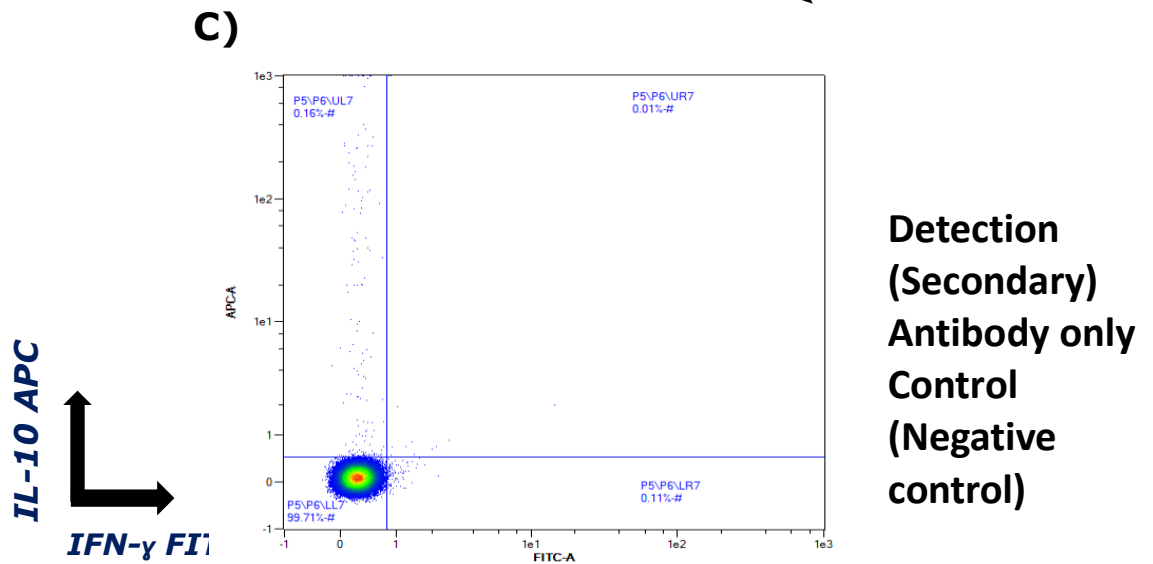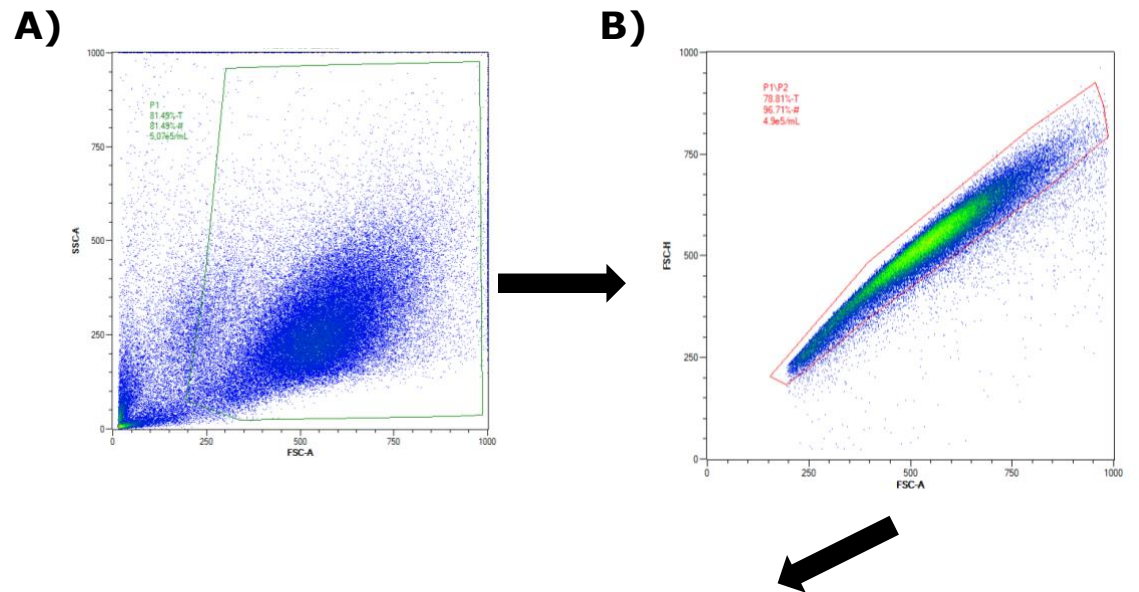
Figure 2.1: Gating strategy for IL-10 and IFN-γ CSA. Determination of IL-10 and IFN-γ secreting cells by Flow cytometry was performed after exclusion of dead cells (A) and doublets (B). Then gating of IL-10$^-$ IFN-γ$^-$, IL-10$^+$ IFN-γ$^-$, IL-10$^+$ IFN-γ$^+$ and IL-10$^-$ IFN-γ$^+$ cells on the basis of negative control (C). The gate selected on the negative control was applied on the test sample (D) to determine the cells which were positive for certain cytokines.

2.10.2   Analysis of Extracellular and Intracellular staining of cells

The data of extracellular and intracellular marker staining were analysed in a similar way as described in the previous section (in section 2.12.1). However, after excluding dead cell and doublets, the background fluorescence level was determined by selecting gates on isotype control which served as the negative control. Then the gate which had been set on the isotype control was applied to the test samples to determine the level of expression of marker of interest.

A)

Isotype Control PE
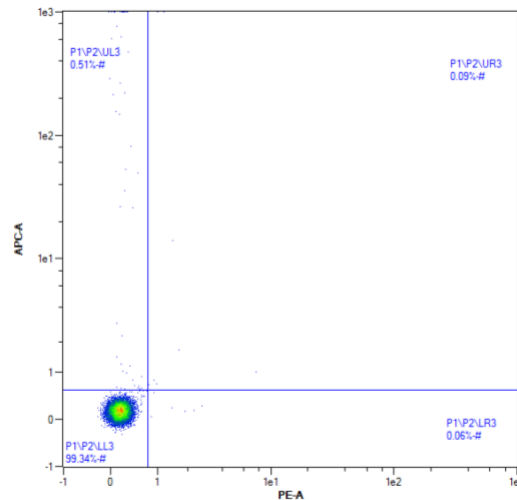
Negative control stained with the Isotype Control

B)
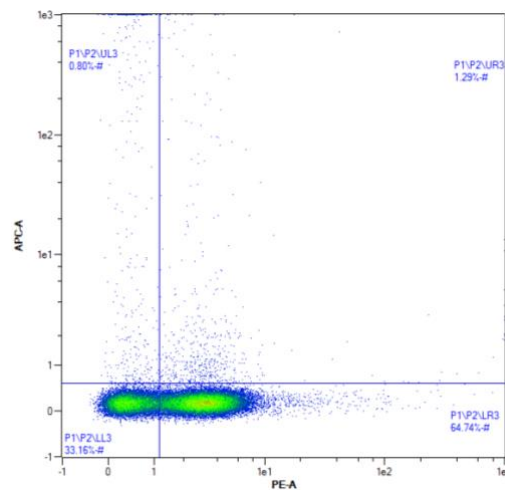
Test sample stained with the antibody specific for the marker of interest

Figure 2.2: Gating strategy for IL-10 CSA and intracellular staining. A) Expression of both extracellular and intracellular markers were determined by selecting gates based on the background fluorescence detected in the negative control (cells stained with isotype antibody). B) The gates from the isotype control were applied on the test sample to identify the cells which expressed the molecule of interest.

2.10.3 Analysis of Cell proliferation assay

Fluorescent cell proliferation dye CellTrace™ Violet was used to determine the effect of various experimental conditions on the proliferative response of the cells by Flow cytometry. Cells were assessed for proliferation after 72 hours of stimulation with either CD3/CD55 or CD3/CD28 in presence or absence of immune-modulators. Unlabelled cells as well as unstimulated CellTrace™ Violet labelled cells were used a negative control to determine the extent of proliferation of the test samples. The unstained cells as a control to estimate the highest number of cell cycle that could be accurately determined with the optimised experimental design whereas the unstimulated labelled cells were used to identify the cells which did not go through any cell division in response to different stimuli.
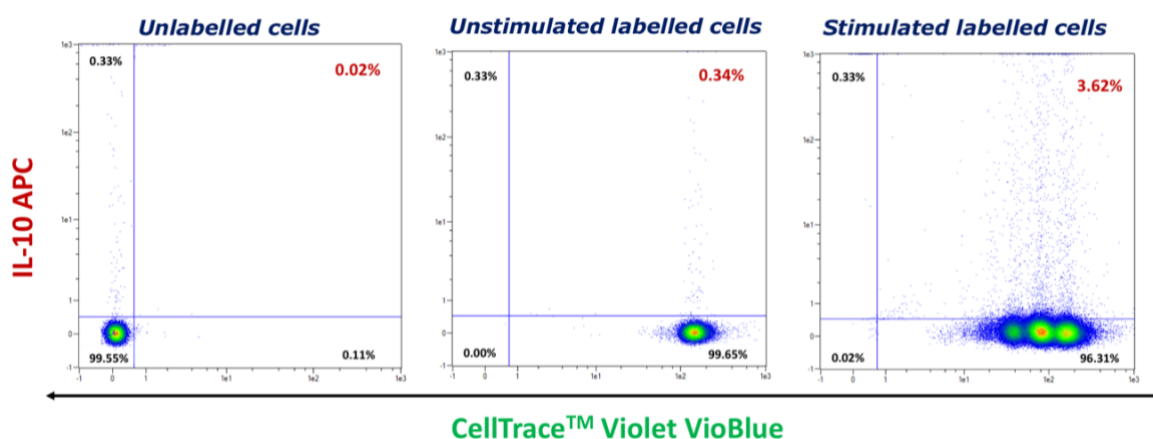
Figure 2.3: Determining cell proliferation by using cell proliferation dye. The proliferative response of cells in response various stimuli was monitored by using CellTrace™ Violet dye. Unstained cells along with unstimulated labelled cells were used to evaluate the proliferation of the test sample

3 Chapter 3: The comparative effect of CD55 and CD28 co-stimulation on the differentiation of Naïve CD4$^+$ T-cells and IL-10 production

3.1 Introduction:

The necessity of a "Second signal" accompanying the primary T-cell receptor signal, later termed co-stimulatory signal, to initiate a T-cell response was first proposed in the "Two-signal model" by Bretscher & Cohn [463] in order to explain a mechanism underlying immune response to self and non-self antigen as well as T-cell anergy. In the following years, Lafferty and colleagues [464-466] demonstrated that costimulatory signal between T-cell and Antigen Presenting Cells (APC) determined T-cell activation, cytokine production and proliferation upon encounter with cognate antigen and failure to provide appropriate costimulatory signal by chemically modified APC led to T-cell unresponsiveness [467, 468]. It has also been demonstrated that naïve T-cells not only require higher TCR signal compared to effector cells [366], they also differ in their absolute requirement for costimulatory signal. As signalling via only TCR results in apoptosis and Activation Induced Cell Death (AICD) [469, 470].

Costimulatory potential of specific signalling receptors has been defined by their ability to induce differentiation of naïve cell to effector cells, induce IL-2 production, prevent AICD and promote proliferation [358,

359, 471, 472]. Many costimulatory molecules have been discovered in the last few decades [370] and CD28 has emerged as the classical costimulatory molecule which interacts with CD80 (B7-1) and CD86 (B7-2) expressed on the APC [359, 472-478]. The CD28:CD80/CD86 signalling is considered canonical for differentiation and function of CD4+ T-cells as it induces cytokine production including IL-2, IL-4, IL-5, GM-CSF and IFN-γ [479, 480]; upregulates activation markers such as CD25 and CD69; promotes cell proliferation and primes cells for subsequent exposure to antigen [481-484]. Moreover, CD28 signalling is also required for a sustained immune response. CD28 deficiency led to impaired T-cell responses characterised by lack of IL-2 production, markedly reduced proliferation and inability to respond to lower dose of antigen [485-487]. Interestingly, it has been demonstrated that CD28 modulates not only Th1 cells, but also Th2 cells [488, 489] and nTreg [490, 491] while It was suppresses differentiation of naïve T-cells into Th17 cells [492-494].

Costimulatory molecules other than CD28 also mediate T-cell function and the role of these alternative costimulatory molecules has been studied in the recent years. It has been reported that Ox-40, a member of the tumour necrosis factor receptor (TNF-R) family, interacts with its ligand (Ox-40L) on dendritic cell and facilitates the differentiation of Naïve CD4+ T-cells into Th2 cells by enhancing IL-4, IL-5 and IL-13 production [495, 496]. It was also reported that Ox-40 prolongs clonal expansion following primary response and increases the cytokine production by both Th1 and Th2 effector cells [497, 498]. Another

member of TNF-R family, 4-1BB has also been reported to have costimulatory potential. Although it has been suggested that 4-1BB is more important for CD8 T-cell survival [499] and cytolytic ability [500], it has been demonstrated that 4-1BB induces cell proliferation, cytokine production and prevents cell death in CD4$^+$ cells [501-504] and both Ox-40 and 4-1BB might work in a synergistic manner with CD28 in T-cells to attain optimal effector function [497, 505]. These observations highlight the importance of interplay of multiple costimulatory signals and how the dominant effect of certain costimulation could potentially override the influence of other signals.

Alternate costimulatory molecules are also important for the function of regulatory T-cells. It has been reported that some costimulatory receptors can drive the differentiation of inducible Treg cells. CD2 was the first costimulatory receptor that had been reported to induce differentiation of antigen specific Tr1 cells via interaction with its ligand CD58 on APC [428]. Later it was reported that CD2 costimulation is also important for the survival of nTreg as it prevented apoptosis by down-regulating pro-apoptotic factor Bim [506]. Several studies reported that signalling via another alternate costimulatory receptor CD46, in conjunction with CD3 leads to induction of IL-10 production by Type 1 regulatory cells (Tr1) [446, 507-509]. Moreover, our previous work demonstrated that CD55 costimulation induces differentiation of naïve CD4$^+$ T-cells into IL-10$^+$ Tr1 cells [315, 386].

The identification of alternate costimulatory receptors connotes that these molecules might act as response modifiers to the prepotent CD28, 41BB, Ox 40 and other co-stimulation. Given the abundance of costimulatory receptors, it is possible that competition between different stimulation determines the outcome of a T-cell response. We hypothesised that CD28 costimulation would subdue the response of CD55 costimulation, preventing Tr1 development in favour of a Th1 response. In this study, we determined the differential effect of CD55 and CD28 costimulation in the presence of suboptimal CD3 signal and demonstrated that the strength of competing costimulatory signal determines the fate of naïve T-cells.

3.2 Results:

3.2.1   Isolation of Naïve CD4$^+$ T-cells from PBMCs and evaluation of the enriched cells

Naïve CD4$^+$ T-cells, defined as CD4$^+$CD45RA$^+$CD45RO$^-$, were isolated from the blood of healthy individuals as well as MS patients (following the standard protocol described in 2.2). After the isolation of the cells, the purity of the cell population was assessed prior to setting up the experiments. Cells were only used for further experiments if the purity was >97%.
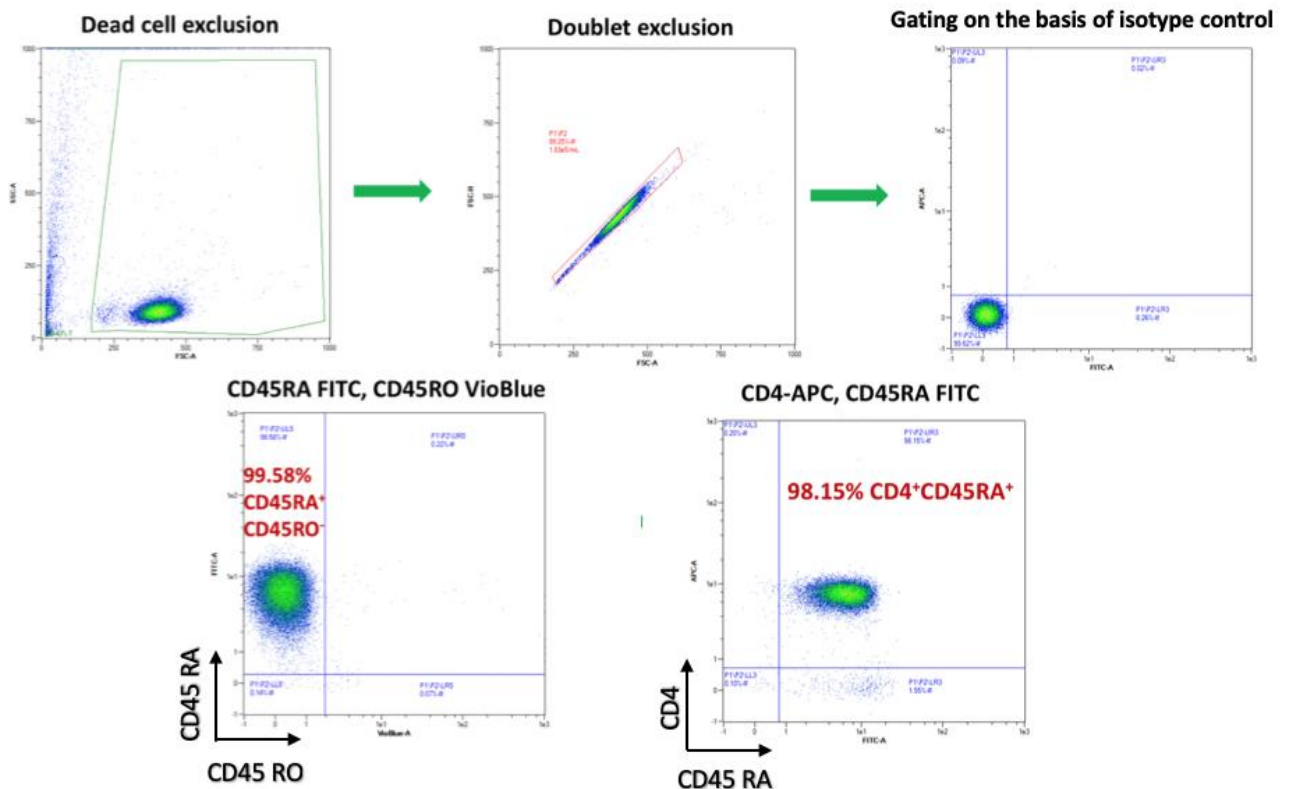
Figure 3.1: Assessment of the purity of isolated naive CD4+T-cells by Flow Cytometry. Dead cells and doublets were excluded before isolated cells were evaluated for the expression of CD4, CD45RA and CD45RO. Isotype control antibodies were used as negative control to determine the expression of the cell surface markers.

3.2.2 The comparative effect of CD3/CD55 and CD3/CD28 co-stimulation on IL-10 production

Costimulatory signals promote differentiation of naïve CD4$^+$ T-cells and results in various cytokine production as well as proliferation [358, 359, 471, 472]. Stimulating naïve CD4$^+$ T-cell with CD55 in the presence of suboptimal TCR signal (by using Anti-CD3 antibody) led to induction of a discrete IL-10$^+$ Tr1 population whereas stimulating cells CD28 resulted in more IFN-γ^+ than IL-10$^+$ cells (Figure 3.2). It was also noted that CD3/CD55 costimulation induced significantly higher amount of IL-10 production compared to CD28 costimulation (Figure-3.3).
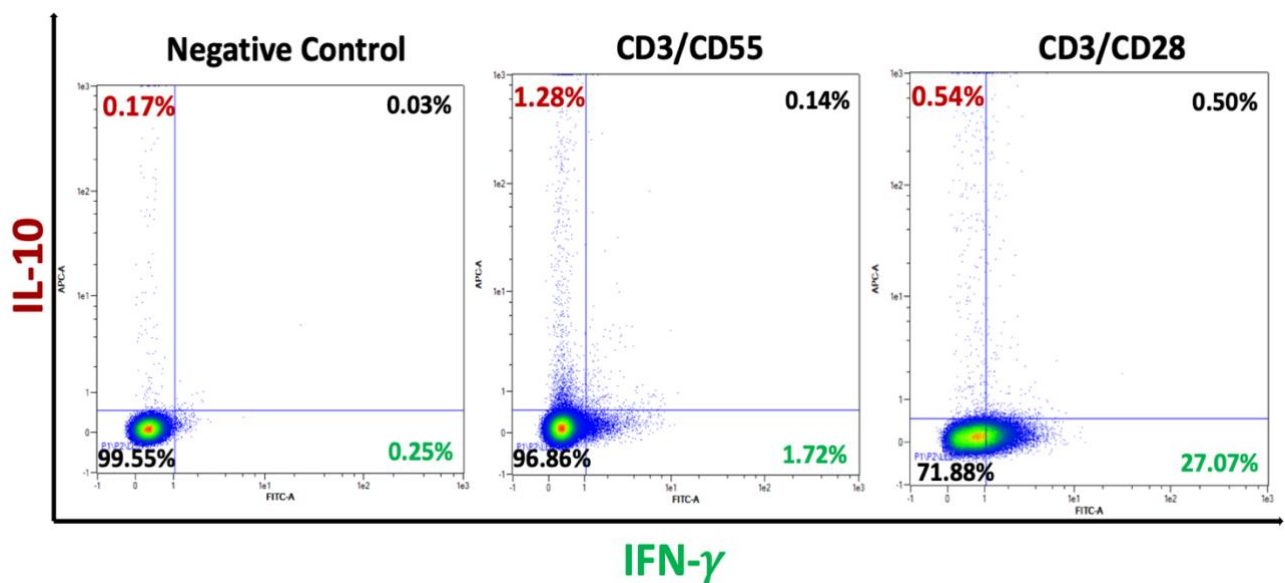
Figure 3.2: Detection of IL-10$^+$ and IFN-γ^+ cells following CD3/CD55 and CD3/CD28 costimulation. Naïve CD4$^+$ T-cells were isolated from PBMCs and stimulated with either CD3/CD55 or CD3/CD28 (1μg/mL of CD3 with 5. μg/mL CD55 or CD28). After 72 hours of cell

stimulation, dual cytokine secretion assay was performed to determine the IL-10 and IFN-γ secreting cells. Data is representative of four independent experiments.

IL-10 production after 72 hours of cell stimulation
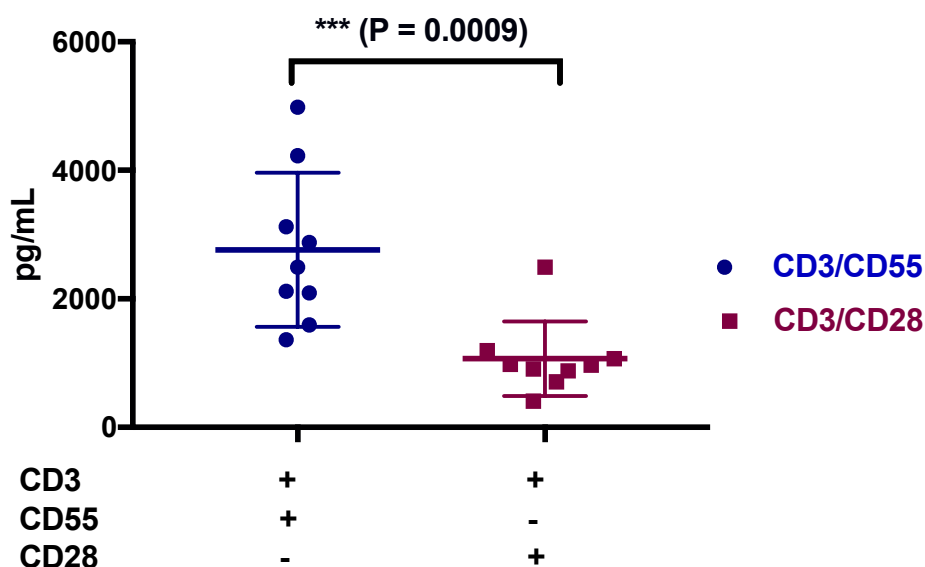
Figure 3.3: Comparison of IL-10 production by CD4 T-cells in response to CD3/CD55 and CD3/CD28 stimulation. Naïve CD4 T-cells were isolated from PBMCs and cells were stimulated with either CD3/CD55 or CD3/CD28 for 72 hours. Then the amount of IL-10 produced was evaluated by measuring the secreted IL-10 in the culture supernatant by ELISA. It was determined that CD3/CD55 costimulation induced significantly higher (P=0.0094) amounts of IL-10 production compared to CD3/CD28. The data represents cumulative results from seven independent experiments and triplicate for each condition was set up for all the donors. Triplicate wells were also used for IL-10 ELISA and average was used for statistical test (paired t-test).

In order to further investigate the differential effect of CD55 and CD28 costimulation on the cytokine production and cell proliferation, naive CD4$^+$ T-cells were stimulated with different combination of CD55 and CD28 in conjunction with constant dose of CD3 (1ug/mL). To determine the effect of CD28 costimulatory signal on the CD55, cells were stimulated with specific concentration of CD3 and CD55 and different concentrations of CD28 were titrated into the selected CD3/CD55 dose combination. Similarly, different concentration of CD55 was added to specific CD3/CD28 dose combination to study the effect of CD55 costimulatory signal on CD28 mediated cytokine production. IL-10 was detected after 72 hours of cell activation with CD3/CD55 and CD3/CD28 and both costimulatory signal induced secretion of IL-10 (Figure 3.4 and 3.5). However, significantly more IL-10 was produced following stimulation with CD3/CD55 compared to CD3/CD28 at higher concentrations (eg.1.00 μg/mL).

A)

CD3	1ug/mL	+	+	+	+	+
CD55	0.03ug/mL	+	-	-	-	-
CD55	0.10ug/mL	-	+	-	-	-
CD55	0.30ug/mL	-	-	+	-	-
CD55	1.00ug/mL	-	-	-	+	-
CD55	3.00ug/mL	-	-	-	-	+

B)

CD3	1ug/mL	+	+	+	+	+
CD28	0.03ug/mL	+	-	-	-	-
CD28	0.10ug/mL	-	+	-	-	-
CD28	0.30ug/mL	-	-	+	-	-
CD28	1.00ug/mL	-	-	-	+	-
CD28	3.00ug/mL	-	-	-	-	+
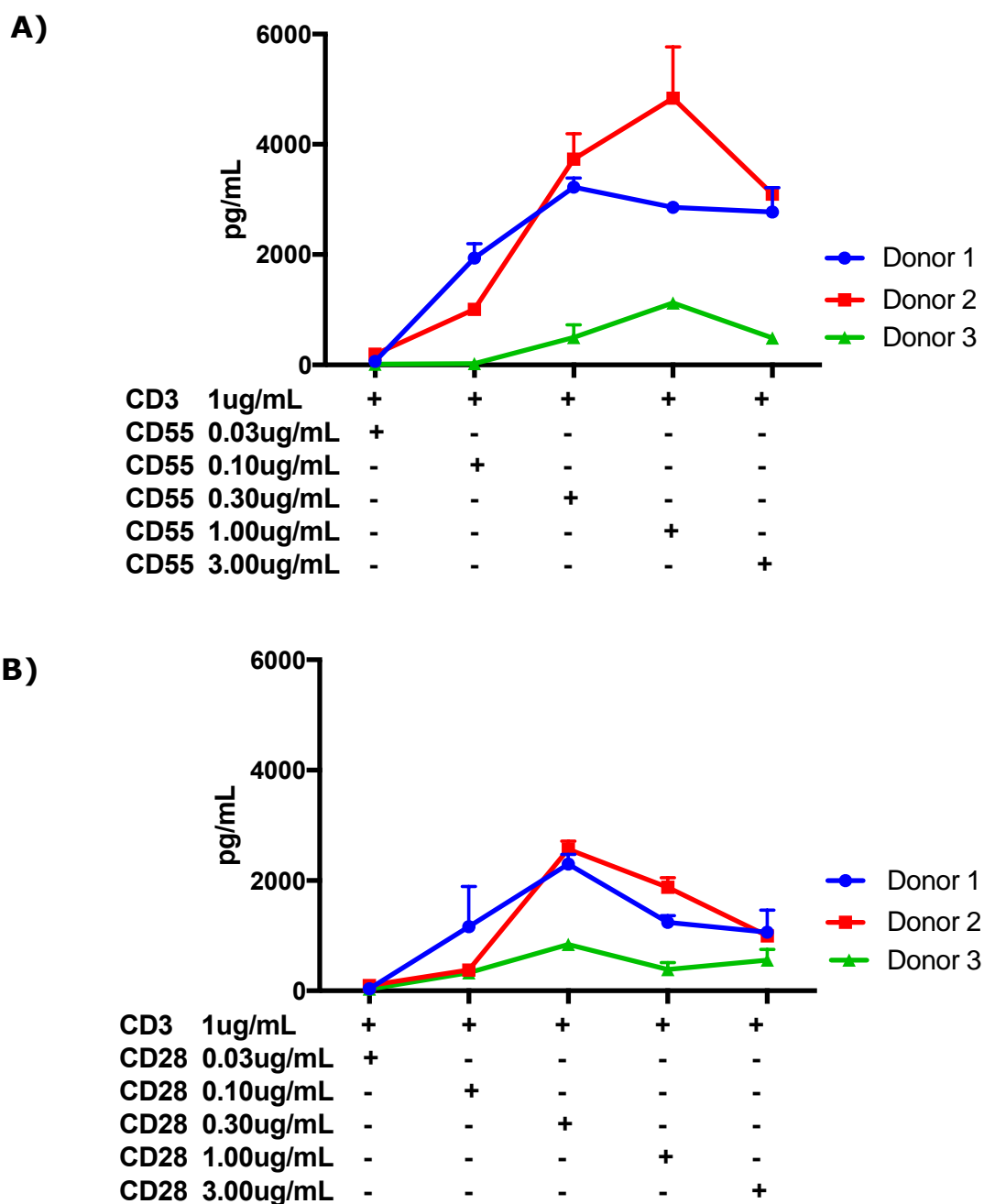
Figure 3.4: Dose dependent effect of CD55 (A) and CD28 (B) costimulation on IL-10 production. In the presence of constant dose of CD3 (1.00 μg/mL), naïve CD4$^+$ T-cells were stimulated with various concentrations of CD55 and CD28, ranging from 0.03 -3.00 μg/mL. After 72 hours of cell stimulation, the IL-10 production was evaluated by ELISA. Data represents cumulative results from three independent experiments.

3.2.3 Inhibition of CD55 costimulation mediated IL-10 production by CD28 costimulatory signal

The transformation of naïve CD4^{+} T-cell to effector cell requires antigen recognition by TCR as well as costimulatory signal which ensure T-cell survival and differentiation. However, naïve T-cells express multiple costimulatory receptors [370, 510] and synergy in costimulatory signals is important to attain optimum T-cell response [511]. Conversely, competition between costimulatory molecules which facilitate different phenotypes could potentially alter the T-cell response where the dominant costimulatory signal would suppress the effect of another costimulatory signal. CD55 costimulation leads to Tr1 phenotype by inducing IL-10 production and CD28 costimulation predominantly results in IFN-γ producing Th1 cells. In this study, we investigated the effect of CD28 costimulation on CD55 induced IL-10 production by titrating in increasing amount of CD28 to constant concentration CD3 and CD55 in order to determine if these costimulatory molecules work in synergy or if one costimulatory signal is dominant over the other one.

Naïve CD4^{+} T-cells were stimulated with CD3/CD55/CD28 antibody of varying concentration combination for 72 hours and IL-10 secretion by cells activated under different conditions were assessed to evaluate the effect of CD28 on CD55 costimulation (Figure 3.5). It was determined that CD28 suppresses CD55 mediated IL-10 production even at lower concentrations and the highest dose of CD28 (3.00 μg/mL) markedly inhibited the IL-10 production. In our experimental setting, the IL-10 secretion induced by highest concentration of CD55 (3.00 μg/mL) in

conjunction with CD3 was reduced more than 50% by ten-times lower concentration of CD28 (0.03 μg/mL) (Figure 3.6). CD28 costimulation demonstrated dominant effect over CD55 costimulation as it significantly decreased (****P<0.0001, paired t-test, n=3) CD3/CD55 mediated IL-10 production even at 10-times lower concentration (0.03 μg/mL) to that of CD55 (3.00 μg/mL) (Figure 3.6).

IL-10 production after 72 hours of cell stimulation

A)

CD3 1ug/mL	+	+	+	+	+	+
CD55 0.03ug/m	+	+	+	+	+	+
CD28 0.03ug/mL	-	+	-	-	-	-
CD28 0.10ug/mL	-	-	+	-	-	-
CD28 0.30ug/mL	-	-	-	+	-	-
CD28 1.00ug/mL	-	-	-	-	+	-
CD28 3.00ug/mL	-	-	-	-	-	+

B)

CD3 1ug/mL	+	+	+	+	+	+
CD55 0.10ug/m	+	+	+	+	+	+
CD28 0.03ug/mL	-	+	-	-	-	-
CD28 0.10ug/mL	-	-	+	-	-	-
CD28 0.30ug/mL	-	-	-	+	-	-
CD28 1.00ug/mL	-	-	-	-	+	-
CD28 3.00ug/mL	-	-	-	-	-	+

C)

CD3 1ug/mL	+	+	+	+	+	+
CD55 0.30ug/m	+	+	+	+	+	+
CD28 0.03ug/mL	-	+	-	-	-	-
CD28 0.10ug/mL	-	-	+	-	-	-
CD28 0.30ug/mL	-	-	-	+	-	-
CD28 1.00ug/mL	-	-	-	-	+	-
CD28 3.00ug/mL	-	-	-	-	-	+

D)

CD3 1ug/mL	+	+	+	+	+	+
CD55 1.00ug/m	+	+	+	+	+	+
CD28 0.03ug/mL	-	+	-	-	-	-
CD28 0.10ug/mL	-	-	+	-	-	-
CD28 0.30ug/mL	-	-	-	+	-	-
CD28 1.00ug/mL	-	-	-	-	+	-
CD28 3.00ug/mL	-	-	-	-	-	+

E)

CD3 1ug/mL	+	+	+	+	+	+
CD55 3.00ug/m	+	+	+	+	+	+
CD28 0.03ug/mL	-	+	-	-	-	-
CD28 0.10ug/mL	-	-	+	-	-	-
CD28 0.30ug/mL	-	-	-	+	-	-
CD28 1.00ug/mL	-	-	-	-	+	-
CD28 3.00ug/mL	-	-	-	-	-	+

Figure 3.5: Dose dependent effect of CD28 on CD3/CD55 mediated IL-10 secretion. Specific concentration of CD55 (0.03 μg/mL, 0.10 μg/mL, 0.30 μg/mL, 1.00 μg/mL and 3.00 μg/mL shown in A-E respectively) was kept constant along with CD3 (1 μg/mL) and increasingly higher amount of CD28 was added to different combination CD3/CD55 and naïve CD4^{+} T-cells were activated with various CD3/CD55/CD28 stimulation. After 72 hours of cell stimulation, IL-10 production was determined by ELISA. CD28 decreased the IL-10 production by C3/CD55 costimulation in a dose dependent manner. Data shown are representative of three independent experiments and the cytokine production was determined by calculating the average of duplicate wells stimulated under same condition.

IL-10 secretion after 72 Hours of cell stimulation
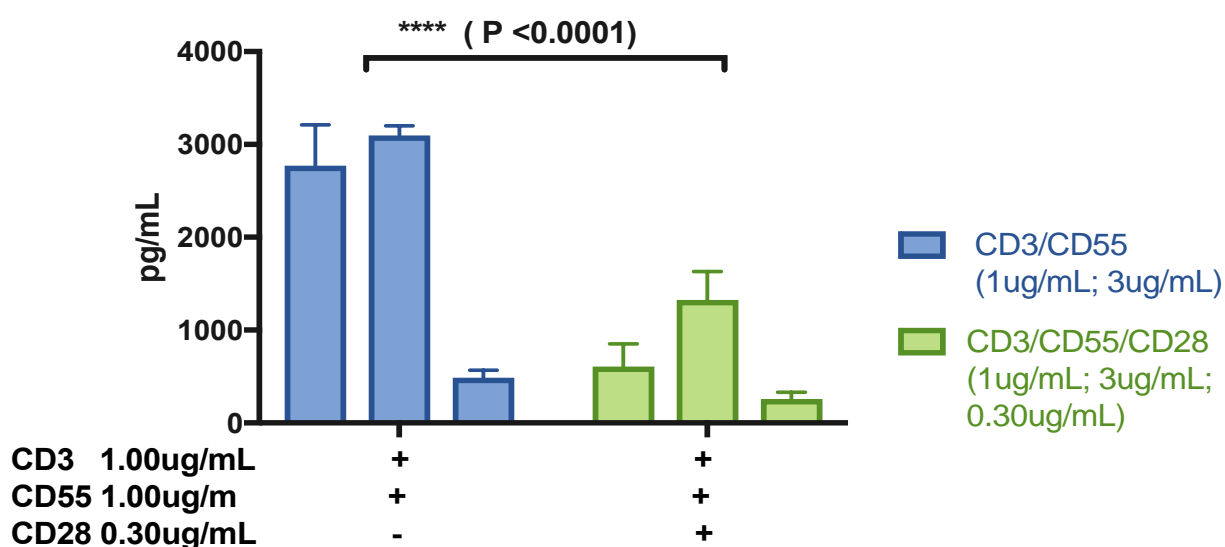
Figure 3.6: Suppression of CD3/CD55 induced IL-10 production by CD28 costimulation. Naive CD4^{+} T-cells were stimulated with either CD3/CD55 (1μg/mL; 3μg/mL) or CD3/CD55/CD28 (1 μg/mL; 3μg/mL; 0.3μg/mL) for 72 hours and IL-10 production by the activated cells were determined by ELISA. CD3/CD55 stimulate IL-10 production by cells and the presence of CD28 significantly reduced the CD3/CD55 mediated IL-10 production (****$P<0.0001$, paired t-test). Data demonstrated here is cumulative results obtained from three independent experiments.

In the next stage, we determined if CD55 had the ability to induce IL-10 in the CD3/CD28 stimulated cells. So CD55 was added at increasingly higher concentration to constant concentration of CD3/CD28. CD55 elevated IL-10 in a dose dependent manner only if the CD28 concentration was low (0.03 and 0.10 μg/mL) (Figure 3.7 and Figure 3.8). However, even the highest concentration of CD55 did not to enhance IL-10 production in the presence of higher doses of CD28 (0.3 , 1.0 and 3.0 μg/mL). These observations became more evident when CD28 was titrated in to constant CD3/CD55 (1 μg/mL, 1μg/mL) and CD28 reduced IL-10 production in dose dependent manner whereas titrating in CD55 to constant CD3/CD28 (1 μg/mL, 1μg/mL) did not modulate IL-10 secretion. At the highest concentration, CD28 reduced IL-10 production by more than 70% in the CD3/CD55 stimulated cells (Figure 3.8). These findings suggest that CD28 costimulatory signal has a dominant effect over CD55 costimulatory signal and cells might not respond to CD55 costimulation by differentiating to Tr1 cells in the presence of CD28 costimulation, even though the CD28 signalling might be lower than CD55. This study has demonstrated that competition between CD28 and CD55 could potentially determine the fate of the cell phenotype. Depending on the strength of CD55 at the time of TCR engagement, naïve CD4^{+}cells might differentiate to IL-10 producing Tr1 cells but it would diverge to Th1 cells if CD28 signalling overrides the effect CD55 costimulatory signal.

IL-10 production after 72 hours of cell stimulation

A)

CD3 1ug/mL	+	+	+	+	+	+
CD28 0.03ug/m	+	+	+	+	+	+
CD55 0.03ug/mL	-	+	-	-	-	-
CD55 0.10ug/mL	-	-	+	-	-	-
CD55 0.30ug/mL	-	-	-	+	-	-
CD55 1.00ug/mL	-	-	-	-	+	-
CD55 3.00ug/mL	-	-	-	-	-	+

B)

CD3 1ug/mL	+	+	+	+	+	+
CD28 0.10ug/m	+	+	+	+	+	+
CD55 0.03ug/mL	-	+	-	-	-	-
CD55 0.10ug/mL	-	-	+	-	-	-
CD55 0.30ug/mL	-	-	-	+	-	-
CD55 1.00ug/mL	-	-	-	-	+	-
CD55 3.00ug/mL	-	-	-	-	-	+

C)

CD3 1ug/mL	+	+	+	+	+	+
CD28 0.30ug/m	+	+	+	+	+	+
CD55 0.03ug/mL	-	+	-	-	-	-
CD55 0.10ug/mL	-	-	+	-	-	-
CD55 0.30ug/mL	-	-	-	+	-	-
CD55 1.00ug/mL	-	-	-	-	+	-
CD55 3.00ug/mL	-	-	-	-	-	+

D)

CD3 1ug/mL	+	+	+	+	+	+
CD28 1.00ug/m	+	+	+	+	+	+
CD55 0.03ug/mL	-	+	-	-	-	-
CD55 0.10ug/mL	-	-	+	-	-	-
CD55 0.30ug/mL	-	-	-	+	-	-
CD55 1.00ug/mL	-	-	-	-	+	-
CD55 3.00ug/mL	-	-	-	-	-	+

E)

CD3 1ug/mL	+	+	+	+	+	+
CD28 3.00ug/m	+	+	+	+	+	+
CD55 0.03ug/mL	-	+	-	-	-	-
CD55 0.10ug/mL	-	-	+	-	-	-
CD55 0.30ug/mL	-	-	-	+	-	-
CD55 1.00ug/mL	-	-	-	-	+	-
CD55 3.00ug/mL	-	-	-	-	-	+

Figure 3.7: Dose dependent effect of CD55 on IL-10 production by cells following CD3/CD28 costimulation. Specific concentration of CD28 (0.03 μg/mL, 0.10 μg/mL, 0.30 μg/mL, 1.00 μg/mL and 3.00 μg/mL shown in A-E respectively) was kept constant along with CD3 (1 μg/mL) and increasingly higher amount of CD55 was added to different combination CD3/CD28 and naïve CD4$^+$ T-cells were activated with various CD3/CD55/CD28 combination. After 72 hours of cell stimulation, cell culture supernatant was collected and assessed for IL-10 secretion by ELISA. It was determined that CD55 could enhance IL-10 production only at the presence of low dose of CD28 signalling but did not enhance IL-10 in the presence of higher dose of CD28. Data demonstrated here are representative of three independent experiments and duplicate wells were set up for each condition for all three donors.

A)

IL-10 secretion after 72 Hours of cell stimulation

Donor 1

B)

Donor 2

C)

Donor 3
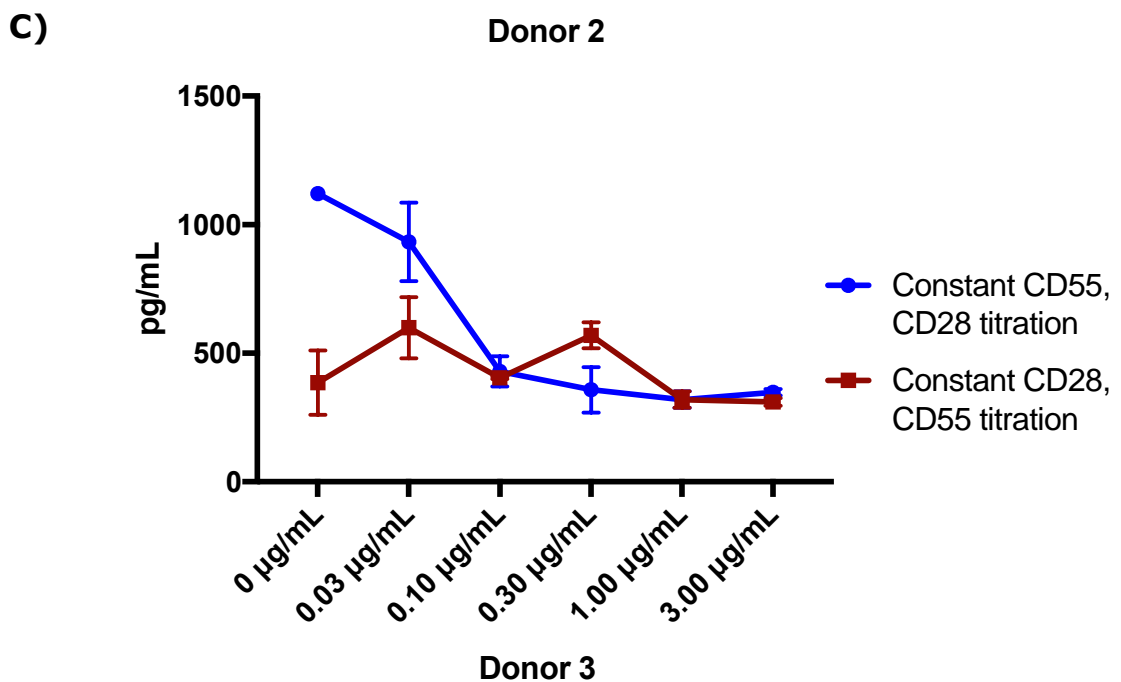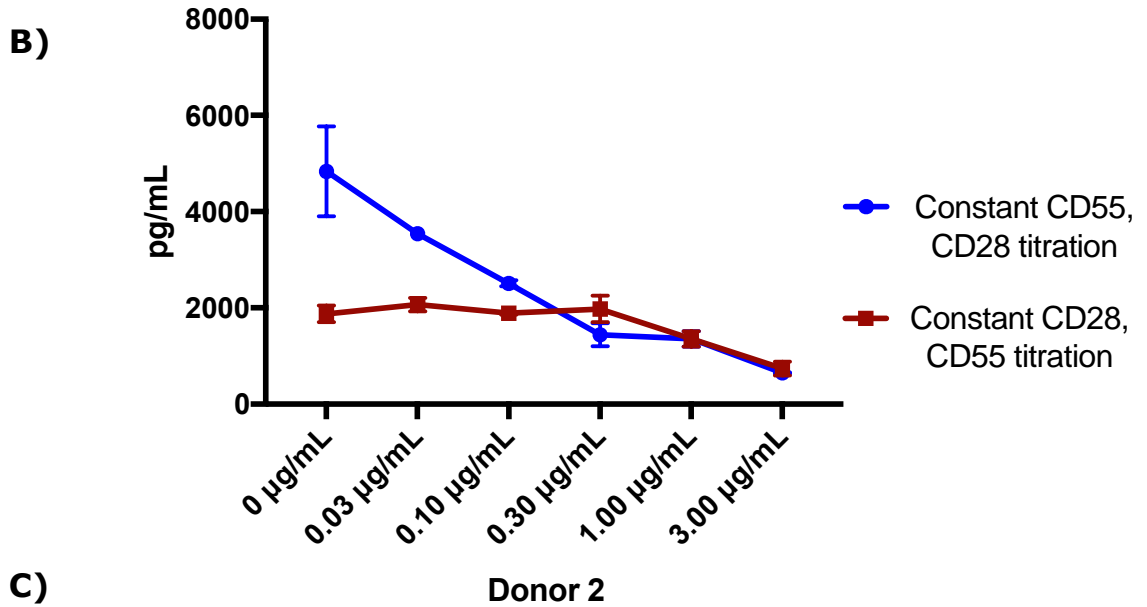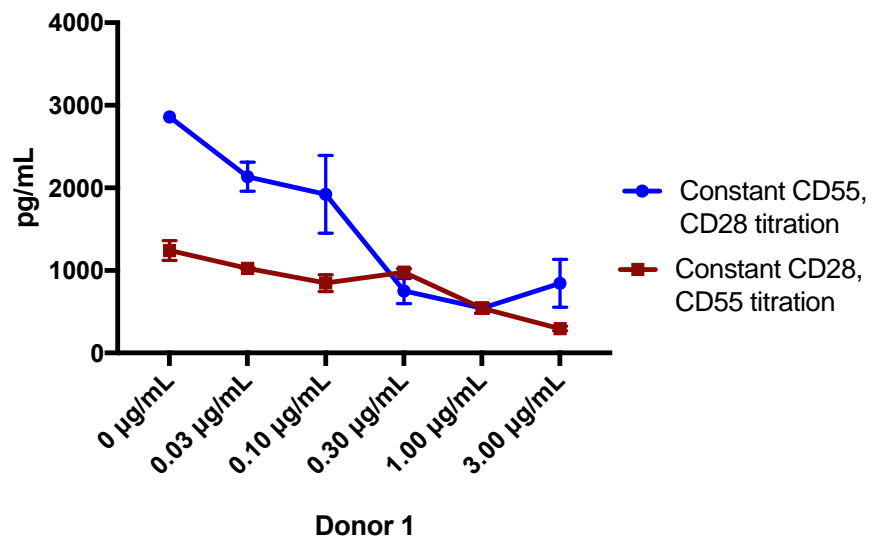
Figure 3.8: Comparative effect of one costimulatory signal on the IL-10 production induced by another. Naïve CD4$^+$ T-cell were stimulated with anti-CD3/CD55 (1 μg/mL, 1μg/mL) and different doses of anti-CD28 (0.03 μg/mL, 0.10 μg/mL, 0.30 μg/mL, 1.00 μg/mL and 3.00 μg/mL) was titrated into the constant CD3/CD55 combination. Similarly, anti-CD55 was titrated into the constant combination of anti-CD3/CD28 (1 μg/mL, 1μg/mL) combination. Naïve CD4 T-cells were stimulated with Anti-CD3/CD55/CD28 combination and the IL-10 production was determined after 72 hours of cell activation by ELISA.). Data demonstrated here is cumulative results obtained from three independent experiments.

3.2.4 The comparative effect of CD3/CD55 and CD3/CD28 co-stimulation on IFN-γ production

CD28 demonstrated the ability of inhibit CD3/CD55 mediated IL-10 production and CD28 could potentially prevent the CD3/CD55 induced differentiation of naïve CD4$^+$ T-cell into Tr1 cells. So, we investigated if CD28 alters the differentiation of CD3/CD55 stimulated naïve CD4$^+$ T-cells to Th1 cells instead of Tr1 cells by promoting IFN-γ secretion. Naïve CD4 T-cells were stimulated with different combination of CD55 and CD28 in conjunction with sub-optimal TCR signalling (1μg/mL of anti-CD3) and IFN-γ secretion was determined after 72 hours of cell activation. Indeed, it was determined that CD28 costimulation enhanced IFN-γ production in the CD3/CD55 stimulated cells.

Both CD3/CD55 and CD3/CD28 costimulation resulted in IFN-γ secretion by activated cells after 72 hours of cell stimulation and both of the costimulatory signal induced IFN-γ in a dose dependent manner (Figure 3.9 A). However, at the higher concentrations, CD3/CD28 costimulation induced more IFN-γ production compared to CD3/CD55 (Figure 3.9 B). The addition of CD28 to CD3/CD55 stimulated cells, with lower concentrations of CD3/CD55 (0.03 and 0.10 μg/mL), increased the IFN-γ production compared to only CD3/CD55 stimulated cells (Figure 3.10 A-C). However, at highest dose of CD55 costimulation (3.00μg/mL), lower doses of CD28 did not enhance IFN-γ and only the higher concentrations of CD28 demonstrated the ability to enhance IFN-γ secretion (Figure 3.10 E).

In order to determine if CD55 suppresses the IFN-γ production following CD3/CD28 stimulation, CD55 was added at various doses to constant CD3/CD28 costimulation (Figure 3.11). Highest dose of CD55 decreased IFN-γ production only in the presence of lower doses of CD28. However, CD55 did not reduce IFN-γ secretion when cells were stimulated with higher concentration of CD28. IFN-γ production remained consistent when cells were stimulated with highest concentration of CD28 and even the highest amount of CD55 costimulation did not alter IFN-γ production.

These findings corroborate that CD28 costimulation could interfere with CD3/CD55 induced differentiation of naïve CD4^{+} T-cells into Tr1 cells by not only inhibiting IL-10 production but also enhancing IFN-γ production and the dominant effect of CD28 costimulation could potentially promote the differentiation of cells into Th1 cells even in the presence of CD3/CD55 stimulation depending on the strength of CD28 signalling.

A) **IFN-γ production after 72 hours of cell stimulation**
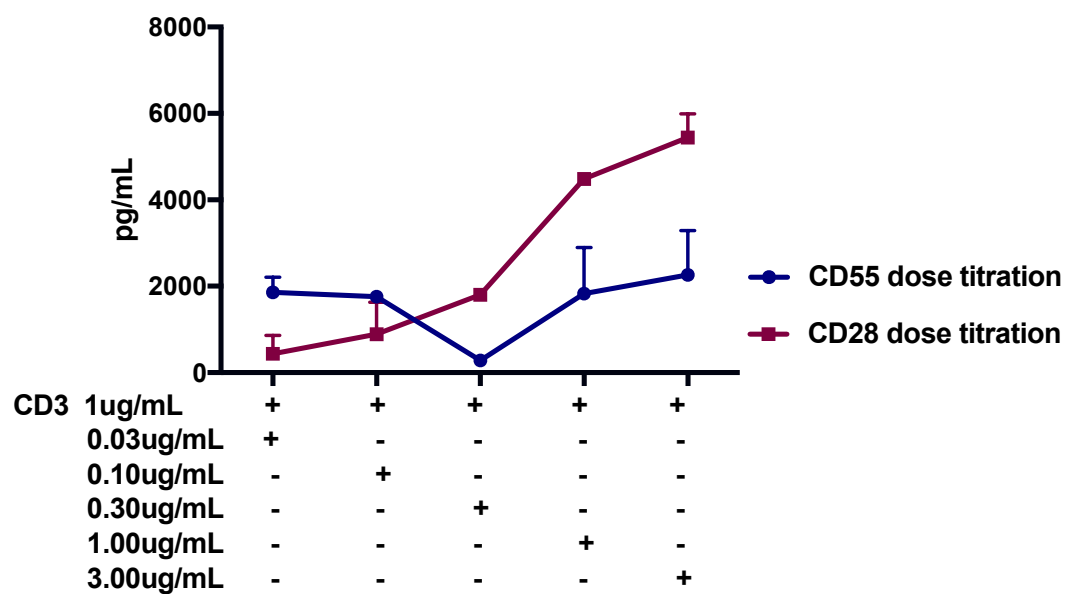
CD3	1ug/mL	+	+	+	+	+
	0.03ug/mL	+	-	-	-	-
	0.10ug/mL	-	+	-	-	-
	0.30ug/mL	-	-	+	-	-
	1.00ug/mL	-	-	-	+	-
	3.00ug/mL	-	-	-	-	+

- CD55 dose titration
- CD28 dose titration

B) **IFN-γ production after 72 hours of cell stimulation**

*** (P = 0.0005)

CD3	1.00ug/mL	+	+
CD55	3.00ug/m	+	-
CD28	3.00ug/mL	-	+
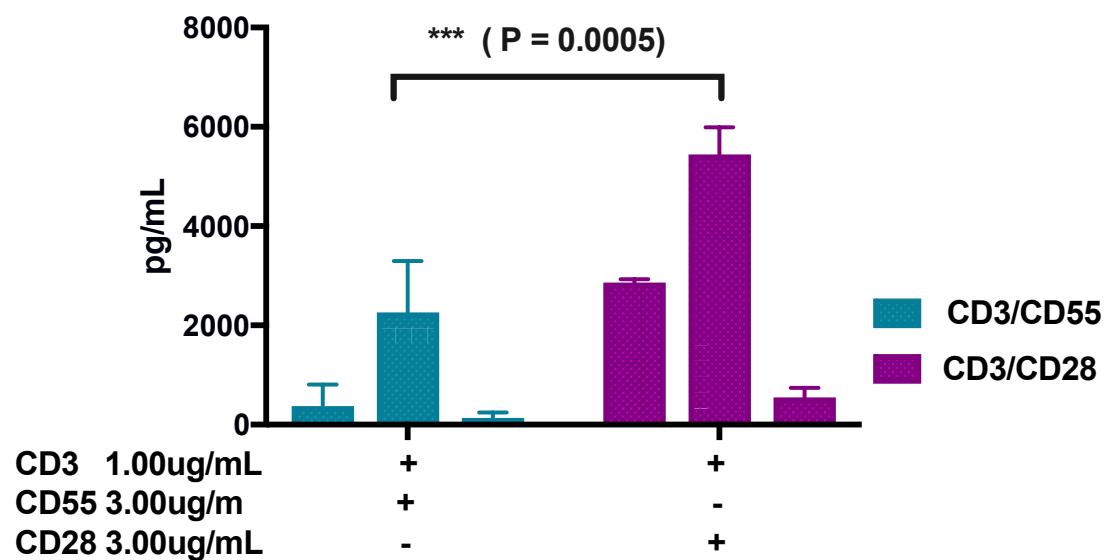
- CD3/CD55
- CD3/CD28

Figure 3.9: IFN-γ production in response to CD3/CD55 and CD3/CD28 costimulation. A) Naïve CD4^{+} T-cells were stimulated with 0.03 μg/mL, 0.10 μg/mL, 0.30 μg/mL, 1.00 μg/mL and 3.00 μg/mL of either CD55 or CD28 in the presence of 1 μg/mL of anti-CD3. After 72 hours of cell stimulation, culture supernatant was assessed for IFN-γ production by ELISA. B) In the presence of 1.00 μg/mL of anti-CD3, naïve CD4 T-cells stimulated with 3 μg/mL of anti-CD28 produced significantly higher (P=0.0005, paired t-test) amount of IFN-γ compared to cells stimulated with similar concentration of anti-CD55. Data shown in (A) is representative of three independent experiments whereas data demonstrated in (B) is cumulative summary of specific condition from those three experiments.

IFN-γ production after 72 hours of cell stimulation

A)

CD3 1ug/mL	+	+	+	+	+	+
CD55 0.03ug/m	+	+	+	+	+	+
CD28 0.03ug/mL	-	+	-	-	-	-
CD28 0.10ug/mL	-	-	+	-	-	-
CD28 0.30ug/mL	-	-	-	+	-	-
CD28 1.00ug/mL	-	-	-	-	+	-
CD28 3.00ug/mL	-	-	-	-	-	+

B)

CD3 1ug/mL	+	+	+	+	+	+
CD55 0.10ug/m	+	+	+	+	+	+
CD28 0.03ug/mL	-	+	-	-	-	-
CD28 0.10ug/mL	-	-	+	-	-	-
CD28 0.30ug/mL	-	-	-	+	-	-
CD28 1.00ug/mL	-	-	-	-	+	-
CD28 3.00ug/mL	-	-	-	-	-	+

C)

CD3 1ug/mL	+	+	+	+	+	+
CD55 0.30ug/m	+	+	+	+	+	+
CD28 0.03ug/mL	-	+	-	-	-	-
CD28 0.10ug/mL	-	-	+	-	-	-
CD28 0.30ug/mL	-	-	-	+	-	-
CD28 1.00ug/mL	-	-	-	-	+	-
CD28 3.00ug/mL	-	-	-	-	-	+

D)

CD3 1ug/mL	+	+	+	+	+	+
CD55 1.00ug/m	+	+	+	+	+	+
CD28 0.03ug/mL	-	+	-	-	-	-
CD28 0.10ug/mL	-	-	+	-	-	-
CD28 0.30ug/mL	-	-	-	+	-	-
CD28 1.00ug/mL	-	-	-	-	+	-
CD28 3.00ug/mL	-	-	-	-	-	+

E)

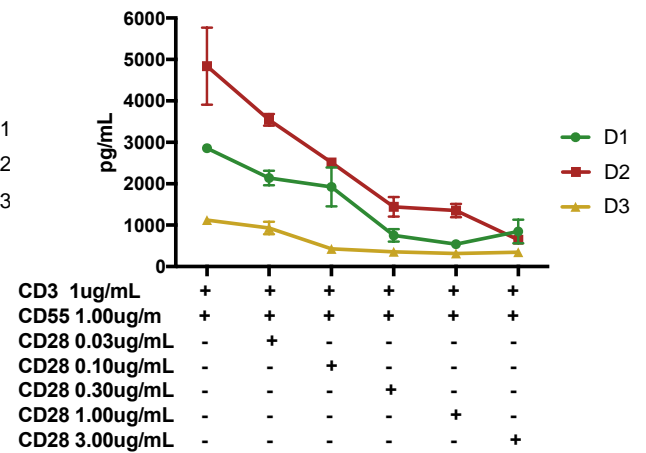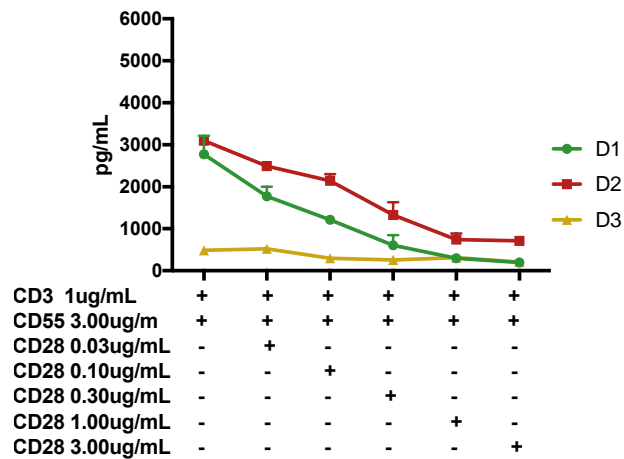| CD3 1ug/mL	+	+	+	+	+	+
CD55 3.00ug/m	+	+	+	+	+	+
CD28 0.03ug/mL	-	+	-	-	-	-
CD28 0.10ug/mL	-	-	+	-	-	-
CD28 0.30ug/mL	-	-	-	+	-	-
CD28 1.00ug/mL	-	-	-	-	+	-
CD28 3.00ug/mL	-	-	-	-	-	+

Figure 3.10: Dose dependent effect of CD28 on CD3/CD55 mediated IFN-γ secretion. Specific concentration of CD55 (0.03 μg/mL, 0.10 μg/mL, 0.30 μg/mL, 1.00 μg/mL and 3.00 μg/mL shown in A-E respectively) was kept constant along with CD3 (1 μg/mL) and increasingly higher amount of CD28 was added to different combination of CD3/CD55 and naïve CD4^{+} T-cells were activated with various CD3/CD55/CD28 stimulation. After 72 hours of cell stimulation, IFN-γ production was determined by ELISA. CD28 enhanced IFN-γ production by C3/CD55 costimulation in a dose dependent manner. Data are representative of three independent experiments.

IFN-γ production after 72 hours of cell stimulation

A)
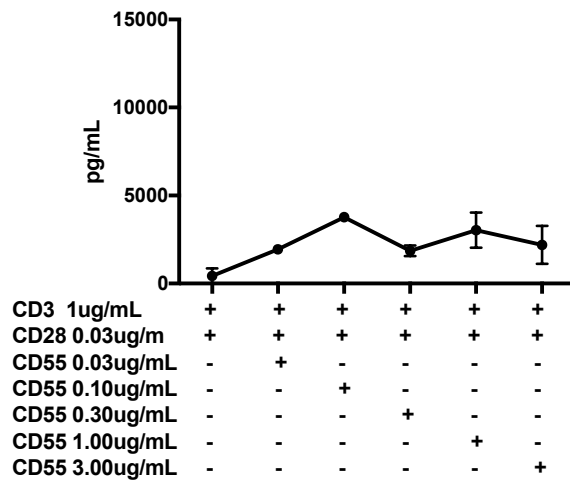
B)
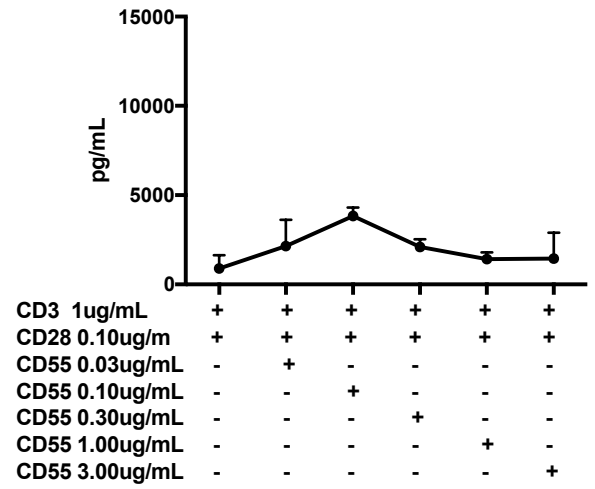
C)
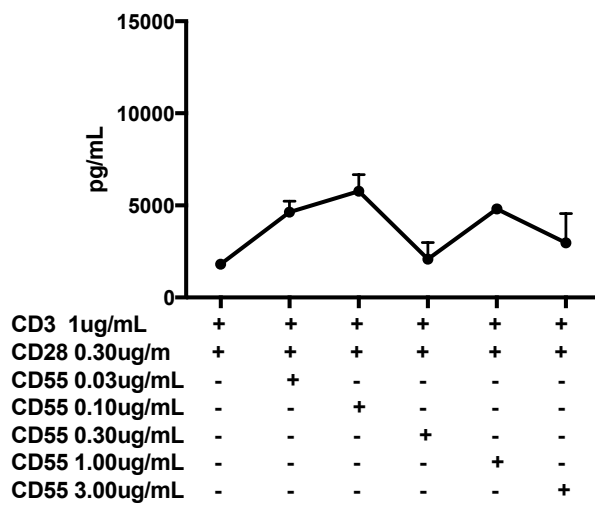
D)
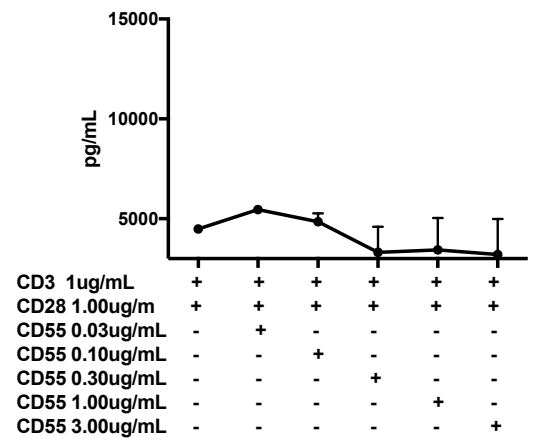
E)
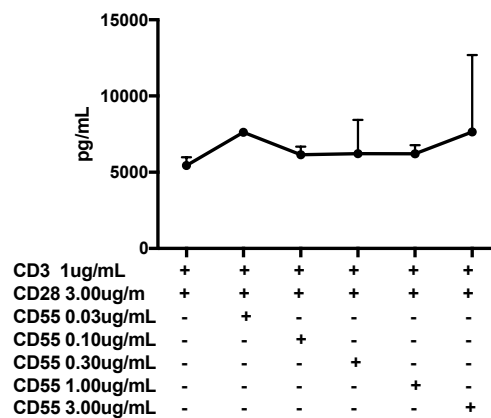
Figure 3.11: Dose dependent effect of CD55 on IFN-γ production by CD3/CD28 stimulated Naïve CD4⁺ T-cells. Specific concentration of CD28 (0.03 μg/mL, 0.10 μg/mL, 0.30 μg/mL, 1.00 μg/mL and 3.00 μg/mL shown in A-E respectively) was kept constant along with CD3 (1 μg/mL) and increasingly higher amount of CD55 was added to different combination CD3/CD28 and naïve CD4⁺ T-cells were activated with various CD3/CD55/CD28 stimulation. After 72 hours of cell stimulation, IFN-γ production was determined by ELISA. CD55 did not significantly alter IFN-γ production by cells stimulated with higher dose of CD3/CD28. Data are representative of three independent experiments.

3.2.5 Dose dependent effect of CD55 and CD28 costimulation on CD4$^+$ T-cell proliferation

The ability to induce proliferation is one of the compulsory requirements for a signalling receptor to be considered as a costimulatory molecule [358, 359, 370, 468]. Naïve CD4$^+$ T-cells were stimulated with various combination of CD3, CD55 and CD28 for 72 hours and thymidine incorporation assay was performed to evaluate the costimulation induced cell proliferation. Both CD55 and CD28 costimulatory signal, in conjunction with CD3, demonstrated the ability to induce robust cell proliferation in a dose dependent manner compared to cells stimulated with only CD3 (Figure 3.12). We also determined if CD28 costimulation could alter CD3/CD55 induced cell proliferation (Figure 3.13). CD28 enhanced the cell proliferation in a dose dependent manner only in presence of lowest concentration of CD55 (0.03 μg/mL). However, CD28 did not significantly alter cell proliferation following CD3/CD55 stimulation at higher doses even though the presence of highest concentration of both CD28 and CD55 reduced the proliferation in comparison to cells stimulated with only the highest dose of CD55. However, the decrease in proliferation was not statistically significant (data not shown). Thus, both CD55 and CD28 costimulation, in conjunction with CD3 signalling, demonstrated comparatively similar ability to induce proliferation in naïve CD4$^+$ T-cells.

Cell proliferation after 72 hours stimulation of cell stimulation

Donor 1

CPM

800000
600000
400000
200000
0

CD3
CD3/CD55
CD3/CD28

Donor 2

CPM

800000
600000
400000
200000
0

CD3 only
CD3/CD55
CD3/CD28

Donor 3

CPM

800000
600000
400000
200000
0

CD3 only
CD3/CD55
CD3/CD28

CD3	1ug/mL	+	+	+	+	+	+
	0.03ug/mL	-	+	-	-	-	-
	0.10ug/mL	-	-	+	-	-	-
	0.30ug/mL	-	-	-	+	-	-
	1.00ug/mL	-	-	-	-	+	-
	3.00ug/mL	-	-	-	-	-	+
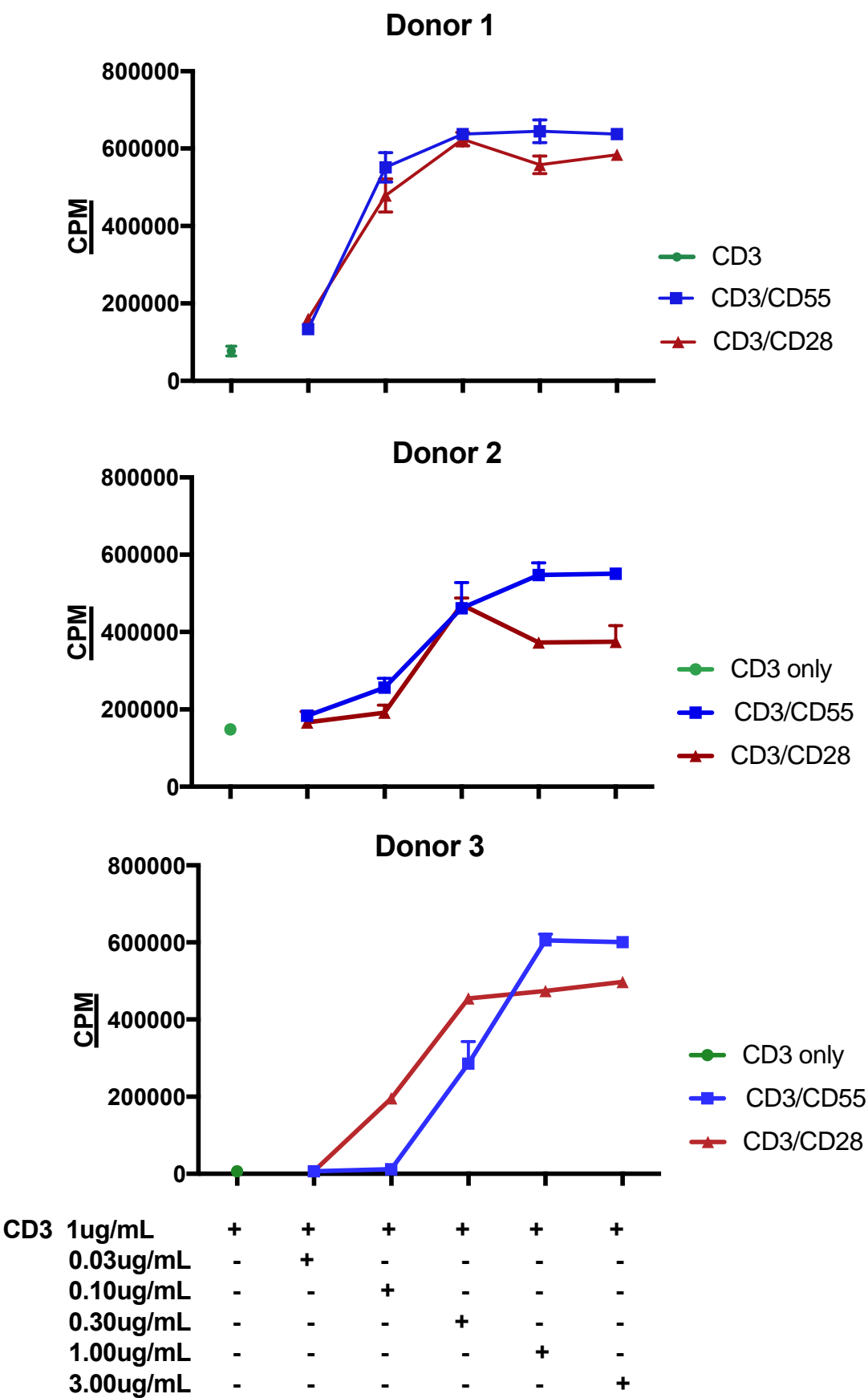
Figure 3.12: Evaluation of CD55 and CD28 costimulatory signal induced cell proliferation. Naïve CD4^{+} T-cells were stimulated with CD3 (1.00 μg/mL) in conjunction with various doses of either CD55 or CD28 (0.03 μg/mL, 0.10 μg/mL, 0.30 μg/mL, 1.00 μg/mL and 3.00 μg/mL). After 72 hours cell activation, thymidine was added to cells for another 16 hours of cell culture and the amount of incorporated thymidine was determined to evaluate cell proliferation. Only CD3 stimulated cells were used as negative control to determine basal cell proliferation. Data shown here was obtained from three independent experiments. (CPM, counts per minute)

A)

CPM

CD3 1ug/mL	+	+	+	+	+	+	+
CD55 0.03ug/mL	-	+	+	+	+	+	+
CD28 0.03ug/mL	-	-	+	-	-	-	-
CD28 0.10ug/mL	-	-	-	+	-	-	-
CD28 0.30ug/mL	-	-	-	-	+	-	-
CD28 1.00ug/mL	-	-	-	-	-	+	-
CD28 3.00ug/mL	-	-	-	-	-	-	+

B)

CPM

CD3 1ug/mL	+	+	+	+	+	+	+
CD55 0.10ug/mL	-	+	+	+	+	+	+
CD28 0.03ug/mL	-	-	+	-	-	-	-
CD28 0.10ug/mL	-	-	-	+	-	-	-
CD28 0.30ug/mL	-	-	-	-	+	-	-
CD28 1.00ug/mL	-	-	-	-	-	+	-
CD28 3.00ug/mL	-	-	-	-	-	-	+

C)

CPM

CD3 1ug/mL	+	+	+	+	+	+	+
CD55 0.30ug/mL	-	+	+	+	+	+	+
CD28 0.03ug/mL	-	-	+	-	-	-	-
CD28 0.10ug/mL	-	-	-	+	-	-	-
CD28 0.30ug/mL	-	-	-	-	+	-	-
CD28 1.00ug/mL	-	-	-	-	-	+	-
CD28 3.00ug/mL	-	-	-	-	-	-	+

D)

CPM

CD3 1ug/mL	+	+	+	+	+	+	+
CD55 3.00ug/mL	-	+	+	+	+	+	+
CD28 0.03ug/mL	-	-	+	-	-	-	-
CD28 0.10ug/mL	-	-	-	+	-	-	-
CD28 0.30ug/mL	-	-	-	-	+	-	-
CD28 1.00ug/mL	-	-	-	-	-	+	-
CD28 3.00ug/mL	-	-	-	-	-	-	+

E)

CPM

CD3 1ug/mL	+	+	+	+	+	+	+
CD55 1.00ug/mL	-	+	+	+	+	+	+
CD28 0.03ug/mL	-	-	+	-	-	-	-
CD28 0.10ug/mL	-	-	-	+	-	-	-
CD28 0.30ug/mL	-	-	-	-	+	-	-
CD28 1.00ug/mL	-	-	-	-	-	+	-
CD28 3.00ug/mL	-	-	-	-	-	-	+

138

Figure 3.13: Dose dependent effect of CD28 on CD3/CD55 induced cell proliferation. Specific concentration of CD55 (0.03 μg/mL, 0.10 μg/mL, 0.30 μg/mL, 1.00 μg/mL and 3.00 μg/mL shown in A-E respectively) was kept constant along with CD3 (1 μg/mL) and increasingly higher amount of CD28 was added to different combination CD3/CD55 and naïve CD4^{+} T-cells were activated with various CD3/CD55/CD28 stimulation. After 72 hours of cell stimulation, thymidine was added to the cells for the last 16 hours of cell culture to determine cell proliferation. Data demonstrated here are representative of three independent experiments. (CPM, counts per minute)

3.3 Discussion:

Costimulatory signal emerged as a mechanism that ensured appropriate T-cell response to self and non-self antigen and supported cell survival and expansion of activated cells by evading apoptosis [358, 463, 464, 469]. Further research revealed the prominent role of costimulatory signal in cellular function and phenotype [370, 510]. The discovery of many costimulatory molecules expressed by the T-cells raised the question about how multiple costimulatory signal orchestrate the development of specific T-cell response. Also, identification of alternate costimulatory molecules, which mediate differentiation of naïve CD4$^+$ T-cells to regulatory cells instead of conventional Th1 and Th2 effector cells, emphasizes that interplay of costimulatory signals during antigen recognition could potentially determine the fate of activated cells. In the previous studies on costimulatory signal, it has been demonstrated that signalling via several molecules such as CD5, CD9, CD2, CD44 or CD11a in conjunction with sub-mitogenic dose of CD3 was able to activate naïve cells and induce low amount of proliferation. However, as opposed to CD28 costimulation, signalling through these molecules did not result in sustained cell function and survival. In fact, signalling via CD9 and CD11a led to increased apoptosis. The lack of ability to promote cell function and survival by these costimulatory molecules was attributed to their inability to induce IL-2 [512-514]. These observations indicate that costimulatory molecules differ in their potential to induce differentiation of naïve T-cells and further supported the notion that competition between different costimulatory signal could influence the function and phenotype of the cells depending on strength of each signal. In our previous study, it was

determined that CD55 costimulatory signal induced IL-2 production to the same extent as CD28 signalling and the level of cell proliferation in response to these costimulatory signals were similar. The potential of CD55 and CD28 costimulatory molecules to influence the cellular function in presence of each other has not been studied before. In this study, we have investigated the comparative effect of two costimulatory signal CD28 and CD55, which have previously been demonstrated to promote differentiation of naïve CD4$^+$ T-cells into Th1 and Tr1 cells respectively. We have demonstrated that CD28 and CD55 have varied potency as costimulatory signal and induction of IL-10 production. CD28 costimulation modulates response to CD55 costimulation and markedly reduces CD3/CD55 induced IL-10 production which could potentially alter the phenotype of the activated cells.

CD3/CD55 stimulation promotes differentiation of naïve CD4$^+$ T-cells to IL-10$^+$ Tr1 cells. The effect of CD55 costimulation is dose dependent where higher doses of CD55 resulted in enhanced anti-inflammatory IL-10 production. While CD28 also induced IL-10 secretion, it was significantly lower than the CD55 mediated IL-10 production. However, the presence of CD28 reduced IL-10 production following CD3/CD55 costimulation. Interestingly, CD28 was able to exert inhibitory effect on the highest dose of CD55 even at a concentration ten-times lower than that of CD55 (3 μg/mL of CD55 ; 0.30 μg/mL of CD28). Conversely, CD55 also elevated IL-10 production in CD3/CD28 stimulated cell but only at

the presence of lower doses of CD28. CD55 did not enhance IL-10 production in cells which were stimulated with higher doses of CD28.

IFN-γ is a proinflammatory cytokine which is induced by costimulatory signal and it is key factor in Th1 immune response. Co-stimulating naïve CD4$^+$ T-cells with CD28 in conjunction with CD3 resulted in IFN-γ production in a dose dependent manner. Similarly, CD55 costimulation also induced IFN-γ. But at the highest dose, CD55 costimulation led to significantly lower amount of IFN-γ compared to CD28. When the effect of CD28 signalling on CD3/CD55 costimulation was investigated, It was noted that CD28 had the ability to increase IFN-γ secretion even by cells stimulated with higher dose of CD55. So CD28 conferred a significant impact on IFN-γ production by CD3/CD55 stimulated cells while CD55 did not alter IFN-γ secretion by CD3/CD28 stimulated cells except in cells which received higher CD55 stimulation in presence of low CD28 costimulation.

Another hallmark of costimulatory signal is the potential to induce proliferation by activated cells. Both CD55 and CD28 costimulatory signal resulted in robust cell proliferation in a dose dependent manner. While CD28 alter the cytokine profile of CD3/CD55 stimulated cells, it did not alter the CD55 mediated cell proliferation. These observations indicate that cumulative costimulatory signal from CD28 and CD55 supports the activated cells to sustain proliferation but depending on the strength of specific costimulatory signals at the time of TCR signalling, the cellular

function might vary with its associated phenotype following differentiation.

The impact of simultaneous costimulatory signals accompanying TCR signalling and the underlying mechanism of coordination of molecular signalling mediated by various costimulatory receptor which configure the cellular phenotype is yet to be elucidated. The underlying molecular signalling resulting in CD55 induced differentiation of Tr1 cells has not been determined yet. However, previous studies reported that CD55 signalling is associated with p56^{lck} -a protein tyrosine kinase related to Src, as determined by immunoprecipitation. CD55 is a GPI-anchored protein which contributes to its function as a signalling molecule because removal of the GPI-anchor abolished the protein kinase activity [515-517]. It was also observed that cross linking of CD55 by monoclonal antibody led to induction of several other protein tyrosine kinases such as fyn, in T-cells. In addition, it was demonstrated that despite not being sufficient to elevate intracellular calcium level and tyrosine phosphorylation of PLC-gamma in CD3$^+$ Jurkat cells, cross-linking of only CD55 was capable of inducing phosphorylation of tyrosine residues on p56lck, the TCR-zeta chain as well as ZAP-70. The addition of cytoplasmic domain of TCR-ζ chain to CD55 signalling resulted in T-cell activation which indicates that CD55 signalling is dependent on CD3-TCR complex [518]. In contrast, signalling via CD28 leads to binding of phosphatidylinositide 3-kinase (PI3K) to the pYMNM on the cytoplasmic domain of CD28. The activation of PI3K pathway induces phosphorylation of the kinases glycogen synthase kinase 3α (GSK3α) and GSK3β which

requires PtdIns (3,4,5)P3-activated AKT kinase. The involvement of AKT kinase in downstream signalling cascade of CD28 associates it with several other molecular pathway including NF-κB pathway and GSK3α/GSK3β and some of these pathways have been reported to be required for IL-2 production, expression anti-apoptotic protein BCL-X and induction of antigen specific response [519]. The difference in the molecular pathways activated by CD55 and CD28 could potentially contribute to development of alternate cellular phenotype and also influence their potency as costimulatory molecules.

Our data has provided an insight about how certain costimulatory signal could have dominant potency which might suppress or negate the effect of other costimulatory signals. We have demonstrated for the first time that the dominant effect of CD28 over alternate costimulatory signal via CD55 alter the IL-10 production which is a crucial immuno-suppressive cytokine for the function of Tr1 cells. CD55 costimulation demonstrated the ability to promote differentiation of naïve CD4+ T-cells to Tr1 cells despite its ineptitude to confer prepotent effect in the presence of higher strength of CD28 costimulation. These findings denote that the potency of CD28 signalling could be one of the limiting factors in the way of differentiation of Tr1 cells in response to CD55 costimulatory signal and the interplay between costimulatory signals could potentially impact the development of naïve CD4+ T-cells to either Th1 or Tr1 cells.

4 Chapter 4: The effect of CD55-CD97 interaction between Dendritic Cells and T-cells on the induction of IL-10$^+$ Tr1 cells

4.1 Introduction:

The interaction between dendritic cells (DC) and T-cells is crucial for the initiation of T-cell responses [520]. It has been suggested that dendritic cells act as specialized antigen presenting cells which assist the priming T-cell responses as the T-cell response to antigen was not hampered in absence of other antigen presenting cells such as B-cells [521]. DCs also demonstrated superior efficacy in priming T-cells, via intercellular interaction, in comparison to B-cells [522]. After encountering the antigen and maturation, dendritic cells upregulate cell surface markers including MHC Class-II (HLA-DR), CD80 and CD86. Some of the upregulated markers such as CD80 and CD86 are ligands for costimulatory receptors expressed by T-cells [523, 524]. These are required to successfully conduct cognate interaction between DC and naïve T-cells as they have impact on synapse formation as well as stability and duration of the interaction between DC:T-cell [522, 525-530]. Interestingly, it has also been demonstrated that DC:T-cell interaction precedes induction of both tolerance and immunity and the nature of the interaction determines commitment to either tolerance or immunity [525, 526, 531-533].

The three signal model of T-cell activation and differentiation emphasizes the significant role of Signal 1-TCR signal, Signal 2-Costimulatory signal

and Signal 3-cytokine signal on the induction of T-cell responses [351]. The importance of costimulation, particularly in CD4$^+$ T-cells became evident through early studies which demonstrated that impaired CD28 signal hampers T-cell proliferation, reduces IL-2 receptor (CD25) expression and fails to induce IL-2 production in response to antigen [534]. The paradigm for T cell activation requires CD80/86:CD28 costimulation resulting in pro-inflammatory Th1 responses. While CD28 have been known for decades along with other molecules such as CD40L (CD154) [535] , OX40 [535, 536] and 4-1BB (CD134) [537, 538], only in recent years attention has been drawn to costimulatory signals necessary for the induction of regulatory cells. Alternate costimulatory molecules may favour the induction of anti-inflammatory phenotypes such as Type 1 regulatory T-cells (Tr1) [315, 429]. One such receptor-ligand pair is CD55-CD97. CD55 is expressed by T-cells whereas its ligand CD97 is expressed by dendritic cells. We have previously demonstrated that costimulation through CD3/CD55 differentiates naïve CD4$^+$ T cells into a Tr1 phenotype (defined as IL-10$^+$, IFN-γ^- and IL-4$^-$) [315, 386].

Among the receptor mediated costimulatory signal for IL-10$^+$ Tr1 cells , CD46, a compliment regulatory protein, was identified as one of the first molecules which leads to generation of IL-10$^+$ CD4$^+$ T-cells [384, 429, 539]. Since the discovery of the role of CD46 in T-cells, the dysregulation and defect in CD46 mediated IL-10 production has also been reported in auto-immune diseases such as Rheumatoid Arthritis (RA) [540, 541], Multiple Sclerosis (MS) [385, 441, 508, 542] and systemic lupus erythematosus [543].

Another costimulatory molecule CD26, a cell surface glycoprotein with dipeptidyl peptidase IV activity, interacts with its ligand Caveolin-1 on dendritic cells to induces activation and IL-10 production by CD4$^+$T-cells [544-546]. The CD26 signalling in conjunction to CD3 signalling leads to enhanced IL-10 production following upregulation of transcription factor Early Growth Response 2 (EGR2) through NFAT and AP-1 activation [544]. It has also been demonstrated that CD26: Caveolin-1 signalling axis is altered in various diseases such as rheumatoid synovium [547], multiple sclerosis [548] and Graves' disease [549].

Interestingly, ICOS, a member of immunoglobulin supergene family along with other costimulatory molecule CD28 and coinhibitory molecule CTLA-4 and share a common immunoglobulin like domain [550], has also demonstrated the ability to activate T-cells, induce their proliferation and upregulate IL-10 production without affecting IL-2 secretion by interacting with its ligand ICOS-L expressed by dendritic cells [551-553]. CD4$^+$ICOS$^+$ cells were able to efficiently suppress IFN-γ producing effector cells *in vitro* and *in vivo* in experimental allergic encephalomyelitis (EAE) mouse models [554]. The receptor mediated induction of regulatory T-cells by various alternate costimulatory molecules emphasizes that signalling between costimulatory receptors on T-cells and their ligands on dendritic cells is one of the important mechanisms which maintain tolerance and control immune responses.

In the previous chapter we demonstrated that while there was cooperativity between CD55 and CD28 in the induction of proliferation of naïve T cells the presence of CD28 had a negative effect on the secretion of IL-10. Considering the important role of DC:T-cell interaction in immune responses as well as the role of IL-10 in immune-regulation, we hypothesized that interaction between CD97 on DCs and CD55 on T-cells mediates the induction Tr1 cells and disruption of CD97-CD55 interaction would impact IL-10 production by T-cells.

4.2 Results:

4.2.1 Generation of Monocyte Derived Dendritic Cells from CD14$^+$ monocytes and characterising the phenotype:

The monocyte derived dendritic cells (moDC) were generated from isolated CD14$^+$ monocyte cells and they were assessed for the expression of several dendritic cell markers to ensure that CD14$^+$ monocytes differentiated into moDCs prior to further experiments. A panel of cell surface molecules including CD14, HLA-DR, CD80 and CD86, was selected to characterise monocyte derived dendritic cells. On day 5, after differentiation from CD14$^+$ monocytes to immature moDCs (iDC) in the presence of IL-4 and GM-CSF, the antigen presenting cells downregulate CD14 expression (Figure 4.1). iDCs also express low levels of HLA-DR, CD80 and CD86. The level of expression of cell surface markers change in mature moDC (mDC) following activation through Toll-like receptor 4 (TLR4) signalling by lipopolysaccharides (LPS) treatment. MHC Class-II molecule HLA-DR, which is essential for antigen presentation, is upregulated significantly upon maturation along with the expression of CD80, CD86 (Figure 4.2).

A)

Monocyte Isolation

97% CD14 +ve

B)

MoDC Day 5

98% CD14 -ve
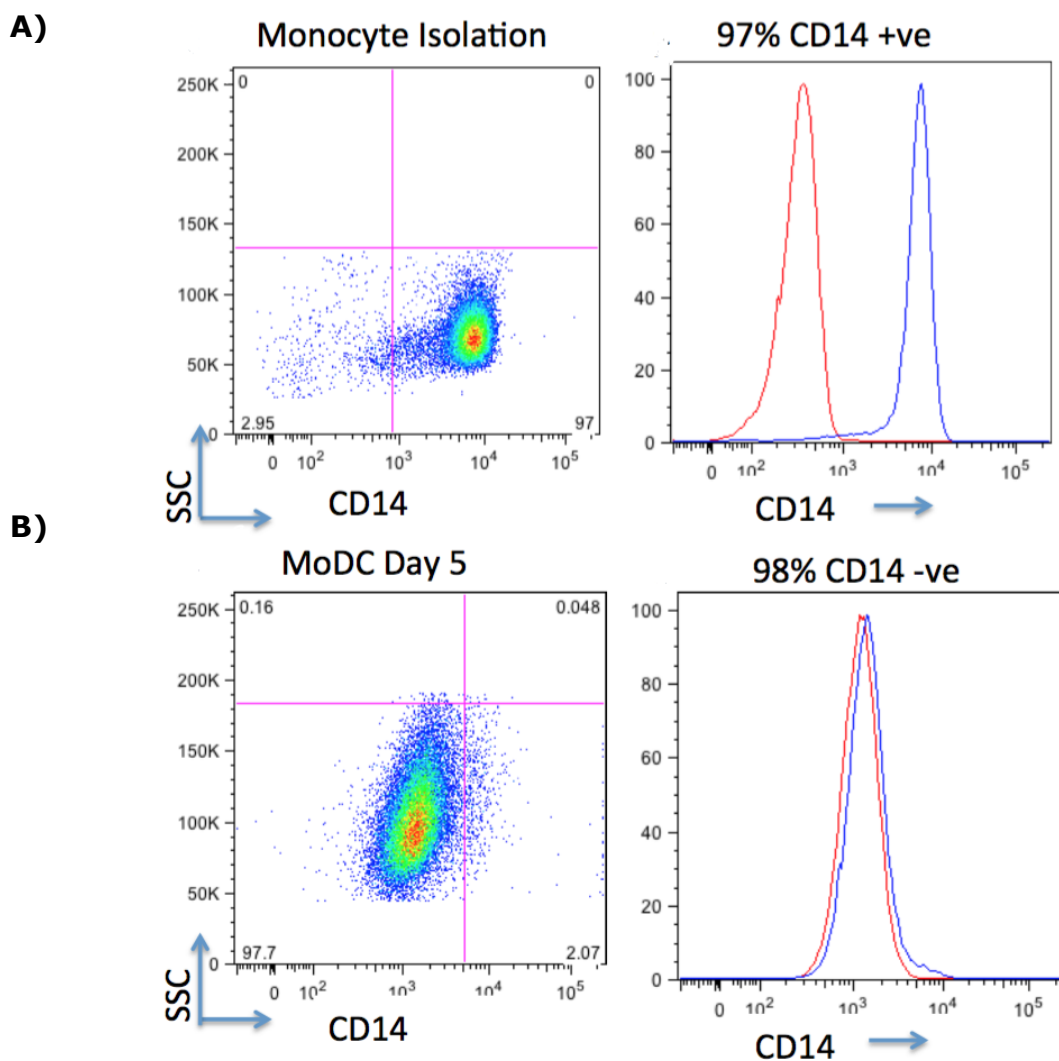
Figure 4.1: Expression of CD14 by monocytes and monocytes derived dendritic cells (moDC). CD14$^+$ expression was determined after isolation of monocytes from PBMCs on Day 0. The highly pure (<95%) cells were cultured with IL-4 and GM-CSF which resulted in the differentiation of CD14$^+$ monocytes into CD14$^-$ immature dendritic cells by Day 5. Data representative of three independent experiments. (In the histograms, red line = isotype control, blue= anti CD14). The level of marker expression was determined of the basis of isotype control on the day 0 and day 5.

A)

CD80

MFI	
Iso	77.2
iDC	947
mDC	6882

CD80 expression by moDC

** (P= 0.0090)

- iDC
- mDC

B)

CD86

MFI	
Iso	115
iDC	134
mDC	857

CD86 expression by moDC

** (P= 0.0026)

- iDC
- mDC

C)

HLA-DR

MFI	
Iso	115
iDC	1528
mDC	3343

HLA-DR expression by moDC

** (P= 0.0017)

- iDC
- mDC
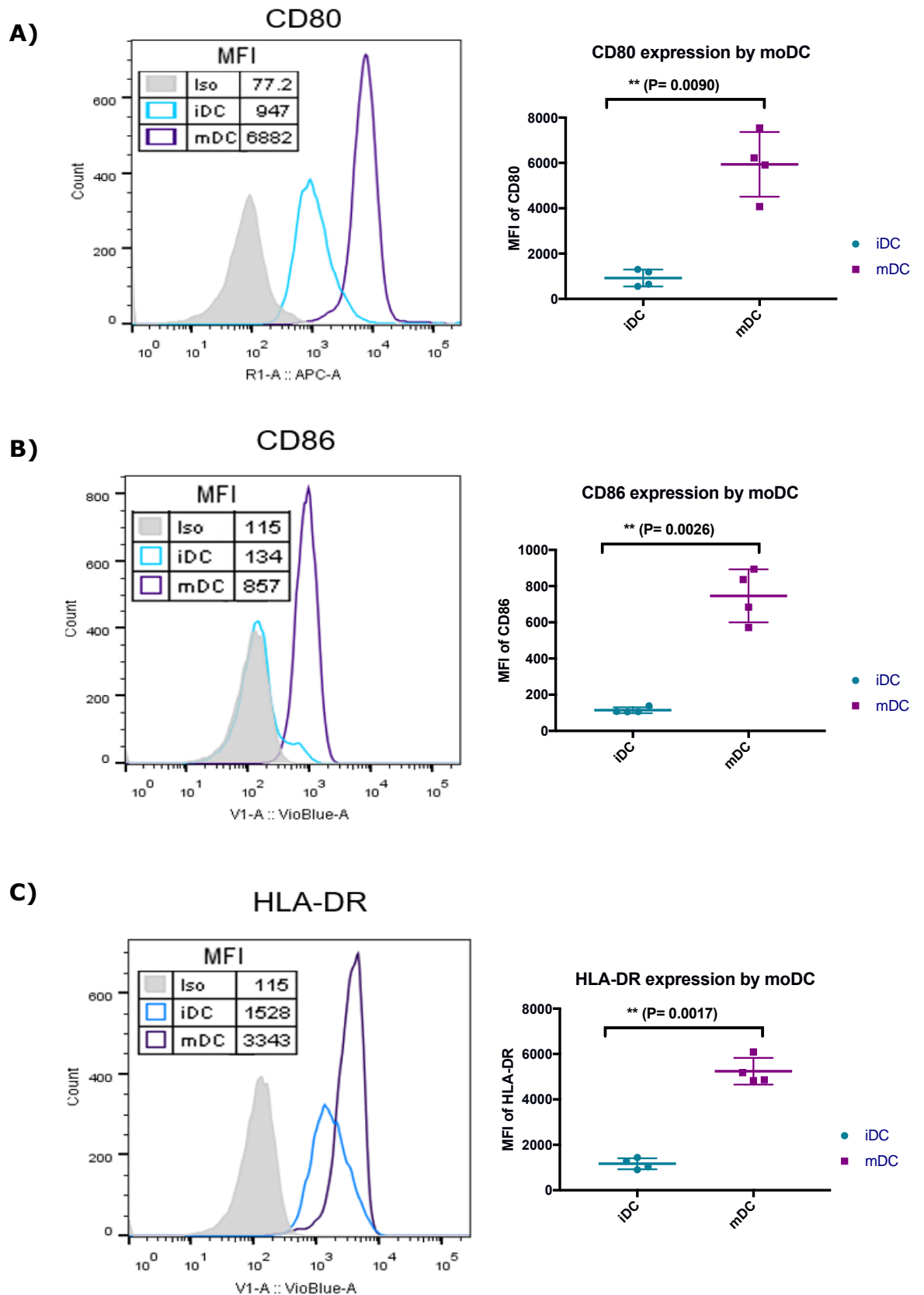
Figure 4.2: Expression of CD80, CD86 and HLA-DR by immature and mature moDC. Monocyte derived dendritic cells change their phenotype following cell activation by LPS treatment. iDCs were harvested on Day 5 and some cells were stimulated with LPS to obtain mature moDCs (mDC) and later expression of various cell surface markers were determined by using flow cytometry. After 24 hours of LPS treatment, in comparison to immature DCs, mature moDCs have significantly higher level of CD80 (A), CD86 (B) and HLA-DR (C) which are upregulated during the maturation. A, B, C represent the Mean Fluorescence Intensity (MFI) for expression of CD80, CD86 and HLA-DR in individual donor along with the summarized data from four independent experiments. Isotype controls were used as negative control to determine the expression level of each cell surface molecule. (The dendritic cell experiments were conducted with the help of Dr. Anna Malecka and Q.F.B. Rubí Misol-Há Velasco Cárdenas, Host Tumour Interface group)

4.2.2 Determination of CD97 expression by Monocyte Derived dendritic cells (moDCs)

It has been previously reported that CD55 and its ligand CD97 are expressed by leukocytes. However, the level of expression of these molecules by different DC subset has not been previously studied extensively. We investigated the expression of CD97 by immature and mature moDC and also developed a whole blood assay (WBA) in order to determine the level of CD97 expression by circulating primary dendritic

cells in blood. Both immature and mature moDCs express CD97 (Figure 4.3). Interestingly, contrary to the expression of other costimulatory ligand such as CD80 and CD86, the expression of CD97 is significantly higher (P=0.0247) in immature moDCs compared to mature moDCs. The difference in the CD97 expression might contribute to the biological function of DC and influence its signalling pathways during interaction with CD55 expressed by CD4 T-cells.

A)

B)
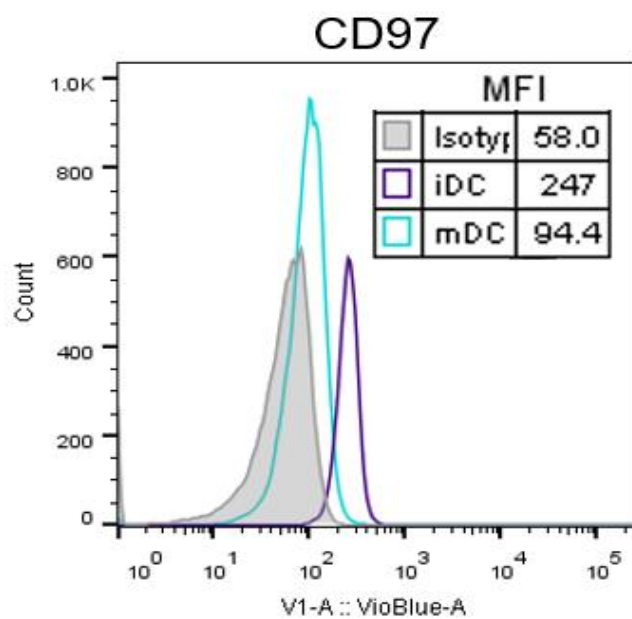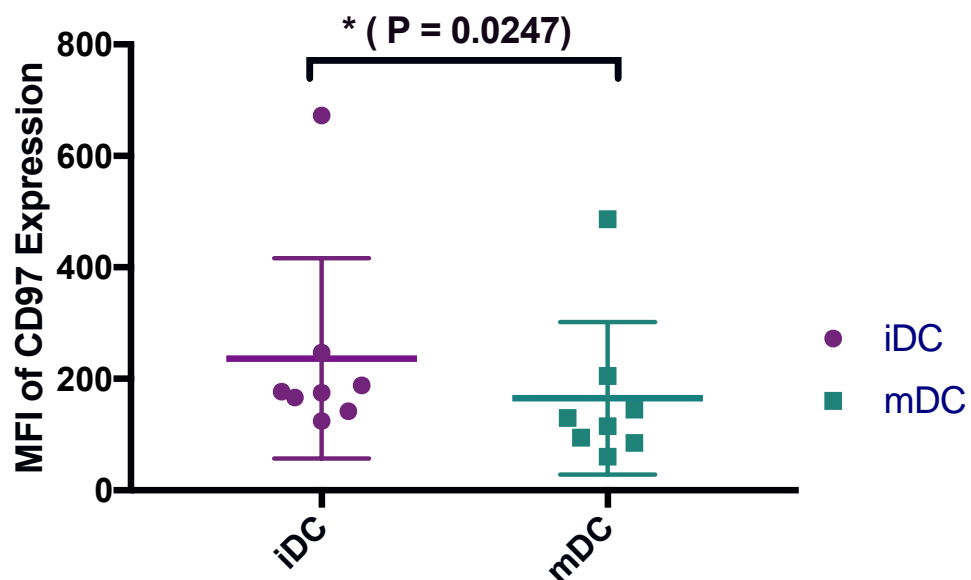
Figure 4.3: The level of CD97 expression by immature and mature moDCs determined by flow cytometry. iDCs were harvested on Day 5. Cells were stimulated with LPS to obtain mature moDCs (mDC) for 24 hours. After 24 hours the expression of CD97 was evaluated, using isotype antibodies as a negative control. The MFI for CD97 expression was compared in both iDC and mDC. iDCs expressed significantly higher CD97 than mDC (P=0.0247; determined by paired T-test). Here, CD97 expression by iDC and mDC for individual healthy donor is shown in (A) and the cumulative data from eight independent experiments is represented in (B).

4.2.3 The effect of CD97-CD55 interaction between Dendritic cell and CD4 T-cells on the induction of IL-10$^+$ Tr1 cells

CD3/CD55 costimulation induces differentiation of naïve CD4 T-cells into IL-10$^+$ Tr1 cells when driven by recombinant receptor (CD97-Fc) or antibody (anti-CD55). CD55 is expressed on the cell surface of CD4 T-cells while CD97 is expressed by the dendritic cells. The changes in expression of CD97 upon activation of monocyte derived DCs might suggest that CD97 has a role in engaging with CD55 on T cells and modulating their activity. The interaction between DC:T-cells via CD97-CD55 could potentially initiate the IL-10 production by Tr1 cells. We investigated role of CD97-CD55 interaction by blocking receptor-ligand binding using a soluble antibody specific to CD55.

We studied both monocyte derived DCs and primary circulating CD1c^{+} dendritic cells isolated from blood to determine if the effect of CD97-CD55 interaction is important for T cell stimulation and induction of IL-10^{+} Tr1 cells. Immature and mature monocyte derived dendritic (moDC) cells were generated and co-culture experiments were conducted by adding peripheral blood lymphocytes (CD14^{+} monocyte depleted PBL from the same donor) at 1:50 ratio (DC:PBL) in the presence or absence of Anti-CD55 antibody (Clone-791T/36). After 24 hours of DC:PBL co-culture, the activation of T cells was determined by evaluating both Interferon gamma and IL-10 production by cytokine secretion assay (CSA) (Figure 4.4).

Notable in both co-culture systems was that IFN-g was not induced in the T cells. However, IL-10 responses were observed. While iDC induced low numbers (less than 1%) of IL-10 producing cells it was significantly greater than the background responses. It was also lower than the response observed with mature DCs (more than 2%). This was consistent across the 4 donors tested. Anti CD55 blocking antibody reduced the number of IL-10^{+} cells to background levels compared to untreated or isotype control antibody. This supports the idea that CD55:CD97 are involved in the induction of the Tr1 phenotype in moDC : T cell co-cultures.

We also investigated this in CD1c$^+$ dendritic cells and T-cells (Figure 4.5). Both CD1c$^+$ and monocyte derived DCs co-cultured with PBL resulted in induction of IL-10 in the CD4+ T cell population. Blocking the interaction between CD97 and CD55 on DC:T-cells abated the induction of IL-10$^+$ Tr1 cells from an average of 3% down to background levels of 1% of IL-10$^+$ CD4 T cells (Figure 4.6). Like moDC, mature CD1c$^+$ DCs induced significantly higher (P=0.0110) numbers of IL-10$^+$ cells in comparison to immature DC (Figure 4.5 and 4.6). When CD97-CD55 interaction was disrupted by using blocking antibody, mature moDCs did not induce IL-10 production by T-cells to the extent as was observed with mature CD1c$^+$ cells. The presence of anti-CD55 antibody significantly reduced the number of IL-10$^+$ cells (P=0.0165) by mature CD1c$^+$ cells. In comparison, isotype control antibody did not similarly affect the induction of IL-10$^+$ cells by mature CD1c$^+$ cells (Figure 4.6). Collectively these data suggest that interaction via CD97-CD55 between DC:T-cell contributes IL-10 production by T-cells and disruption of this alternate costimulatory pair might affect induction of IL-10$^+$ Tr1 cells.

Only PBL

0.20%   0.02%

99.67%   0.11%

iDC +PBL

0.62%   0.07%

99.16%   0.15%

mDC +PBL

2.14%   0.07%

97.54%   0.25%

mDC +PBL+ Blocking Ab

0.42%   0.05%

99.17%   0.36%

mDC +PBL+ Isotype Control Ab

2.41%   0.08%

97.13%   0.38%

IL-10 APC

IFN-γ FITC

157
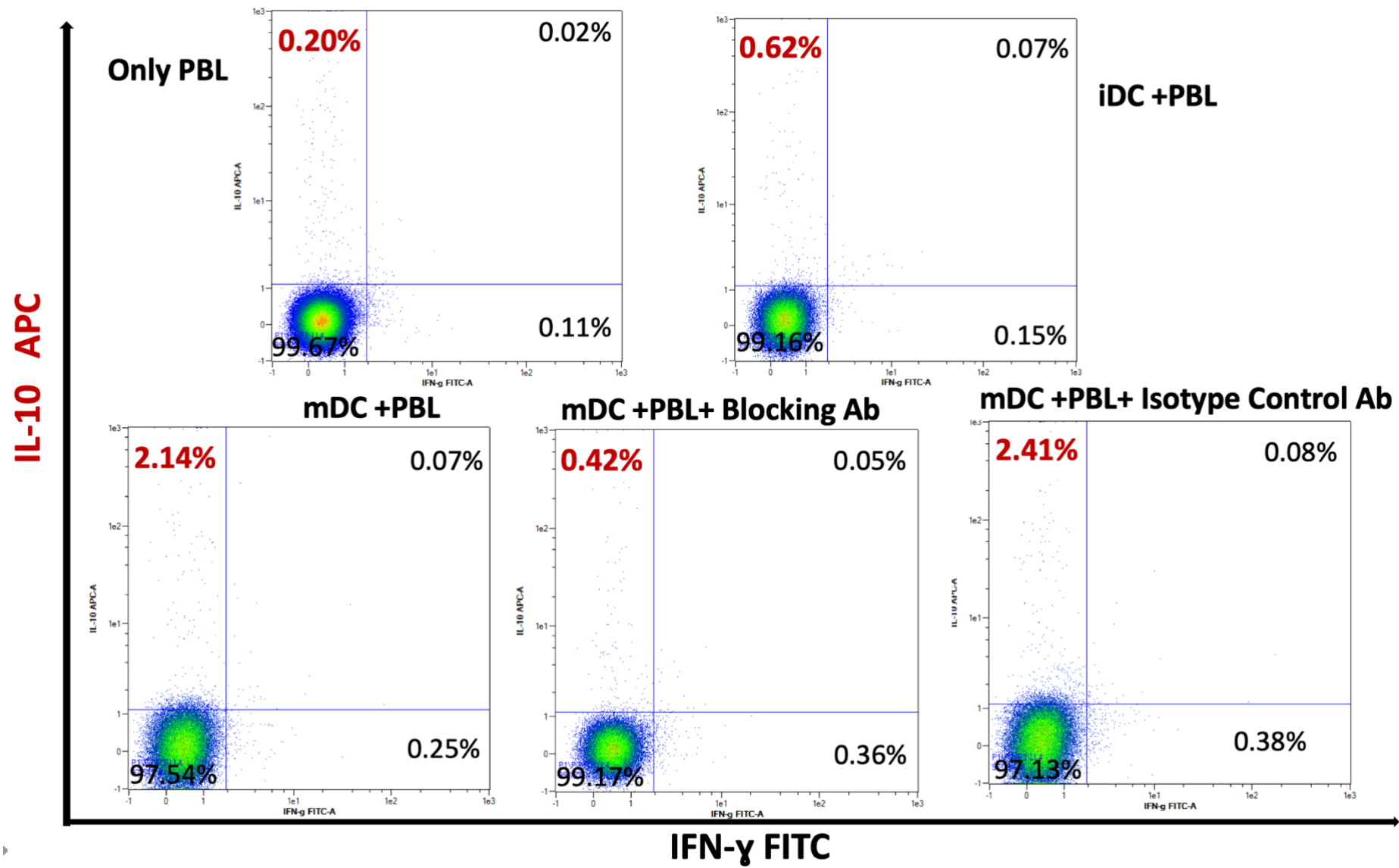
Figure 4.4: Evaluation of induction of IL-10$^+$ Tr1 cells by CD55-CD97 interaction between moDC and T-cells. Monocyte derived dendritic cells were generated and on Day 5. Cells were stimulated with LPS to obtain mature moDC (mDC). After 24 hours, CD14$^+$ cells depleted PBL was added to the iDC and mDC (1:50 of DC:T-cells). Anti-CD55 blocking antibody (791T/36, 1ug/ml) was pre-incubated with the PBL before adding them to DCs. Cultures were incubated for 24 hours before dual cytokine secretion assay for IFN-γ and IL-10 was performed. PBL alone were used as negative control and cytokine production was determined for CD4$^+$ gated population by flow cytometry. Data are representative of four independent experiments.

Only PBL
0.79%   0.02%
99.08%   0.11%

iDC +PBL
1.49%   0.02%
98.27%   0.22%

mDC +PBL
4.62%   0.12%
93.93%   1.33%

mDC +PBL+ Blocking Ab
1.10%   0.03%
98.61%   0.26%

mDC +PBL+ Isotype Control Ab
3.08%   0.18%
96.10%   0.63%

IL-10 APC

IFN-γ FITC
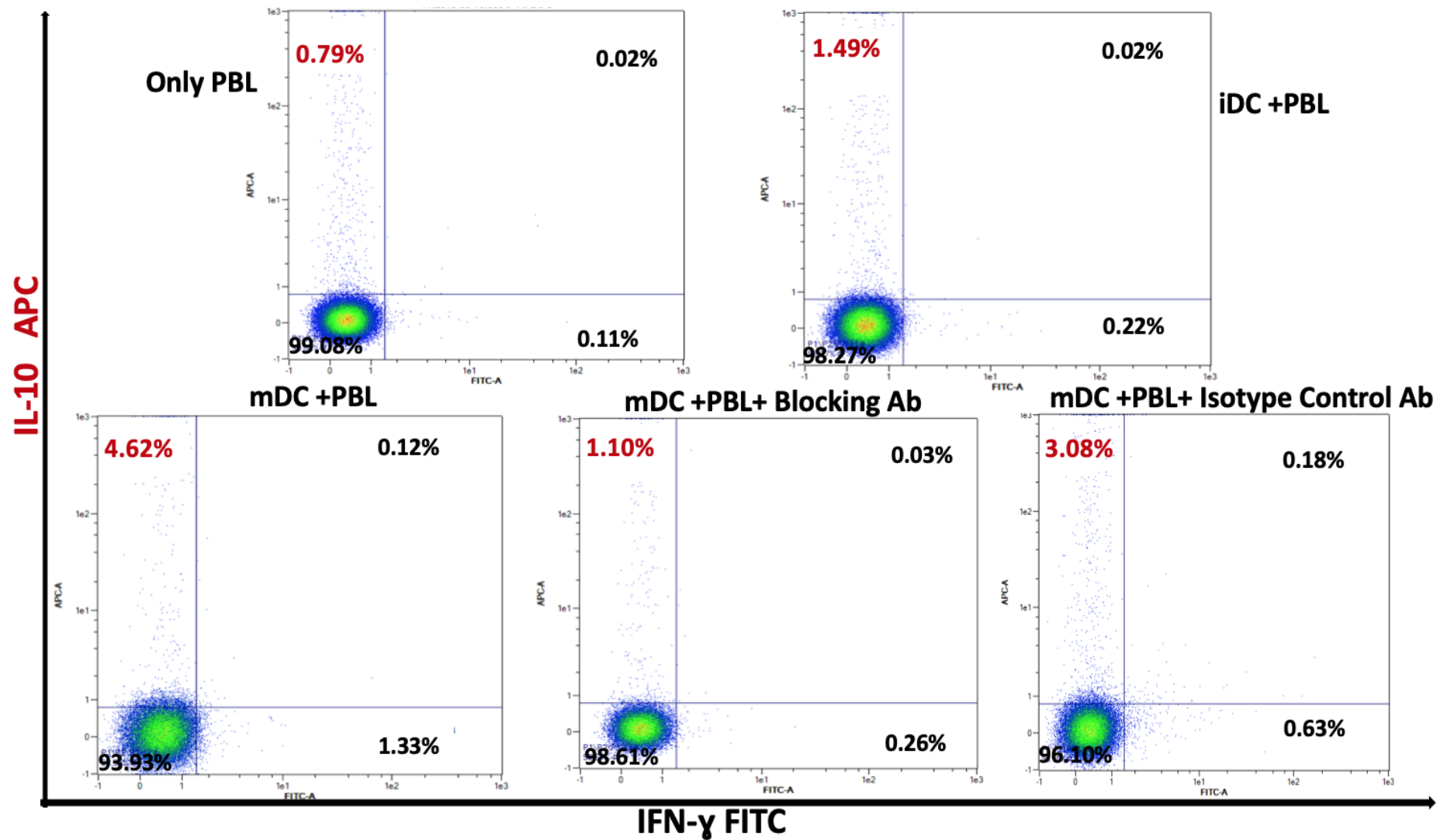
Figure 4.5: Evaluation of induction of IL-10$^+$ Tr1 cells by CD55-CD97 interaction between CD1c$^+$ dendritic cells and T-cells. CD1c$^+$ dendritic cells were isolated from PBMCs and stimulated with Poly I:C and R848 to obtain mature DC (mDC). After 24 hours, CD14$^+$ cells depleted PBL were added to the iDC and mDC (1:50 of DC:T-cells). For CD55-CD97 interaction blocking conditions, Anti-CD55 blocking antibody (791T/36, 1ug/ml) or isotype control antibody was pre-incubated with the PBL before adding them to DCs. Cultures were incubated for 24 hours before dual cytokine secretion assay for IFN-γ and IL-10 was performed to determine IL-10$^+$ Tr1 cells. PBL alone were used as negative control and cytokine production was determined for CD4$^+$ gated population by flow cytometry. Data shown is representative of four independent experiments.
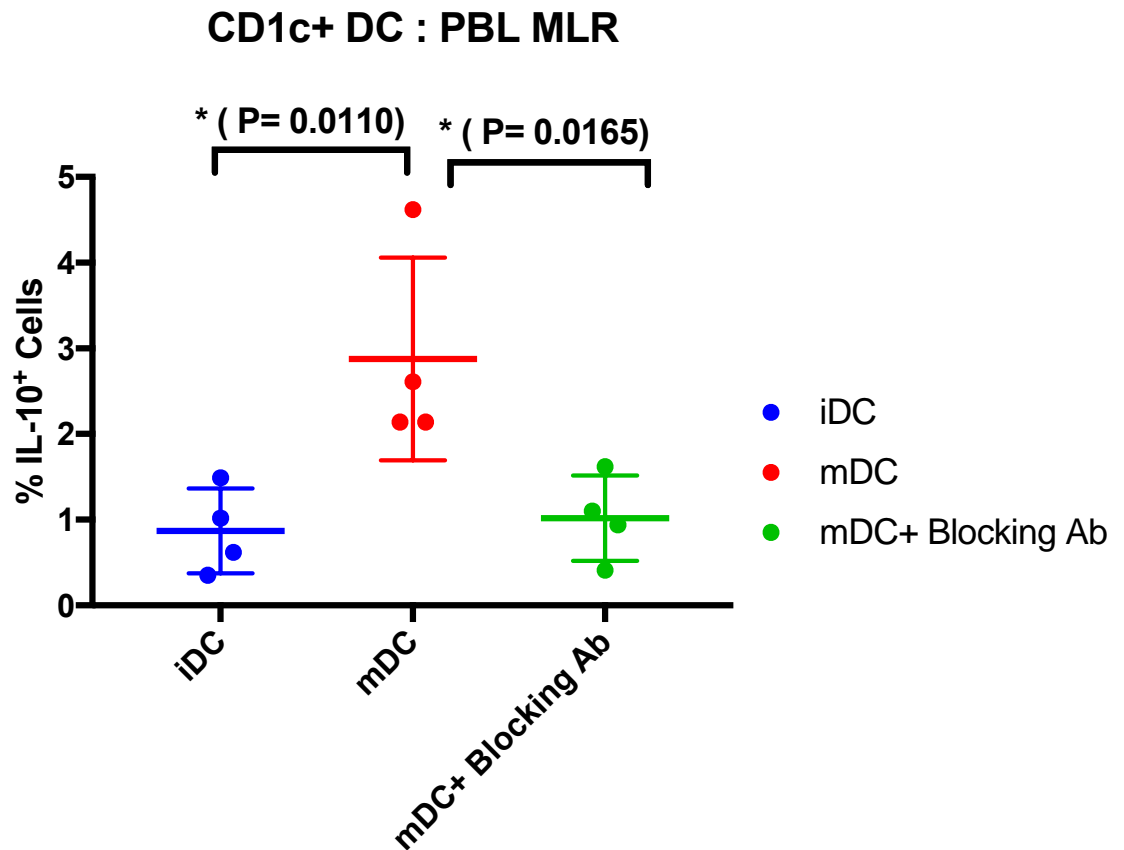
Figure 4.6: Blocking CD55-CD97 interaction suppresses induction of IL-10+Tr1 cells. The effect of CD97-CD55 interaction between CD1c+ dendritic cells and T-cells were determined by evaluating IL-10 secretion by CD4 T-cells following co-culture with DC in presence or absence of anti-CD55 blocking antibody. Mature CD1c+ dendritic cells induced significantly higher amount of IL-10+ Tr1 cells whereas blocking the CD97-CD55 interaction significantly reduced the induction of IL-10+ cells by mature DCs. Data represents the summarized results from four independent experiments.

4.3 Discussion:

Dendritic cells represent a crucial link between innate and adaptive immune system. Dendritic cells orchestrate the host response against immunogens through their unique ability to initiation primary immunity by priming naive CD4 T-cells [377, 555-558] and facilitating the immunological memory. Along with antigen presentation, DC provides costimulatory signals and produce immunomodulatory cytokines to support T-cell function. Interestingly, the DC:T-cell interaction has also been implicated in not only engendering immune response but also in immune-tolerance. It has been demonstrated that stable DC:T-cell interaction is required for the development of immune tolerance *in vivo* [525, 526, 531]. Moreover, it was reported that nTreg interacted with self-antigen presenting dendritic cells prior to exerting suppressive effect on islet antigen–specific $CD4^+CD25^-$ T helper cells in diabetogenic mouse model [532]. These observations suggest that the induction and function of inducible regulatory T-cells such as Tr1 cells could also be influenced by DC:T-cell interaction.

In our study, we investigated the role of CD55-CD97 interaction between DC:T-cell on the induction of $IL-10^+$ $CD4^+$ Tr1 cells and demonstrated that blocking CD55-CD97 interaction leads to significantly reduced induction of Tr1 cells. While immature DC (iDC) expressed higher level of CD97, they induced lower number of Tr1 cells compared to mature DC (mDC). The maturation status of dendritic cells could have potentially influenced the CD55-CD97 interaction which in turn affected the induction of Tr1 cells. It has been previously demonstrated that mature DCs

establish stable, organized and prolonged immune synapses with T-cells while immature DC have short intermittent contacts with T-cells without forming organized immune synapses [559]. So stable immune synapse formation could be one of the prerequisites for the CD55-CD97 interaction between DC:T-cell leading to induction of Tr1 cells.
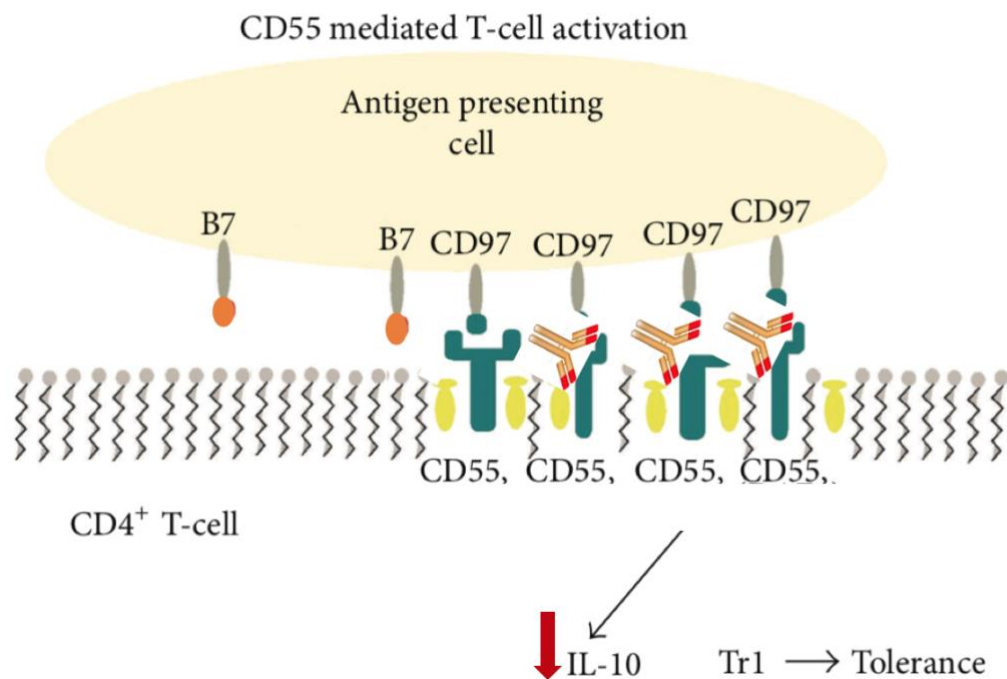
Figure 4.8: Schematic illustration of proposed CD55-CD97 interaction mediated induction of IL-10^{+} Tr1 cells. CD97, expressed on the surface of mature DC could interact with CD55 expressed by CD4 T-cell and lead to the induction of Tr1 cells which contributes to maintain tolerance. Blocking the CD55-CD97 interaction would impede the induction of Tr1 cells and IL-10 secretion.

Immune-homeostasis requires immune-tolerance to self-antigens. The elimination of self-reactive T-cells during T-cells development as well as development of self-antigen nTreg contributes to maintenance of

tolerance. Moreover, recently a tolerogenic subset of primary circulating dendritic cell, termed DC-10, has been identified based on CD141 and CD163 expression and it has been demonstrated that DC-10 cells induce the differentiation of allogenic naïve T-cells into CD49b$^+$LAG-3$^+$ Tr1 cells [560]. The presence of such tolerogenic DCs in vivo indicates that alloantigen-specific Tr1 cells induced in the peripheral immune system following recognition of self-antigen (signal-1) also contributes to immune-tolerance. While induction of Tr1 cells by dendritic cells has been attributed to high level of IL-10 production by these tolerogenic DCs which provides the signal-3 for cell activation, the costimulatory signal (signal -2) has not been determined. In our study, the induction of Tr1 cells by both CD1c$^+$ and moDC without the presence of any foreign antigen (such as viral or bacterial antigen) further supports the notion of development of alloantigen-specific Tr1 cells by a subset of DCs. The decreased induction of Tr1 following the blocking of CD55-CD97 interaction suggests that this co-stimulatory signal promotes the inducible tolerogenic T-cells in the periphery.

In the recent years, several studies demonstrated that tolerogenic dendritic cells- including moDCs differentiated in the presence of IL-10 [561, 562], rapamycin [563], dexamethasone [564], vitamin D3 [565, 566] as well as IL-10 producing primary circulating dendritic cells known as DC-10 [323, 567], can induce regulatory T-cells. Tolerogenic DCs are currently being explored for clinical application such as immune-suppression following organ transplantation and immunotherapy for auto-immune diseases. However, the expression of CD97 by these tolerogenic

DCs has not been studied yet and it would be intriguing to determine if

CD55-CD97 interaction is one of the mechanisms by which tolerogenic

DC induce Tr1 cells and confer immune-suppression.

5 Chapter 5: Effect of different immune-modulators on CD3/CD55 mediated IL-10 production

5.1 Introduction

The role of IL-10$^+$CD4$^+$ Tr1 cells has been explored in various diseases since the existence of these cells *in vivo* were noted in Severe Combined Immuno Deficiency (SCID) patients transplanted with HLA-mismatched hematopoietic stem cells and did not develop Graft vs. Host Disease (GvHD) [568]. The function of Tr1 cells is of particular interest due to its ability to maintain immune homeostasis which is disrupted under disease condition. The prevalence of Tr1 in cancer could potentially suppress anti-tumour immunity whereas the paucity of Tr1 in auto-immune diseases would facilitate the aggravated activity of pro-inflammatory self-reactive immune responses. Indeed, it was reported that highly potent immunosuppressive IL-10$^+$LAP$^+$FoxP3$^-$ CD4$^+$ T-cells were prevalent in tumour infiltrating lymphocytes in Colorectal Cancer (CRC) patients [569, 570]. Moreover, enhanced level of Tr1 were observed in Hodgkin lymphoma patients with Epstein–Barr virus infection along with the upregulation of certain chemokines, including CCL17, CCL4, CCL5, CCL22, CCL20 and CXCL9, which might contribute to the recruitment of the Tr1 cells [571]. It was also determined that tumour infiltrating Plasmacytoid Dendritic cells (pDCs) promote Tr1 in hepatocellular carcinoma and liver metastases from colorectal cancer by upregulating ICOS-L which induce IL-10 production by Tr1 cells [552]. In addition, it was determined that chemotherapeutic agent used for the treatment cancer, such as Cisplatin, induce tolerogenic DCs which in turn enhance

Tr1 cells [572].

In contrast to the augmentation of Tr1 cells in cancer, a lack of Tr1 cells has been observed in several auto-immune diseases. Studies on Multiple Sclerosis (MS) patients identified abnormality in the CD46 mediated Tr1 differentiation and detected lower level of IL-10 production in patients compared to healthy controls [385, 441, 508]. The lower level of IL-10 in secondary progressive MS patients were also found to be associated with higher disability and MRI lesion load which contributed to both pathological process and disease outcome [434]. Similarly, in Rheumatoid Arthritis (RA) patients, the frequency of IL-10$^+$LAG-3$^+$CD4$^+$ were lower compared to healthy individuals and the lower number of regulatory cells were associate with higher Clinical Disease Activity Index scores [573]. It was also reported that allergen specific Tr1 cells, but not thymus derived nTreg defined as CD4$^+$CD25$^+$CD127^{Low} cells, were decreased in persistent allergic rhinitis and there was an inverse correlation between the frequency of Tr1 cells and the clinical symptom scores [574]. Interestingly, in patients with immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome, which is caused by mutation in FoxP3 gene [575], IL-10$^+$CD4$^+$ Tr1 cells could be isolated from patients and expanded *in vitro* in presence of IL-10 and IFN-α [308]. Tr1 cells are also important for patients with organ transplantation where enrichment of Tr1 cells could potentially prolong the survival of graft by suppressing host responses and preventing GvHD [576, 577]. These observations led to studies which investigated the effect of immune-modulators (e.g. immune-suppressive agents, drugs used for treatment for diseases etc.) on Tr1 cells.

Previous studies demonstrated that immuno-modulatory glucocorticoids (GC) such as Dexamethasone, prednisone etc. increased the frequency of FoxP3^{+} nTreg with the ability to suppress effector cells in auto-immune diseases [578-581]. It was also reported that Dexamethasone confer immunosuppressive effect on PBMCs by reducing Th1 type pro-inflammatory cytokine IFN-γ and promoting Th2 cytokine IL-4 along with IL-10 [580]. The effect of Dexamethasone on the differentiation of CD4^{+}T-cells has also been revealed in studies which demonstrated that exposing naïve CD4^{+} T-cells to Dexamethasone and activating them with CD3/CD28 results in higher IL-10^{+} cells instead of IFN-γ^{+} which provides these cells the ability to modulated immune response [582]. A combination of Vitamin D3 and Dex had even more pronounce effect on the reduced IFN-γ production and enhanced IL-10 production following CD3/CD28 stimulation confirming that immune-modulators mediate suppression by controlling Th1 response and promoting regulatory responses [583, 584]. In a separate study in glucocorticoid-resistant asthma patients, it was demonstrated that Vitamin D3 could reinstate of effect of Dex treatment and restore enhanced IL-10 production in the GC-resistant patient to the levels of GC-sensitive patients [585]. Vitamin D3 also modulated IL-10 production via CD3/CD46 pathway in CD4^{+} T-cells and efficiently changed the IL-10^{+}: IFN-γ^{+} to reinstate the immunological balance from pro-inflammatory responses in patients diagnosed with MS [509]. Moreover, it was reported that Dex and IL-10 had synergistic inhibitory effect on CD4^{+} cell proliferation [586]. However, it was later demonstrated that Dex had differential effect on CD4^{+}CD25^{+} nTreg and CD4^{+}CD25^{-} where regulatory cells were more

resistant to Dex mediated cell death indicating Dex treatment confers immune suppression by not only affecting effector cells but also by favouring regulatory cells [587]. Finally, first line treatments of MS such as Interferon-β also demonstrated the ability to elevate IL-10 production in MS patients through which it conferred immune suppression [348, 454, 455]. Currently a wide range of Disease Modifying Treatments (DMTs) are available and these are routinely used in the clinic based on the diagnosis of the disease condition of the patients. Some of these DMTs exert their effect by targeting T cells, regulatory cells, B cells, and cell trafficking into the nervous system. Some of the DMTs include-

i. Alemtuzumab (Anti-CD52 monoclonal Antibody)
ii. Interferon-β (cytokine)
iii. Fingolimod
iv. Cladribine (purine analogue)
v. Daclizumab (Anti-CD25 monoclonal Antibody)
vi. Dimethyl fumarate (methyl ester of fumaric acid)
vii. Glatiramer acetate (4 amino acid polymer)
viii. Natalizumab (Anti-α4 integrin antibody)
ix. Ocrelizumab (Anti-CD20 antibody)

Considering the critical role of immune-modulators on IL-10 production in autoimmune diseases and previously reported dysregulation of the CD46 induced Tr1 cells in MS, we aimed to study the CD55 mediated IL-10 production in MS patients. We hypothesized that induction of CD55 mediated Tr1 cells is defective in MS patients and immune-modulators would enhance CD55 mediated IL-10 production.

5.2 Results:

5.2.1 MS patients produce lower level of IL-10 in response to CD3/CD55 stimulation:

The immune-balance in auto-immune disease patients is considered to be altered due to exaggerated inflammation and inefficacious immune regulatory responses. Previous studies have demonstrated that MS patients responded to antigenic stimulation as well as CD46 costimulatory signal by producing lower level of immune-suppressive cytokine IL-10 compared to healthy individuals [6, 348]. So, in this study, we investigated CD3/CD55 induced IL-10 production in MS patients and compared it with age-and-gender matched heathy individuals.

Naïve $CD4^+$ T-cells were isolated from MS patients as well as healthy donors and stimulated with either CD3/CD55 or CD3/CD28. After 72 hours of cell activation, the IL-10 production in response to costimulatory signals was determined by measuring IL-10 in culture supernatant and performing dual cytokine secretion assay (CSA) for IL-10 and IFN-γ. It was determined that MS patients produced significantly lower (P=0.0124, paired t-test, n=11) amount of IL-10 (on average 841 pg/mL) in response to CD3/CD55 stimulation compared to heathy controls (mean 2140 pg/mL) (Figure-5.1). In contrast, no significant difference was noted in IL-10 production following CD3/CD28 stimulation (Figure-5.1C). In line with these observations, reduced number of IL-10^+ cells were detected by CSA in MS patients following CD3/CD55 stimulation. In some

MS patients, no or less than 0.1% IL-10$^+$ Tr1 cells was observed along with higher number of IFN-γ^+ cells (more than 90% noted in case of MS patient 1 and 2 as shown in Figure 5.2). However, in other patients, including those on various disease modifying treatments, the number of IL-10$^+$ Tr1 cells induced by CD3/CD55 was similar to the number determined in healthy donors (data not shown here). These observations led us investigate the effect of immune-modulators on IL-10 production by CD3/CD55 induced Tr1 cells.

A)

IL-10 production after 72 hours of cell stimulation
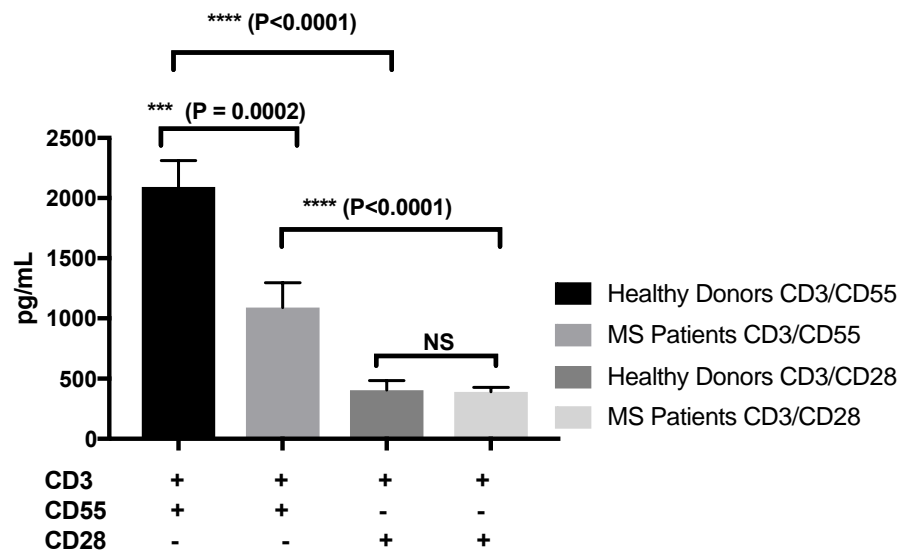
CD3	+	+	+	+
CD55	+	+	-	-
CD28	-	-	+	+

Legend:
- ■ Healthy Donors CD3/CD55
- ■ MS Patients CD3/CD55
- ■ Healthy Donors CD3/CD28
- ■ MS Patients CD3/CD28

B)

IL-10 production after 72 hours of cell stimulation

| CD3 | + | + |
| CD55 | + | + |

- ● Healthy Donors
- ■ MS Patients

C)

IL-10 production after 72 hours of cell stimulation

| CD3 | + | + |
| CD28 | + | + |

- ● Healthy Donors
- ■ MS Patients
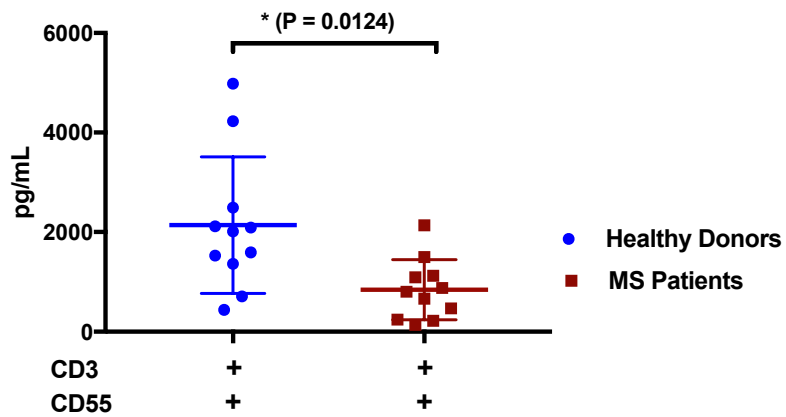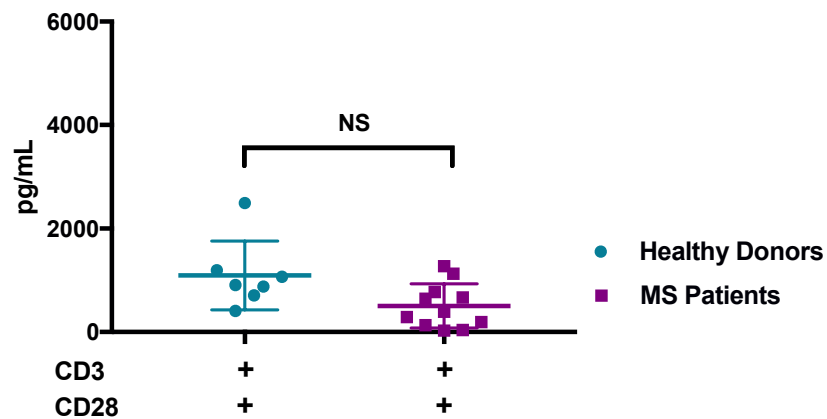
Figure 5.1: Comparison of IL-10 production by CD4 T-cells in response to CD3/CD55 and CD3/CD28 stimulation between MS patients and heathy control. Naïve CD4 T-cells were isolated from blood of both MS patients and healthy donors were stimulated with either CD3/CD55 or CD3/CD28 for 72 hours. Then the amount of IL-10 produced in response to the CD3/CD55 and CD3/CD28 was determined by evaluating by ELISA. IL-10 responses to costimulatory signal in individual MS patient along with the age-and-gender matched heathy donor control is demonstrated in (A). The cumulative data obtained from 11 independent experiments are shown in (B) and (C). In each experiment, triplicate of each condition was measured to determine the IL-10 secretion. Statistical significance was determined by two-way ANOVA for (A) and paired t-test for (B) and (C). [For (B), P=0.0124, 95% confidence interval 347.9 t0 2250, mean difference 1299 pg/mL]
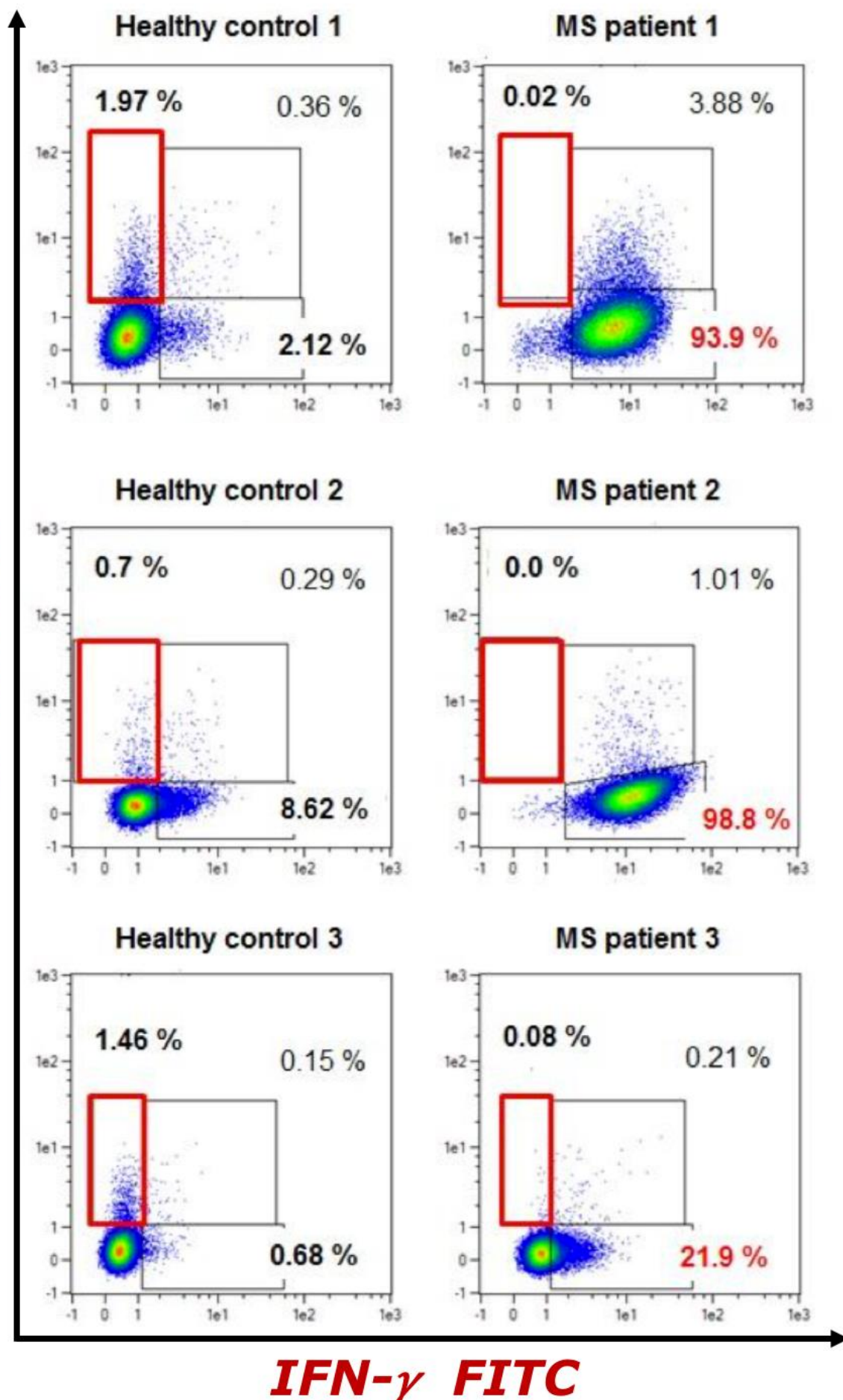
Figure 5.2: Detection CD3/CD55 induced IL-10$^+$ Tr1 cells and IFN-γ^+cells in MS patients and healthy controls. Naïve CD4 T-cells were isolated from blood of both MS patients and healthy donors were stimulated with either CD3/CD55 or CD3/CD28. After 72 hours of cell stimulation, IL-10$^+$ Tr1 cells were determined by performing dual CSA for IL-10 and IFN-γ. Data shown here was obtained from three independent experiments and negative control for specific experiment was used to determine the IL-10$^+$ and IFN-γ^+ cells (not shown here). (Data kindly shared by Dr. Ruhcha Sutavani)

5.2.2 Dose dependent effect of immune-modulators on CD3/CD55 mediated IL-10 production

Naïve CD4$^+$ T-cells were stimulated with CD3/CD55 in presence or absence of different immune-modulators including Dexamethasone (Dex), Vitamin D3 and Interferon-β (IFN-β) (Figure-5.3 to 5.5). All of the immune-modulators significantly enhanced IL-10 production in a dose dependent manner except IFN-β which increased IL-10 production with all the dose tested in our experimental settings (dose ranging from 25 IU/mL to 100IU/mL). Vitamin D3 modulated IL-10 production at a low dose 10^{-8} M/mL and addition of higher dose of Vitamin D3 amplified the effect. In case of Dexamethasone, the effect was more prominent at lower dose compared to higher doses. It has been previously reported that Dexamethasone causes cell cycle arrest and apoptosis at higher doses [588] which could influence the effect on IL-10 production at higher doses of Dex. The effect of immune-modulators was consistent in all the healthy donors. Also, the cytokine production was determined after only 72 hours of CD3/CD55 stimulation in naïve CD4$^+$ cells which represent the early response by these T-cells. These observations denote the prominent modulatory potential of Dex, IFN-β and Vitamin D3 on early responses of cells to CD3/CD55 costimulation and their preferential effect on regulatory T-cells.

IL-10 production after 72 hours of cell stimulation
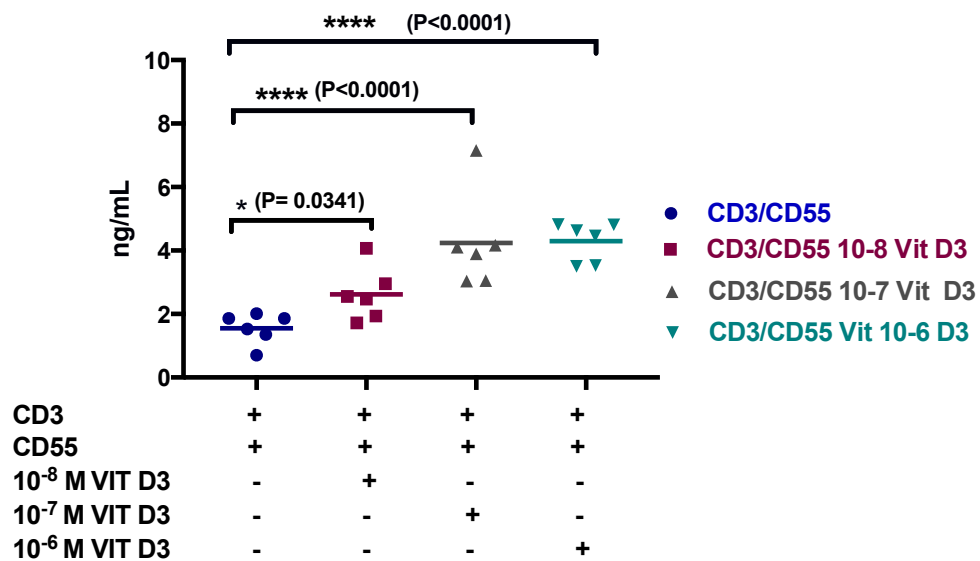
Figure 5.3: Dose dependent effect of Vitamin-D3 on CD3/CD55 induced IL-10 production. Naive CD4^{+} T-cells were stimulated with CD3/CD55 in presence or absence of different doses of Vitamin D3 for 72 hours and the amount of IL-10 production by cells was determined by ELISA. Vitamin D3 enhanced IL-10 production in a dose dependent manner in healthy donors following CD3/CD55 activation. The cumulative data from experiments conducted with samples obtained from a cohort of healthy donors (n=6) is demonstrated here. Statistical significance was determined by two-way ANOVA and data shown here is cumulative results obtained from six independent experiments.

IL-10 production after 72 hours of cell stimulation

CD3	+	+	+	+
CD55	+	+	+	+
10^{-8} M Dex	-	+	-	-
10^{-7} M Dex	-	-	+	-
10^{-6} M Dex	-	-	-	+
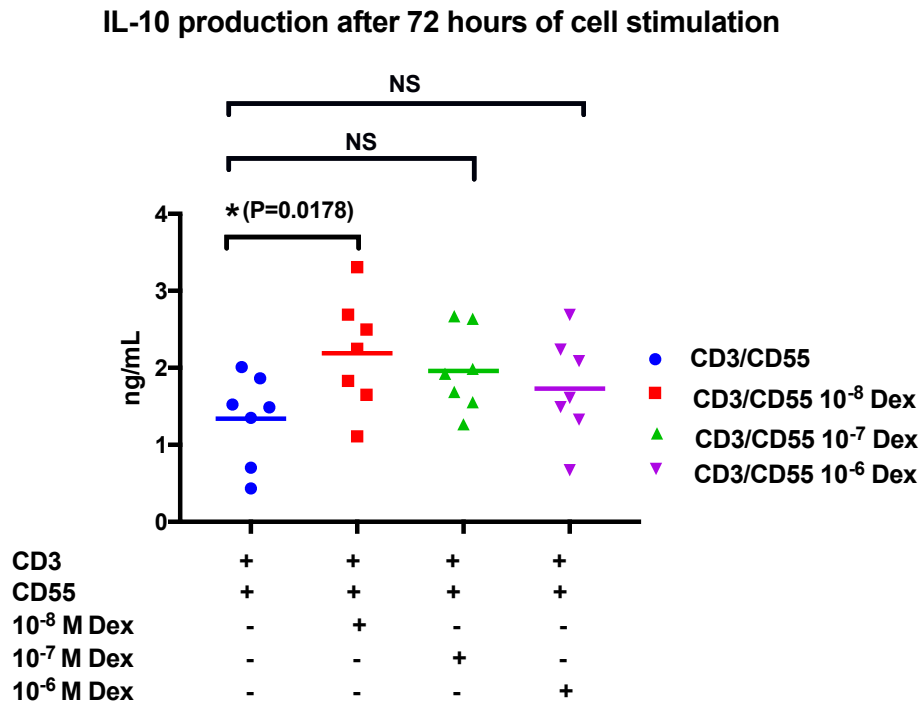
Figure 5.4: Dose dependent effect of dexamethasone on CD3/CD55 induced IL-10 production. Naive CD4^{+} T-cells isolated from healthy individuals were stimulated with CD3/CD55 in presence or absence of different doses of Dexamethasone and culture supernatant was collected after for 72 hours to determine the amount of IL-10 production by cells. The dose dependent effect of dexamethasone on CD3/CD55 mediated IL-10 production from seven independent experiments is demonstrated here. Dexamethasone significantly enhanced CD3/CD55 induced IL-10 production at the lowest dose (10^{-8}M) (P=0.0178, statistical significance was determined by two-way ANOVA, n=7).

IL-10 production after 72 hours of cell stimulation

CD3	+	+	+	+
CD55	+	+	+	+
IFN-B 25 U/mL	-	+	-	-
IFN-B 50 U/mL	-	-	+	-
IFN-B 100 U/mL	-	-	-	+

Legend:
- CD3/CD55
- CD3/CD55 IFN-b 50 U/mL
- CD3/CD55 IFN-b 25 U/mL
- CD3/CD55 IFN-b 100 U/mL

* (P= 0.0202)
* (P= 0.0253)
* (P= 0.0306)
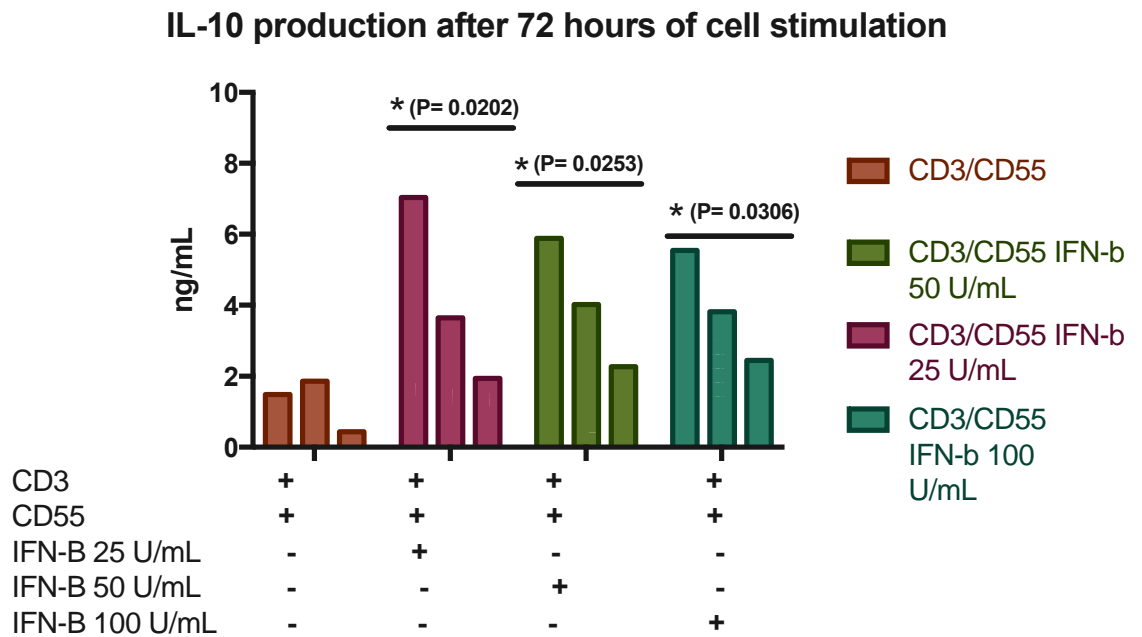
Figure 5.5: Dose dependent effect of IFN-β on CD3/CD55 induced IL-10 production. Naive CD4^{+} T-cells, isolated from healthy individuals, were stimulated with CD3/CD55 in presence or absence of different doses of IFN-β and culture supernatant was collected after for 72 hours to determine the amount of IL-10 production by cells. The dose dependent effect of IFN-β on CD3/CD55 mediated IL-10 production is demonstrated from three independent experiments conducted with healthy donors is demonstrated here. IFN-β significantly enhanced CD3/CD55 induced IL-10 production with all the tested concentration. In each experiment, triplicate of each condition was measured to determine the IL-10 secretion. Statistical significance was determined by two-way ANOVA.

5.2.3 Immune-modulators suppress IFN-γ production in CD3/CD55 activated cells:

Previous studies reported that immune-modulators confer immune-suppression by decreasing pro-inflammatory cytokines such as IFN-γ [589-592]. So, in our study, we investigated the effect of immune-modulators on IFN-γ production by CD3/CD55 stimulated cells. Naïve CD4 T-cells were stimulated with CD3/CD55 in presence or absence of different doses of dexamethasone and Vitamin D3 for 72 hours and culture supernatant was evaluated for determine IFN-γ production. The immune-modulator treated cells produce less amount of IFN-γ following stimulation with CD3/CD55 (Figure-5.6). The decrease of IFN-γ secretion was dose dependent where the highest dose of Vitamin D3 (10^{-6}M) demonstrated the most prominent effect and significantly (P=0.0073, n=5) reduced IFN-γ production compared to the cells which were not treated with immune-modulators. No significant change was observed in IFN-γ production in CD3/CD55 stimulated cells in the presence of lower doses of Vitamin D3 (10^{-8}M) or vehicle only control without any immune-modulators (ethanol + DMSO). Similarly, Dexamethasone also reduced IFN-γ production compared to only CD3/CD55 treated cells although the decrease in IFN-γ secretion was not statistically significant (n=5). The suppressive effect of these immune-modulators on CD3/CD28 activated Th1 cells has been previously demonstrated by several studies [582, 593, 594] but their effect on other alternative costimulatory signals has not been studied extensively. The immune-modulators abrogate IFN-γ production which could limit the self-reactive immune responses in MS

patients and alleviate the disease condition. Considering the enhanced IL-10 production and suppressed IFN-γ secretion after treatment with immune-modulators upon CD3/CD55 stimulation, it could potentially modify IL-10:IFN-γ which in turn would re-establish immune balance in the auto-immune disease patients.

A)

IFN-g secretion after 72 Hours of cell stimulation

** (P=0.0295)

** (P=0.0073)

ng/mL

150 —
100 —
50 —
0 —

- CD3/CD55
- CD3/CD55 10-6 D3
- CD3/CD55 10-7 D3
- CD3/CD55 10-8 D3
- Ethanol+DMSO

CD3	+	+	+	+	+
CD55	+	+	+	+	+
10^{-6} M VIT D3	-	+	-	-	-
10^{-7} M VIT D3	-	-	+	-	-
10^{-8} M VIT D3	-	-	-	+	-
Ethanol+DMSO	-	-	-	-	+

B)

IFN-g secretion after 72 Hours of cell stimulation

NS

NS

NS

ng/mL

150 —
100 —
50 —
0 —

- CD3/CD55
- CD3/CD55 10^{-6} DEX
- CD3/CD55 10^{-7} DEX
- CD3/CD55 10^{-8} DEX

CD3	+	+	+	+
CD55	+	+	+	+
10^{-6} M DEX	-	+	-	-
10^{-7} M DEX	-	-	+	-
10^{-8} M DEX	-	-	-	+
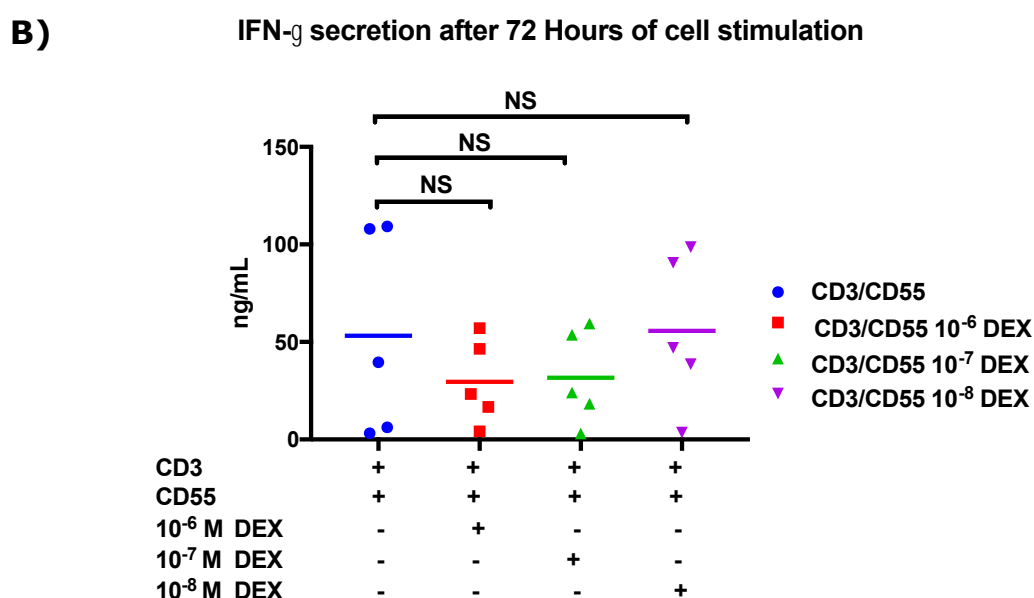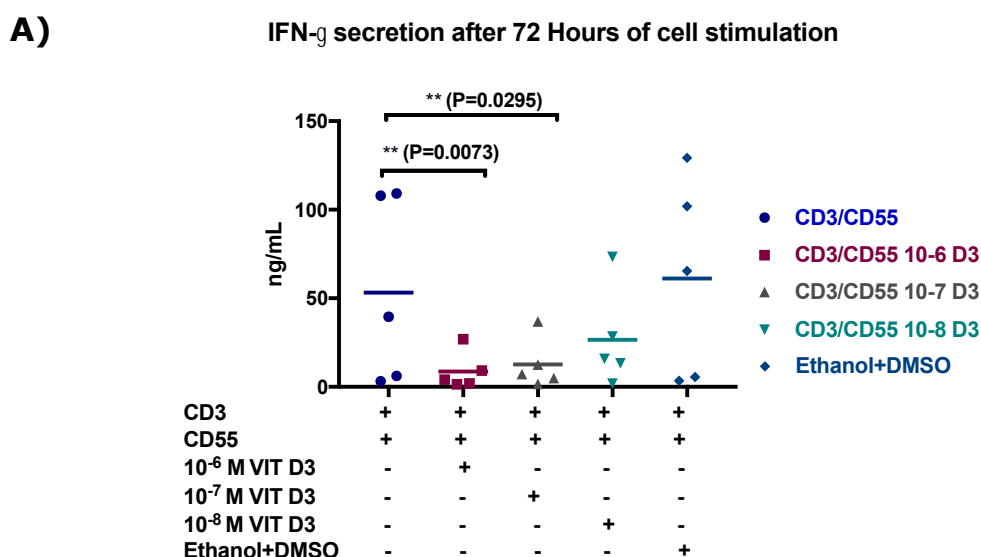
Figure 5.6: Dose dependent effect of vitamin-D3 and dexamethasone on IFN-γ production in response to CD3/CD55 costimulation. Naïve CD4 T-cells were stimulated with CD3/CD55 in presence or absence of different doses of Vitamin-D3 (A) and Dexamethasone (B) after 72 hours of cell activation and culture supernatant was used for ELISA to determine IFN-γ production. Statistical significance was determined by two-way ANOVA and the data shown here is the cumulative results obtained from five independent experiments. In each experiment, triplicate of each condition was measured to determine the IFN-γ secretion.

5.2.4 Immune-modulators preferentially elevate IL-10 production following CD3/CD55 stimulation:

Different costimulatory signals to Naïve CD4+ T-cells leads to activation, differentiation and proliferation of cells. While classical costimulatory signal such as CD3/CD28 results in pro-inflammatory Th1 response, alternative costimulatory CD3/CD55 signal induces anti-inflammatory Tr1 response. In the next stage of our study, we compared the effect of immune-modulators on IL-10 production by cells in response to CD55 and CD28 costimulation. Naïve CD4 T-cells were stimulated with either CD3/CD55 or CD3/CD28 in presence or absence of optimal dose of dexamethasone (5×10^{-8} M) and IFN-β (50 IU/mL) (optimal dose was selected on the basis of the results of the dose titration experiments described in 5.2.2). The presence of IFN-β altered the response in both CD3/CD28 and CD3/CD55 stimulated cells and significantly enhanced IL-10 production (Figure 5.8) whereas Dexamethasone preferentially elevated IL-10 production following only CD3/CD55 but not CD3/CD28 stimulation (Figure-5.7) during the early response (On Day 3). The difference in the ability of the immune-modulators to enhance IL-10 production following CD/CD28 costimulation might indicate that they have varying potential to influence Th1 population. Interestingly, both of the immune-modulator increased the IL-10 production in the CD3/CD55 stimulated cells which raises the possibility that they could be exerting the effect on Tr1 cells via same molecular pathway.

IL-10 secretion after 72 Hours of cell stimulation

CD3	+	+	+	+
CD55	+	+	-	-
CD28	-	-	+	+
Dex	-	+	-	+
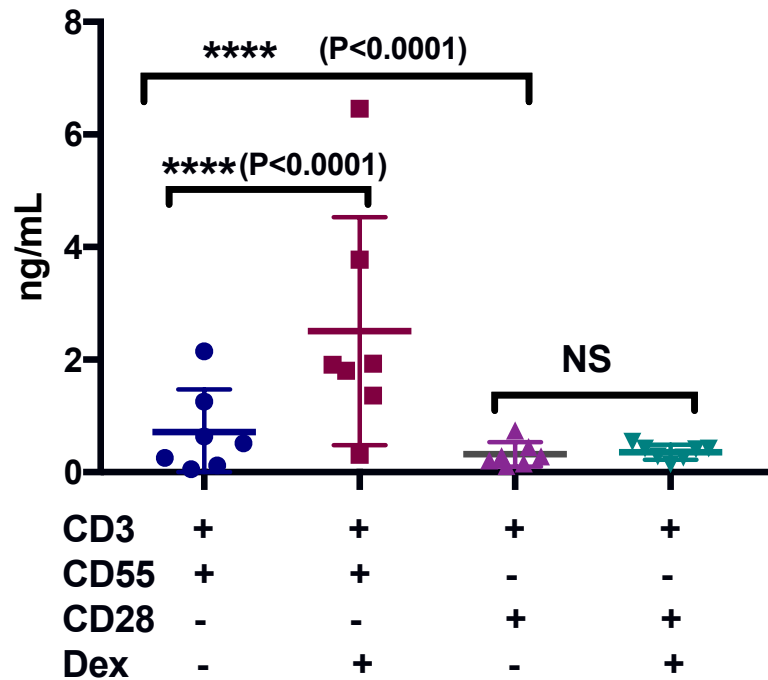
Figure 5.7: Dexamethasone preferentially increases IL-10 production when Naïve CD4^{+} T-cells are stimulated with CD3/CD55 but not CD3/CD28. Dexamethasone preferentially increases IL-10 production when Naïve CD4^{+} T-cells are stimulated with CD3/CD55 but not CD3/CD28. Naïve CD4^{+} T-cells were stimulated with either CD3/CD55 or CD3/CD28 in presence or absence of Dexamethasone ($5X10^{-8}$ M) and the culture supernatant was collected after 72 hours to evaluate IL-10 production by cells in response to different stimuli. CD3/CD55 stimulated cells secreted significantly higher amount of IL-10 ($P<0.0001$) in comparison to CD3/CD28 stimulated cells and the IL-10 production was further enhanced in CD3/CD55 stimulated cells with Dexamethasone treatment compared to the untreated cells ($P<0.0001$). Similar IL-10 enhancing effect was not observed in CD3/CD28 stimulated cells in the presence of dexamethasone. Statistical analysis was done by performing two-way ANOVA and data is representative of cumulative results from seven independent experiments (n=7). In each experiment, triplicate of each condition was measured to determine the IL-10 secretion and mean of the triplicate values was used for statistical analysis.

IL-10 secretion after 72 Hours of cell stimulation
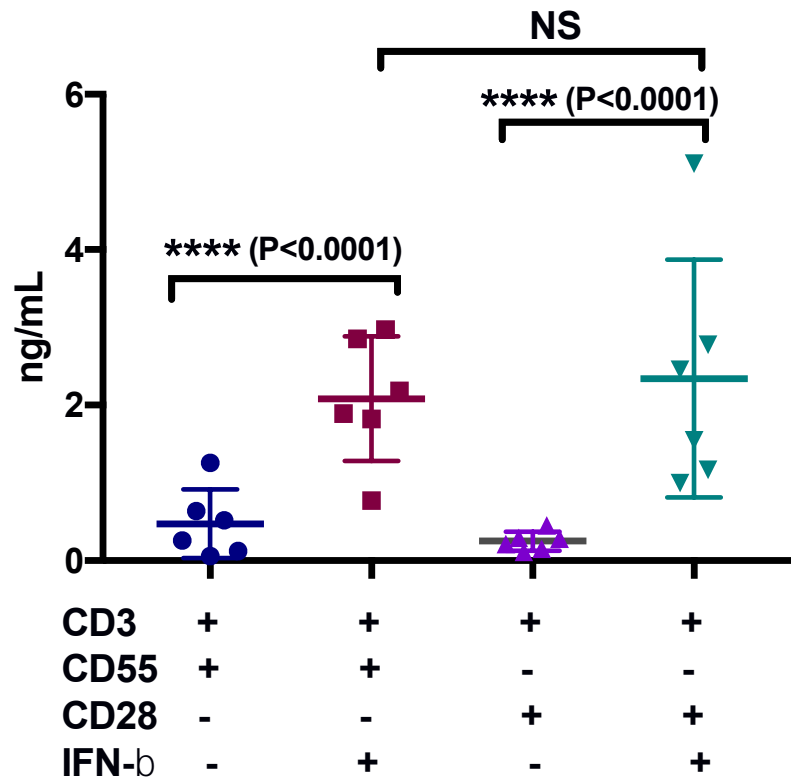
Figure 5.8: Comparison of modulation of IL-10 production in CD3/CD55 and CD3/CD28 stimulated cells by IFN-β. Naïve CD4^{+} T-cells were stimulated with either CD3/CD55 or CD3/CD28 in presence or absence of IFN-β and the culture supernatant was collected after 72 hours to evaluate IL-10 production by cells in response to different stimuli by ELISA. IL-10 production was enhanced in CD3/CD55 stimulated cells in the presence of IFN-β compared to the untreated cells (P<0.0001). Similarly, IFN-β also significantly enhanced IL-10 production in CD3/CD28 stimulated cells compared to untreated CD3/CD28 stimulated cells. No significant difference was noted in IL-10 production between CD3/CD55+IFN-β and CD3/CD28+IFN-β stimulated cells. Statistical analysis was done by performing two-way ANOVA and data is representative of cumulative results from six independent experiments (n=6). In each experiment, triplicate of each condition was measured to determine the IL-10 secretion and mean of the triplicate values was used for statistical analysis.

5.2.5 Immune-modulators exert their effect by inducing IL-10$^+$ Tr1 cells and suppressing IFN-γ^+ cells

CD3/CD55 induces the differentiation of Naïve CD4$^+$ T-cells into a discrete regulatory IL-10$^+$ Tr1 cell population. The addition of immune-modulators resulted in 2-3 fold increase in IL-10 secretion which was determined by ELISA. However, it was unclear whether the immune-modulators enhanced the cytokine production by each cytokine producing cell or they increased the number of cells that produced cytokine. In order to address this question, naïve CD4$^+$ T-cells were stimulated with either CD3/CD55 or CD3/CD28 in presence or absence of immune-modulators and after 72 hours of cell activation, they were assessed for cytokine production by using CSA. It was determined that the immune-modulator treatment led to increased IL-10$^+$ Tr1 cells while it suppressed IFN-γ^+ cells (Figure 5.9). Dexamethasone preferentially modulated and significantly increased (P=0.0021, n=5) the percentage of IL-10 producing cells following CD3/CD55 costimulation. In contrast, IFN-β modified responses to both CD3/CD55 and CD3/CD28 stimulation and amplified the number of IL-10$^+$ cells (Figure-5.10). Both Dexamethasone and IFN-β demonstrated immune-suppressive potential which was partially exerted by reducing IFN-γ^+ cells. The ability to regulate both IL-10 and IFN-γ production renders immune-modulators the efficacy to shift immune response from pro-inflammatory to anti-inflammatory.

IL-10 (vertical axis label, left side)

IFN-γ (horizontal axis label, bottom)

CD3/CD55
- 2.26%
- 1.19%
- 78.80%
- 17.75%

CD3/CD28
- 0.95%
- 2.69%
- 47.51%
- 48.85%

CD3/CD55 + Dex
TM2017-05-22.057\P1\P2
- 6.10%
- 0.80%
- 89.81%
- 3.29%

CD3/CD28 + Dex
TM2017-05-22.061\P1\P2
- 2.25%
- 0.80%
- 87.33%
- 9.62%

CD3/CD55 + IFN-β
TM2017-05-22.059\P1\P2
- 4.52%
- 0.89%
- 87.51%
- 7.08%

CD3/CD28 + IFN-β
TM2017-05-22.063\P1\P2
- 4.75%
- 2.06%
- 77.42%
- 15.78%

187
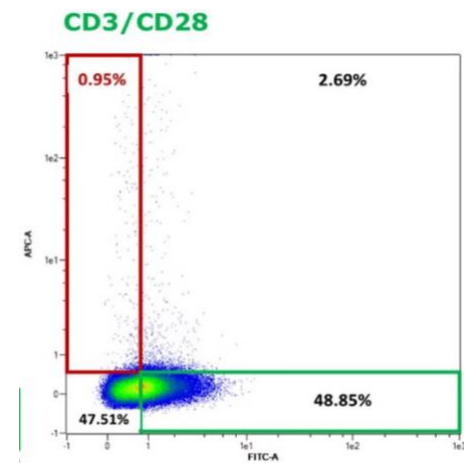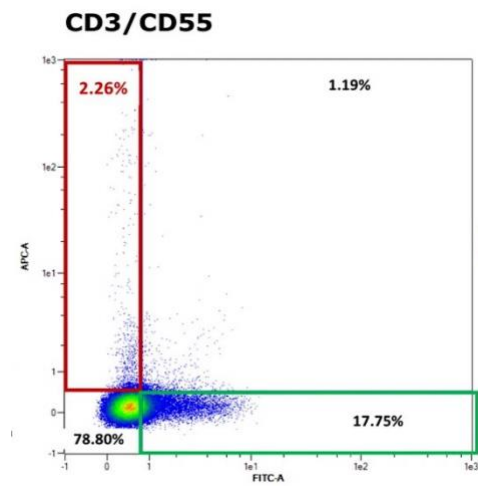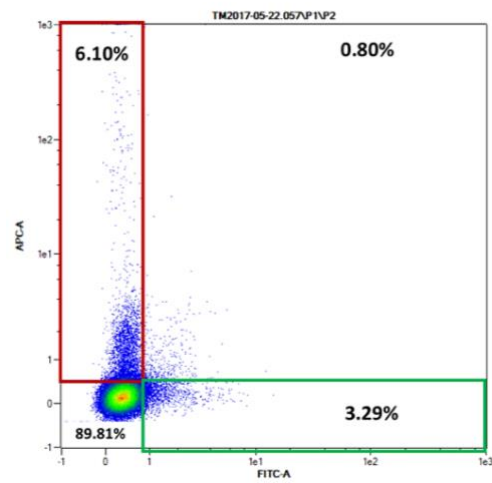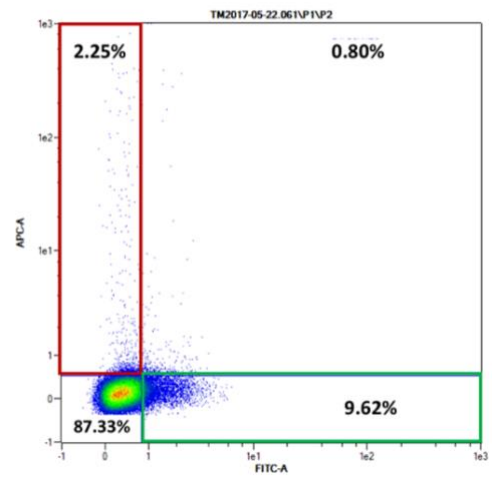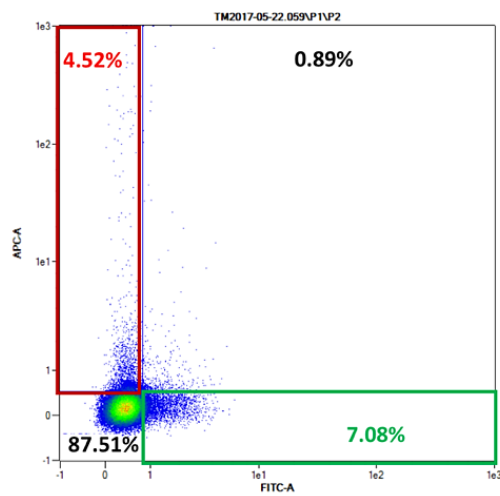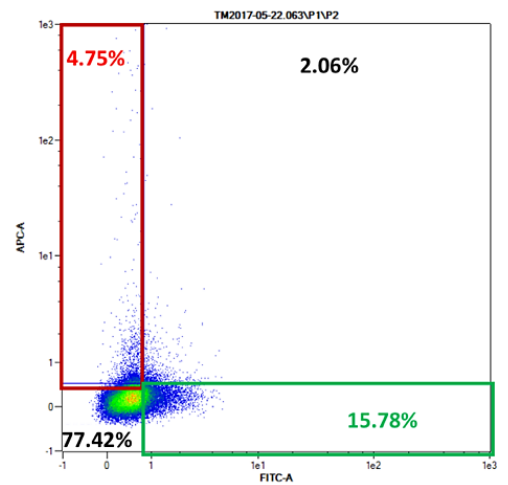
Figure 5.9: The effect of immune-modulators on induction of IL-10$^+$ and IFN-γ^+ cells in response to costimulatory signals. Naïve CD4 T-cells were stimulated with either CD3/CD55 or CD3/CD28 in the presence or absence of dexamethasone (5X10^{-8} M) and IFN-β for 72 hours and dual CSA was performed to determine IL-10$^+$ as well as IFN-γ^+ cells. Data is representative of five independent experiments.
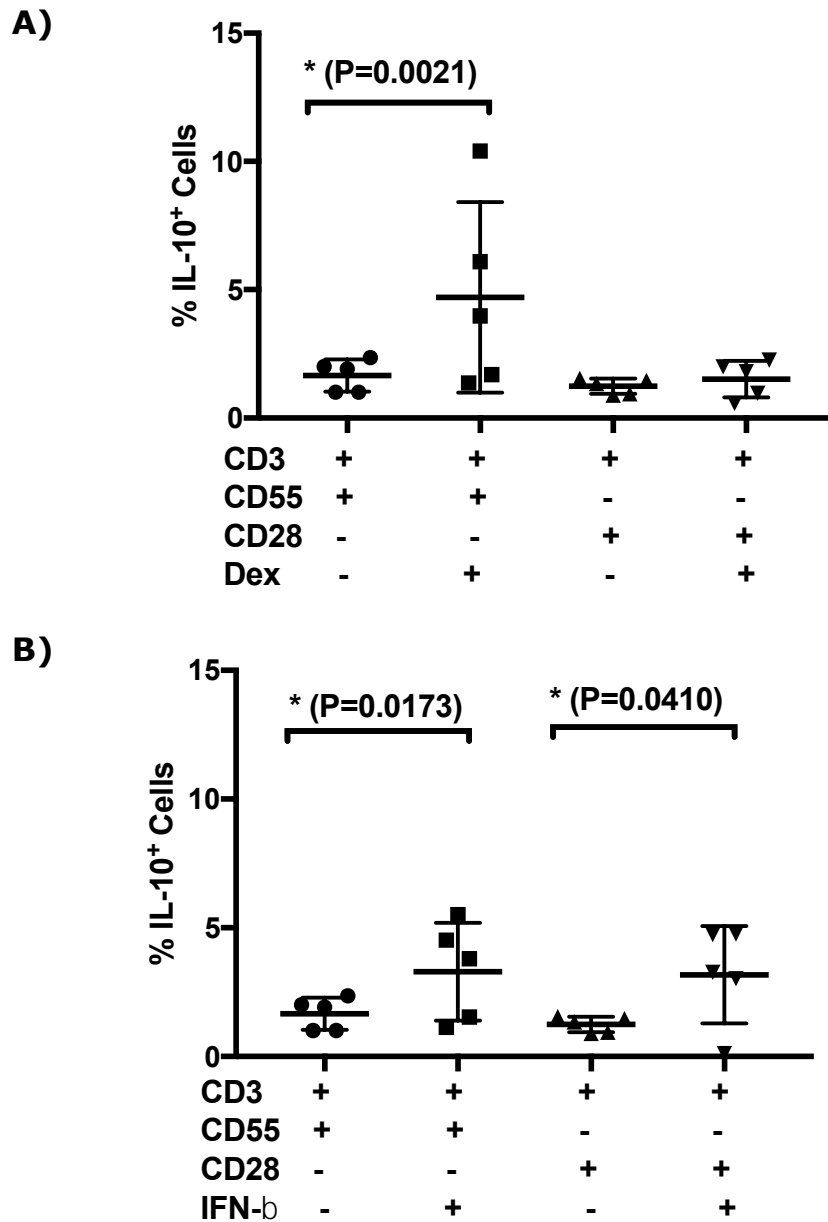
Figure 5.10: The percentage of IL-10⁺ cells under the influence of immune-modulators following CD3/CD55 and CD3/CD28 stimulation. Naïve CD4 T-cells were stimulated with either CD3/CD55 or CD3/CD28 in the presence or absence of (A) dexamethasone ($5X10^{-8}$ M) and (B) IFN-β for 72 hours and dual CSA was performed to determine IL-10⁺ as well as IFN-γ⁺ cells. Statistical significance was determined by two-way ANOVA and data shown here is cumulative results obtained from five independent experiments.

Fold change in percentage of IL-10⁺ Tr1 cells and IFN-γ⁺ in the presence of Dexamethasone
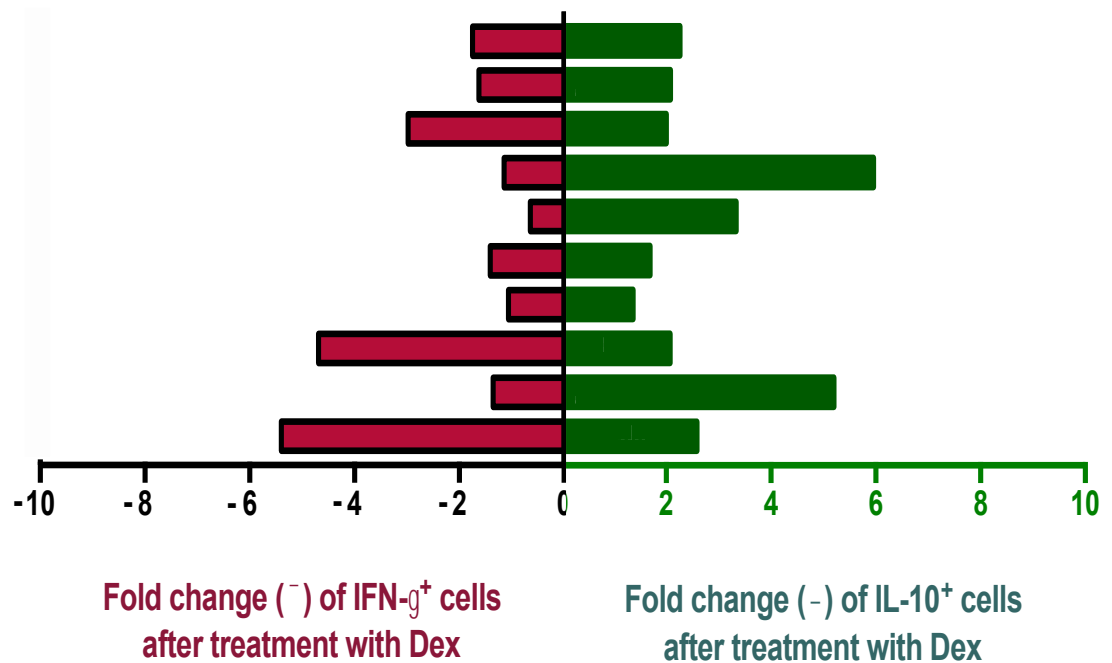
Figure 5.11: Fold change in percentage of IL-10+ Tr1 cells and IFN-γ⁺ in the presence of Dexamethasone. Naïve CD4 T-cells were stimulated with CD3/CD55 in the presence or absence of dexamethasone (5×10^{-8} M) for 72 hours and dual CSA was performed to determine IL-10⁺ as well as IFN-γ⁺ cells. The fold change in IL-10⁺ and IFN-γ⁺ were determined by comparing the dexamethasone treated cells with the untreated cells. The presence of Dex resulted in augmentation of 2-6 fold (mean 2.85 fold, n=10) higher Tr1 cells compared to CD3/CD55 control. Also, Dex suppresses the IFN-γ upto 2-5 fold (mean 2.20 fold, n=10).

5.2.6   The effect of immune-modulators on the cell cycle of the CD3/CD55 stimulated cells:

Immune-modulators (e.g. Glucocorticoids) have been reported to be anti-proliferative as they cause cell cycle arrest and apoptosis [595, 596]. In order to determine if the exposure of CD3/CD55 stimulated cells to various immune-modulators affect their cell division, cell cycle and cell proliferation were evaluated by Propidium Iodide (PI) staining for DNA content, thymidine incorporation assay as well as cell proliferation dye CellTrace^{TM} Violet. In the first stage, naïve CD4 T-cells were stimulated with CD3/CD55 in the presence of various doses of immune-modulators and cell cycle status was determined by PI staining after 72 hours of cell activation. CD3/CD55 stimulated cells without any immune-modulator was used as control and only CD3 stimulated cells were used as negative control to determine cell cycle arrest (data shown in Figure-5.12 and table-5.1, n=3). Only at the highest dose of dexamethasone (10^{-6}M and 10^{-7}M) and vitamin-D3 (10^{-6}M), cell cycle arrest (sub-G_1 and G_0/G_1) was noted and it was similar to that observed in cells stimulated with only CD3 and without any costimulatory signal (Figure-5.12 A and C). Higher percentages of cells were also observed in sub-G_1 and G_0/G_1 phase when cells were stimulated in the presence of highest dose of IFN-β (100 IU/mL). Similar percentages of cells were observed in S phase and G_2/M phase when cells were stimulated with only CD3/CD55 control and CD3/CD55 stimulated cells in the presence of selected optimal doses in this study- dexamethasone Vitamin D3 (10^{-7}M) and IFN-β (optimal dose 50 IU/mL).

A) CD3 only

<2N: 13.4%
2N: 43.8% [μ: 29184, cv: 50.7%]
S: 5.81%
4N: 25.2% [μ: 88768, cv: 40.5%]
Ratio: 3.04
>4N: 11.8%

B) CD3/CD55

<2N: 0%
2N: 23.3% [μ: 23488, cv: 127%]
S: 2.37%
4N: 61.4% [μ: 112896, cv: 43.6%]
Ratio: 4.81
>4N: 13.0%

C) CD3/CD55+ 10^{-6} M Dex

<2N: 8.64%
2N: 56.9% [μ: 26496, cv: 40.6%]
S: 15.1%
4N: 3.69% [μ: 65280, cv: 9.20%]
Ratio: 2.46
>4N: 15.7%

D) CD3/CD55+ 10^{-7} M Dex

<2N: 10.9%
2N: 41.5% [μ: 27072, cv: 43.0%]
S: 13.5%
4N: 3.37% [μ: 61568, cv: 6.26%]
Ratio: 2.27
>4N: 30.8%

E) CD3/CD55+ 10^{-8} M Dex

<2N: 0%
2N: 26.0% [μ: 26368, cv: 129%]
S: 7.25%
4N: 53.6% [μ: 132160, cv: 36.2%]
Ratio: 5.01
>4N: 13.2%

F) CD3/CD55+ 10^{-9} M Dex

<2N: 0%
2N: 21.5% [μ: 31424, cv: 131%]
S: 0%
4N: 61.5% [μ: 122560, cv: 40.9%]
Ratio: 3.90
>4N: 17.0%

192

Figure 5.12: Cell cycle analysis of CD3/CD55 stimulated cells in presence or absence of dexamethasone.

Naive CD4 T-cells were stimulated with CD3/CD55 in the presence or absence of various doses of dexamethasone ranging from 10^{-6} to 10^{-9}M. Only CD3 stimulated cells were used as negative control. After 72 hours of cell stimulation, PI staining for DNA was performed to determine the cell cycle by flow cytometry and the data was analysed by FlowJo (version X). Data is representative of three independent experiments. [Blue: Cells arrested in G_0/G_1 phase; Green: Cells in S phase and Orange: Cell in G_2/M phase].

Table 5.1: Cell cycle analysis with PI staining following CD3/CD55 stimulation in presence or absence of different doses of dexamethasone, vitamin-D3 and IFN-β (data representative of three independent experiments).

Experimental Condition	Sub-G1	G0/G1	S	G_2/M
CD3 only	13.40%	43.80%	5.80%	37.00 %
CD3/CD55	0.00%	23.30%	2.37%	74.40%
+10^{-6}M Dex	8.64%	56.90%	15.10%	19.39%
+10^{-7}M Dex	10.90%	41.50%	13.50%	34.17%
+10^{-8}M Dex	0.00%	26.00%	7.25%	66.80%
+10^{-9}M Dex	0.00%	21.50%	0.00%	78.50%
+10^{-6}M VitD3	**29.10%**	**51.50%**	**0.00%**	**25.68%**
+10^{-7}M VitD3	0.00%	29.70%	0.00%	75.80%
+10^{-8}M VitD3	13.20%	1.76%	21.90%	63.20%
+10^{-9}M VitD3	0.00%	21.00%	6.42%	72.60%
+100IU/ml IFNβ	**17.60%**	**2.10%**	**15.20%**	**69.20%**
+50IU/ml IFNβ	0.00%	26.10%	8.89%	65.10%
+25IU/ml IFNβ	0.00%	17.40%	9.10%	73.60%

5.2.7 Evaluation of the effect of immune-modulators on the proliferation of CD3/CD55 stimulated cells using Thymidine Incorporation Assay

The overall cell proliferation following CD3/CD55 stimulation of Naïve CD$^+$ T-cells in presence or absence of different doses of immune-modulators was determined by thymidine incorporation assay which was performed after 72 hours of cell activation. Radioactive thymidine [^{3}H] was incorporated in the DNA of the cells that went through cell division and it was determined that cell proliferation of the CD3/CD55 stimulated cells were significantly higher (P=0.0317, n=5) than the cells which were stimulated with only CD3 (with the sub-optimal concentration of 1μL/mL to measure the basal level of cell proliferation). Interestingly, there was no significant difference in cell proliferation with immune-modulator treatment (both Vit-D3 and Dexamethasone), even at the highest concentration (Figure 5.13). These findings contradicted the observations of the cell cycle analysis with PI which demonstrated that higher dose of Dex and VitD3 (10^{-6}M and 10^{-7}M) resulted in accumulation of cells in Sub-G_1 and G_0/G_1 phase (table 5.1 and 5.2) which would have led to lower thymidine uptake by cells activated under those conditions. However, the discrepancy in the measurement of cell proliferation between two different assays could be explained by the limitation of thymidine incorporation assay where the [^{3}H] uptake by certain proliferating cell population of the immune-modulator treated cells might result in comparable overall [^{3}H] uptake to the immune-modulator untreated cells.

Also, it could not be determined if the CD3/CD55 induced IL-10$^+$ Tr1 cells proliferated in presence of the immune-modulators by either PI staining for DNA content or [^{3}H] incorporation assay as both of the assays evaluated the cell proliferation of the total cell population and were not appropriate to measure the proliferation of the cytokine producing cells. In order to address these questions, we further investigated the cell proliferation of CD3/CD55 induced Tr1 cells with Flow cytometry by using cell proliferation dye (section 5.2.8)

A) The effect of Vitamin D3 on CD3/CD55 induced cell proliferation

CD3	+	+	+	+	+
CD55	-	+	+	+	+
10^{-8} M VitD3	-	-	+	-	-
10^{-7} M VitD3	-	-	-	+	-
10^{-6} M VitD3	-	-	-	-	+

B) The effect of Dexamethasone on CD3/CD55 induced cell proliferation

CD3	+	+	+	+	+
CD55	-	+	+	+	+
10^{-8} M Dex	-	-	+	-	-
10^{-7} M Dex	-	-	-	+	-
10^{-6} M Dex	-	-	-	-	+
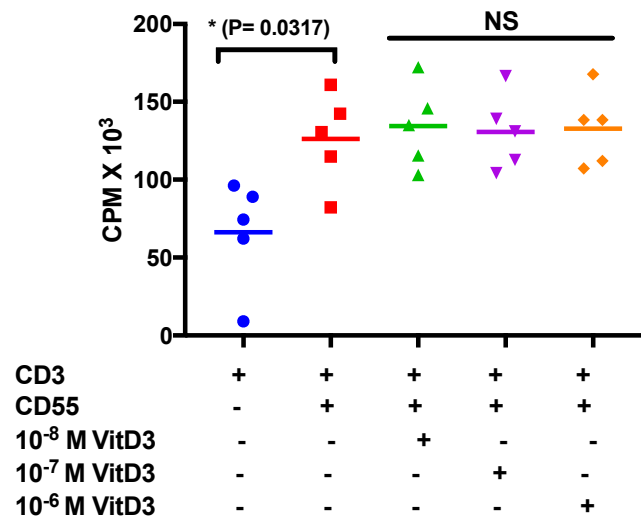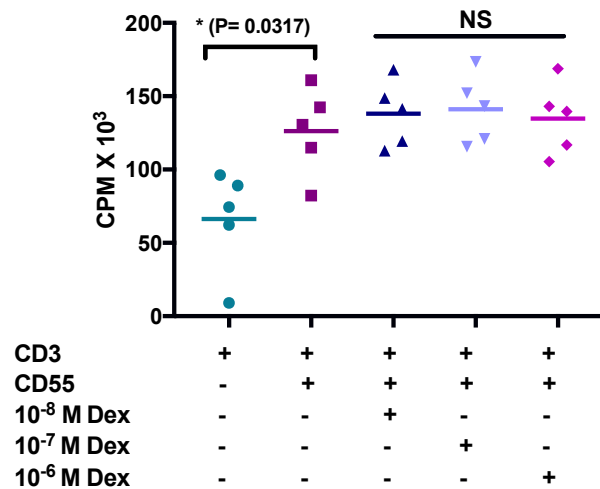
Figure 5.13: Evaluation of dose dependent effect of vitamin-D3 and dexamethasone on CD3/CD55 costimulation induced cell proliferation Naïve CD4 T-cells were stimulated with CD3/CD55 in the presence and absence of different doses of (A) vitamin-D3 and (B) dexamethasone for 72 hours. Then thymidine was added for the last 16 hours of cell culture before the cell proliferation was evaluated by measuring the amount of incorporated thymidine. The graphs represent combined results obtained from five individual experiments and statistical significance was determined by two-way ANOVA.

5.2.8 Determining the proliferative response of CD3/CD55 induced IL-10$^+$ Tr1 cells in presence of immune-modulators

The early studies on Tr1 cells reported that these cells were anergic [597-599]. So, in this study, we investigated the proliferative capacity of CD3/CD55 induced Tr1 cells in presence or absence of immune-modulators by using cell proliferation dye. Naïve CD4 T-cells were stained with Cell Trace Violate prior to stimulation with CD3/CD55 and proliferation of Tr1 cells were determined following detection of IL-10$^+$ by performing CSA after 72 hours. Some cells were stimulated with CD3/CD28 to compare two costimulatory signal induced cell proliferation and only CD3 stimulated cells were used as negative control to determine basal level of proliferation in absence of costimulation. It was determined that both CD3/CD55 and CD3/CD28 stimulation resulted in 3-4 round of cell division (Figure-5.15, 5.16 A and C). Importantly, IL-10$^+$ Tr1 cells demonstrated proliferative capacity similar to that of IL-10$^-$ cells. The presence of dexamethasone and vitamin D3 enhanced the IL-10$^+$ Tr1 cells in CD3/CD55 stimulated cells but not with CD3/CD28 cells and did not alter the cell proliferation. The Tr1 cells differentiated with CD3/CD55 and dexamethasone or vitamins D3 also proliferated in a similar way to those differentiated with only CD3/CD55 costimulation (Figure-5.15; 5.16 A and B) and these cells divided 3-4 times within a period of 72 hours which demonstrated that CD3/CD55 induced Tr1 cells are not anergic.

3rd cell division   **2nd cell division**   **1st cell division**

Cell Trace Violet labelled cells

Cell activation by CD3/CD55 signal
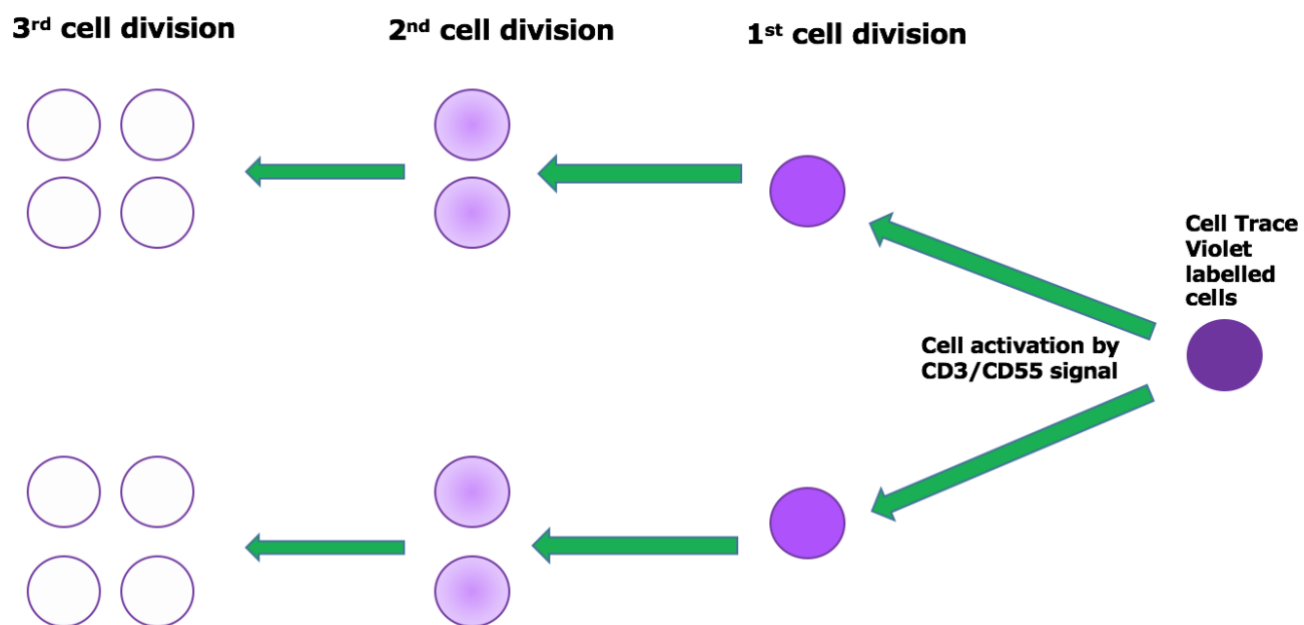
Figure 5.14: The schematic representation of evaluating cell proliferation by the cell proliferation dye CellTrace^{TM} Violet (CTV). The cells of interest are labelled with fluorescent CTV which diffuses into the cells and covalently binds to the intracellular amines. When CTV stained cells are stimulated under various condition, they proliferate in response to the stimuli and starts to lose the fluorescence by half with each round of cell division they go through as the amount of CTV dye is distributed between the daughter cells
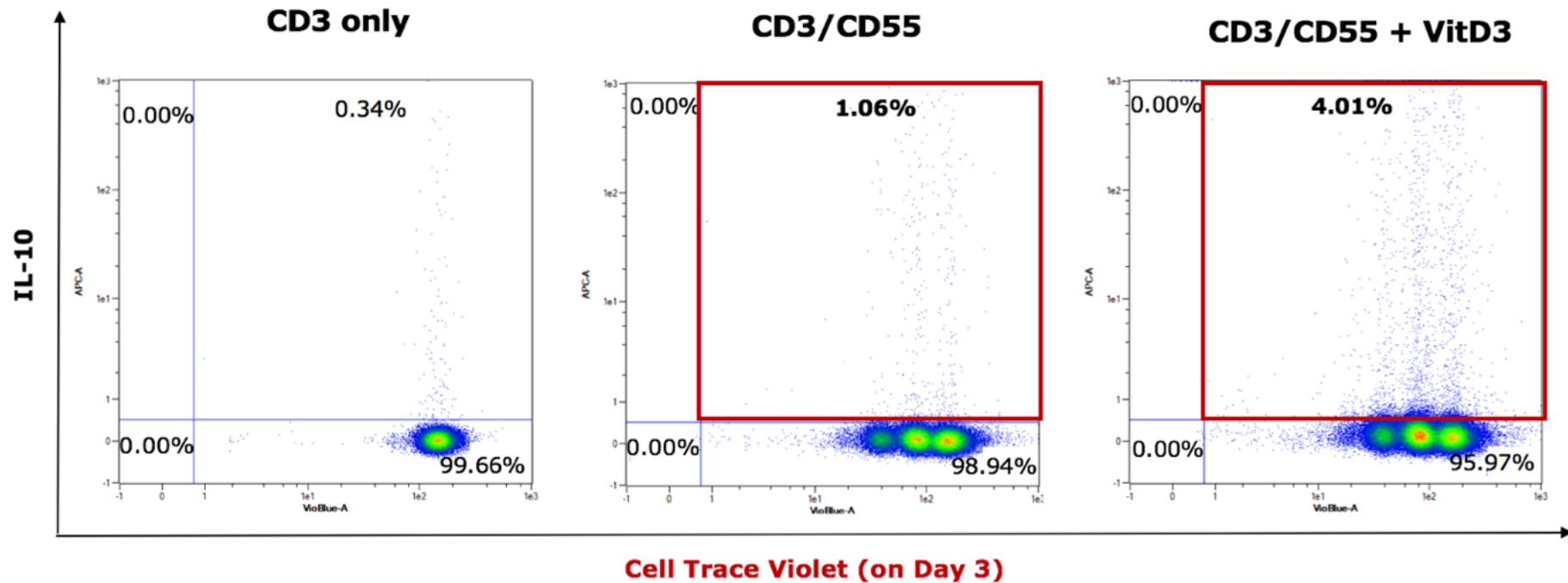
Figure 5.15: Determination of cell proliferation of CD3/CD55 induced IL-10$^+$ tr1 cells in presence or absence of Vitamin-D3. Naïve CD4 T-cell were stained with cell trace violate and stimulated with only CD3, CD3/CD55 and CD3/CD55 + Vitamin D3 (10^{-7}M/mL) for 72 hours. Then IL-10 cytokine secretion assay was performed to detect Tr1 cells before cell proliferation was determined by flow cytometry. Data is representative of three independent experiments.

A)

CD3/CD55

IL-10 APC / IFN-γ FITC

| 1.80% | 0.24% |
| 97.49% | 0.47% |

0.00% | 1.61%
0.03% | 98.36%

0.00% | 0.94%
0.03% | 99.03%

B)

CD3/CD55 + Dex

| 3.41% | 0.19% |
| 95.87% | 0.53% |

0.00% | 3.60%
0.07% | 96.32%

0.00% | 1.36%
0.06% | 98.58%
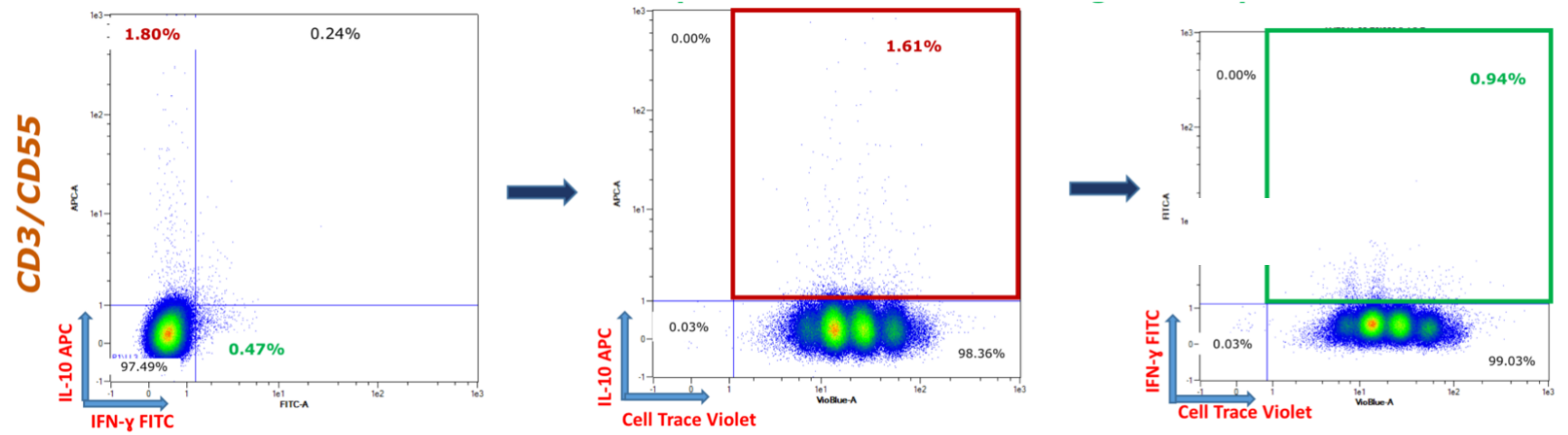
C) *CD3/CD28*
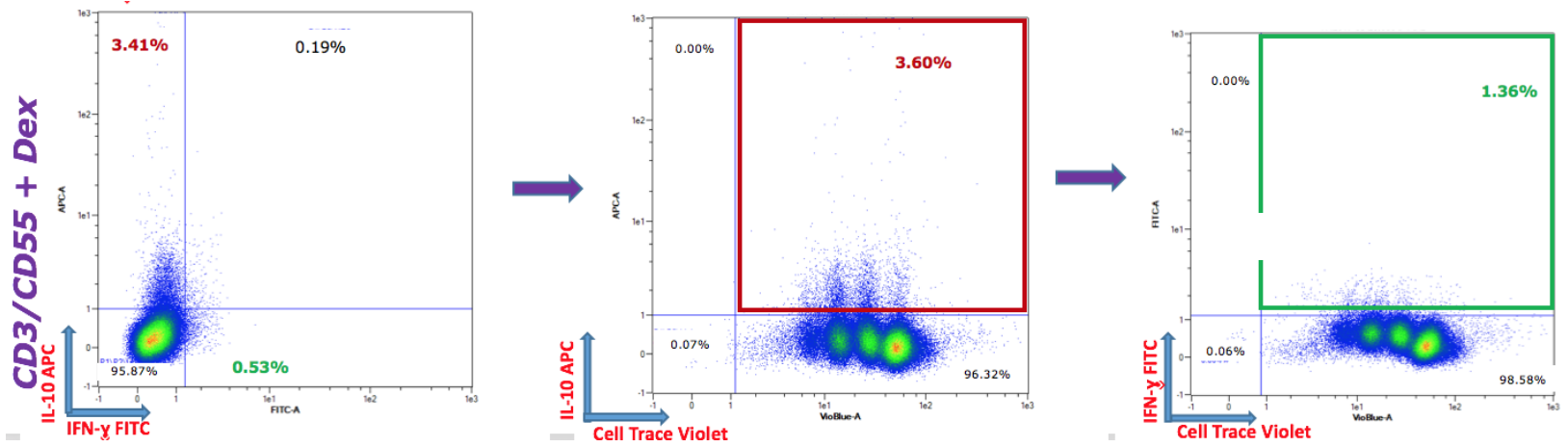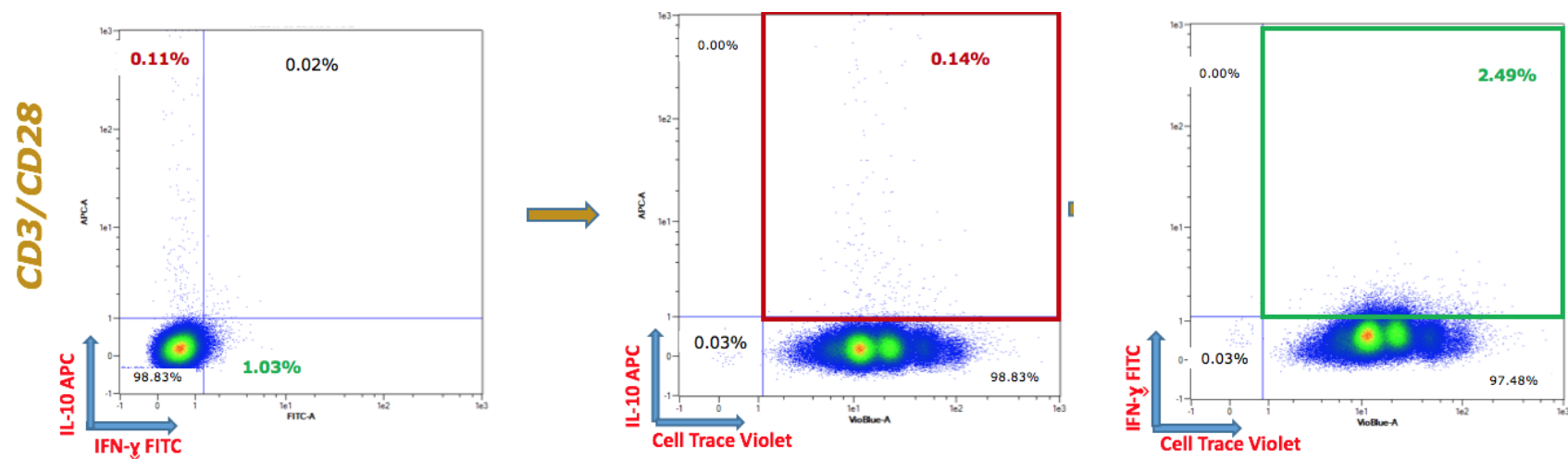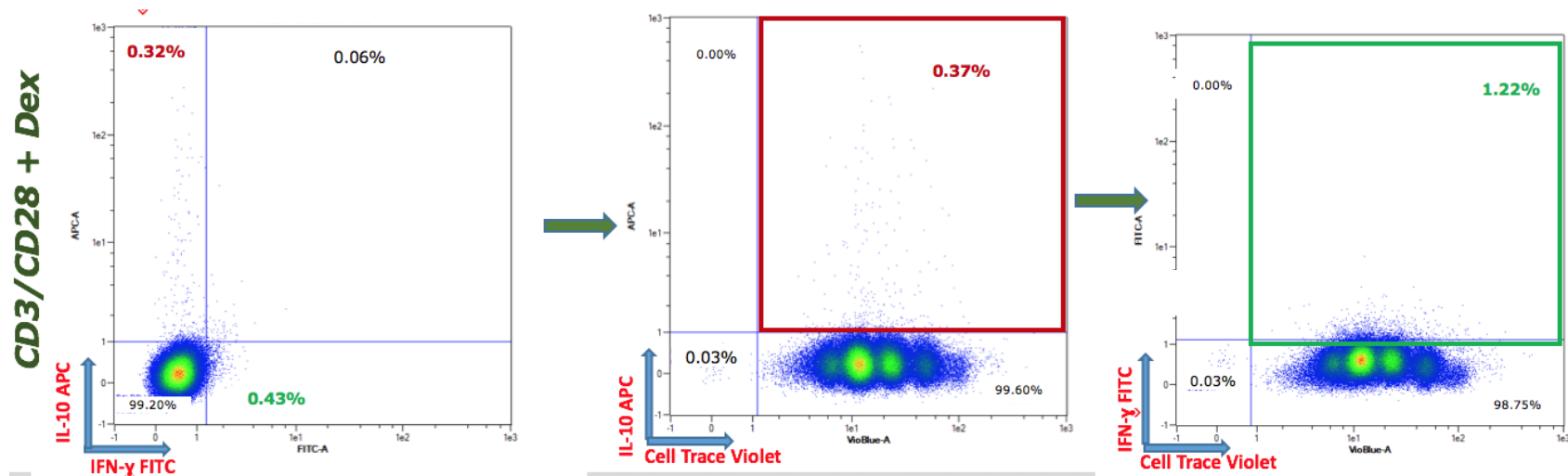
D) *CD3/CD28 + Dex*

Figure 5.16: Determination of cell proliferation of CD3/CD55 and CD3/CD28 induced IL-10$^+$ and IFN-γ$^+$ cells in presence or absence of dexamethasone. Naïve CD4 T-cell were stained with cell trace violate and stimulated with (A) CD3/CD55, (B) CD3/CD55 + dexamethasone (5X10^{-8}M), (C) CD3/CD28 and (D) CD3/CD28 +dexamethasone (5X10^{-8}M) for 72 hours. Then IL-10 cytokine secretion assay was performed to detect Tr1 cells before cell proliferation was determined by flow cytometry. Data is representative of three independent experiments. The data is representative of three individual experiments

5.2.9   Immune modulators have a lasting effect on Tr1 differentiation, through multiple rounds of stimulation

The CD3/CD55 costimulation induces differentiation of Naïve CD4$^+$ T-cells into IL-10$^+$ Tr1 cells and the presence of immune-modulator such as dexamethasone led to augmentation of IL-10$^+$ Tr1 cells. However, it was unclear if the enhanced production of IL-10 was restricted to the presence of immune-modulator and if the characteristics observed during the primary stimulation would be recapitulated upon restimulation of the cells. To address these questions, naive CD4 T-cells were primed with CD3/CD55 in presence or absence of dexamethasone and rested before they were restimulated with either CD3/CD55 or CD3/CD28 (figure-5.19). The Tr1 cells differentiated with CD3/CD55 + dexamethasone remained functional upon secondary stimulation with CD3/CD55 even in the absence of further dexamethasone treatment. The retention of function as well as the lack of requirement of further immune-modulator indicated that Dex modulated cell differentiation towards regulatory Tr1 phenotype. Interestingly, in a manner similar to CD3/CD55 differntiated cells, Dex treated CD3/CD55 primed cells did not completely respond to CD3/CD28 costimulation during secondary activation which further confirms their differentiated state. It could be stipulated from this observation that Dex modulated IL-10$^+$ Tr1 cells could confer immune-suppression to maintain tolerance in long term which is required to achieve clinically-quiescent state in auto-immune disease patients.

Primary Stimulation (Day 3)

Secondary Stimulation (Day 12)

CD3/CD55

1.40%   0.06%

98.31%   0.23%

CD3/CD55 + DEX

4.67%   0.8%

94.89%   0.36%

CD3/CD55

1.33%   0.3%

95.76%   2.76%

CD3/CD55

6.74%   1.36%

88.60%   3.30%

CD3/CD28

0.32%   0.12%

97.44%   2.12%

CD3/CD28

2.90%   0.74%

92.89%   3.47%

IL-10 APC

IFN-γ FITC

205
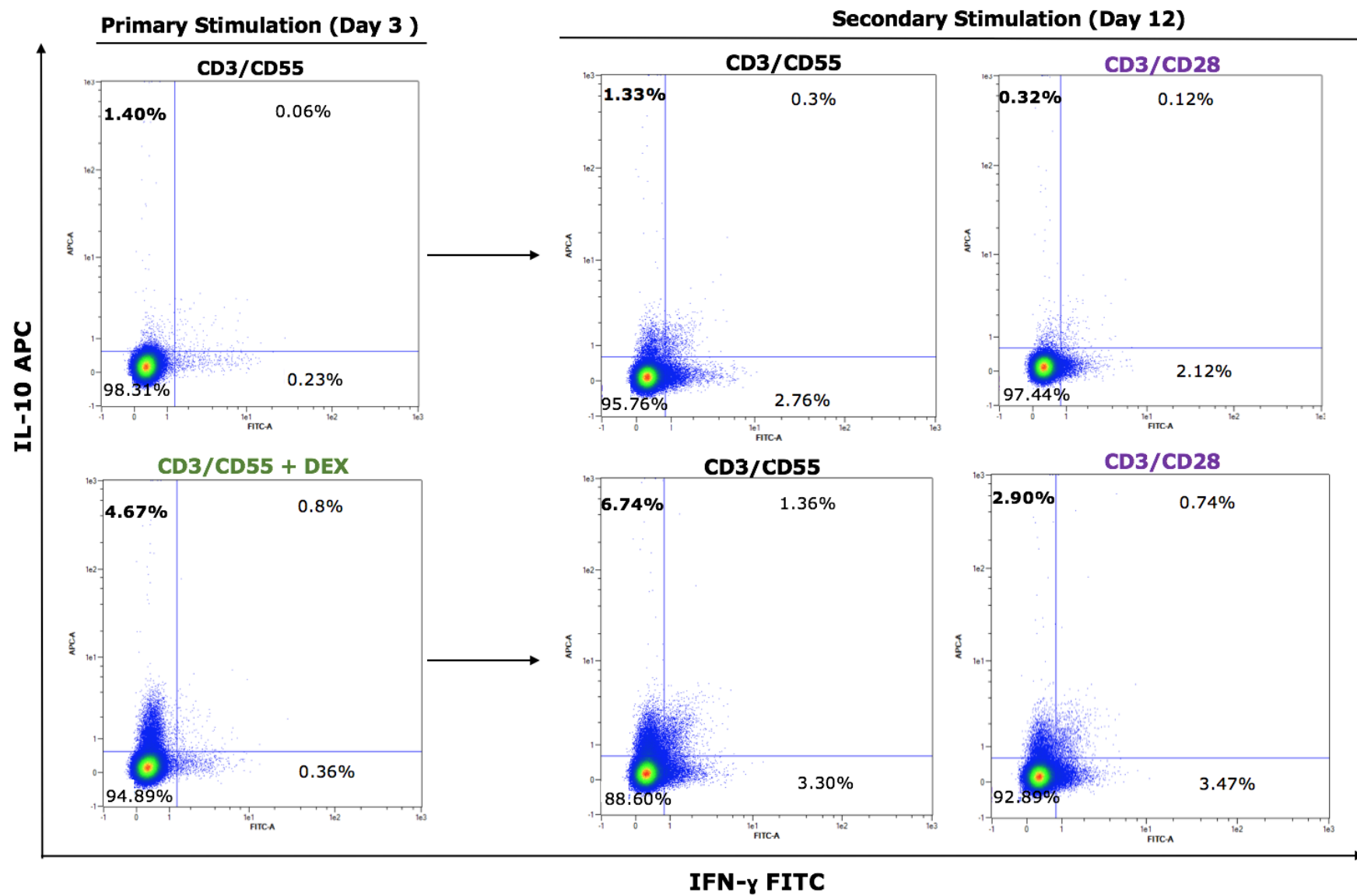
Figure 5.17: Evaluation of cell function of CD3/CD55 induced IL-10$^+$ Tr1 cells primed in presence of immune-modulators upon secondary restimulation. Naïve CD4 T-cells were stimulated with CD3/CD55 and CD3/CD55 +dexamethasone (5X10^{-8}M) for 72 hours and dual CSA for IL-10 as well as IFN-γ was performed to determine the Tr1 cells following primary stimulation. Then, cells were rested for 7 days. On day10, cells were restimulated with either CD3/CD55 or CD3/CD28 for 36 hours before dual CSA was performed to determine the induction of Tr1 cells upon restimulation. Data is representative of three independent experiments

5.3 Discussion:

Immune homeostasis requires an intricate balance between pro-inflammatory and anti-inflammatory response modulated by various immune cells. Although regulatory cells constitute a small fraction of the total cell population, they efficiently restrain proinflammatory Th1 cells to prevent immunopathology. By-stander suppression via IL-10 production is one of the significant mechanisms of action of regulatory T-cells [600-602]. In fact, enforced expression of IL-10 in CD4$^+$ T-cells confers regulatory function to them and they retain their function in vivo to provide protection against GvHD in xeno-graft transplanted mouse models [603]. Moreover, IL-10 plays a critical role in maintaining self-tolerance as lower level of IL-10 has been reported in several auto-immune diseases which might contribute to the severity of disease [348, 455, 604-608]. Various immuno-modulatory agents including IFN-β and Dimethyl fumarate seem to impart their effect by rescuing IL-10 production as augmentation of IL-10 was noted following treatment [348, 609, 610]. Interestingly, the level of IL-10 producing cells was deficient during relapse phase but increased during stable disease condition in clinically-quiescent MS patients to a comparable level to the healthy subjects [608]. These observations suggest that IL-10 production by regulatory T-cells might be defective during active disease phase in auto-immune disease patients. Therapeutic intervention could re-establish IL-10 production to reinstate the immunological balance and ameliorate pathological consequences of the disease.

Defects in costimulatory signals that govern the generation of Tr1

responses may also account for the pro-inflammatory bias observed in MS patients [508]. The CD3/CD55 costimulatory pathway regulates IL-10 production by Tr1 cells and in this study was defective in relapsing MS patients. While stimulation of naïve CD4^{+} T-cells resulted in induction of a small population of IL-10^{+} Tr1 cells, the cells from untreated MS patients failed to respond in a similar manner. The overall IL-10 production was also significantly lower in MS patient compared to age-and–gender matched heathy controls, confirming previous observations of lower levels of IL-10 in MS patients [508]. The treatment with various immune-modulators enhances the IL-10 production in a dose dependent manner in healthy subject and could potentially redress the deficiency of IL-10 production by Tr1 cells in MS patients. Also, at the optimal dose concentration, these immune-modulators do not affect the proliferation but only affect the conversion of cells to IL-10^{+} Tr1 cells while suppressing IFN-γ production. More importantly, these immune-modulators affect differentiation stage of Tr1 cells and these retain their 'altered/enhanced Tr1' function upon secondary restimulation. This occurs even in the absence of the drugs which indicates the stable modification of IL-10^{+} cells with the ability to provide long term tolerance. Both Dexamethasone and IFN-β are already used in the clinics to treat MS patients and have demonstrated the ability to alleviate the severity of the disease. This study has shown for the first time that these immune modulators not only diminish the Th1 response but importantly enhance the number and potency of the regulatory Tr1 response in conjunction with CD3/CD55 signaling. These observations would suggest that modulation of CD3/CD55 induced Tr1 is a potential mechanism of

action of these immune-modulators. Our identification of the defective

CD55 mediated induction of Tr1 cells MS patients as well as the immune-

suppression potential enhancing effect of immune-modulators on CD55

induced Tr1 cells provides us with an immuno-therapeutic intervention to

restore the immune-balance in MS patients by adoptive cell transfer

therapy.

6 Chapter 6: The effect of immune modulators on the phenotype of CD3/CD55 induced IL-10$^+$ Tr1 cells

6.1 Introduction:

Type 1 Regulatory T-cells (Tr1) were initially defined by their functional characteristics of imparting immune suppression in an IL-10 dependent manner [427]. In the recent years, many studies have focused on identifying a Tr1 phenotype on the basis of cell surface molecule expression in order to monitor the generation of cells in the peripheral immune system as well as to isolate them for clinical application. Gagliani et al. [431] reported the first Tr1-associated markers and demonstrated that Lymphocyte-activation protein 3 (LAG-3) and CD49b along with CD226 were expressed by IL-10$^+$ cells in both human and mice. Several other Inhibitory Receptors (IR) including ICOS, TIM-3, TIGIT and PD-1 [329, 611-613] as well as IL-10Rα [614] have been reported as makers of Tr1 cells. However, as with other leukocyte subsets, none of the reported markers are exclusively expressed by Tr1 cells. Discrepancies in the level of expression of these markers in different studies could be attributed to different methods of generating Tr1 cells which includes differentiating Tr1 cells from Naïve CD4 T-cells under the influence of co-stimulatory signal including CD46, CD55; or cytokines such as IL-27 and co-culture of CD4 T-cells with dendritic cells in the presence of IL-10. Similarly, differences in the time at which surface markers were analysed and also the kinetics of cytokine production may account for the differences reported in levels of markers and production of cytokines. Moreover, unlike nTreg cells, which are determined by expression of

transcription factor Foxp3 [615, 616], no transcription factor has been identified that acts as a master regulator for inducible regulatory T-cells.

As a result, multiple markers have been used to identify Treg populations. This can cause confusion and mislabelling of cells as some of these have been shown to be transiently expressed on other populations, upon activation [617-625]. We therefore investigated the expression of various surface markers and transcription factors on the IL-10 single positive and negative populations of CD3/CD55 induced Tr1 cells.

Considering our observations in Chapter 5 which demonstrated that various immune-modulators enhance the number of CD3/CD55 induced Tr1 cells, we hypothesized that the characteristics of CD3/CD55 induced Tr1 cells would have a similar phenotype both in presence or absence of immune-modulators.

6.2 Results:

6.2.1   Expression of Tr1-associated markers by CD3/CD55 induced IL-10$^+$cells

6.2.1.1   CD226

CD226 or DNAX accessory molecule-1 (DNAM-1) was identified as an accessory adhesion molecule required for the cytotoxic T lymphocyte (CTL)–mediated cytotoxicity [626]. It has been reported to be highly expressed by Tr1 cells [431, 612] and involved in Tr1 mediated killing of myeloid APCs [332].

CD226 is expressed by T-cells (both $\alpha\beta$ and $\gamma\delta$ T-cells), NK cell, subset of B-cells and monocytes [626]. It interacts with CD155 (Polio Virus Receptor, PVR) and CD112 (Nectin-2 δ/α) [627] which are expressed by dendritic cells [628]. It is known to compete with two co-inhibitory receptors, i) T-cell immunoglobulin and ITIM domain protein (TIGIT) for its ligand CD122, ii) CD96 for its ligand CD155 [629-631]. Interestingly, it has been reported that a nonsynonymous single nucleotide polymorphism (nsSNP) rs763361/Gly307Ser in *CD226* gene is associated with predisposition to several auto-immune diseases including Multiple Sclerosis (MS), Type 1 Diabetes (T1D) and Rheumatoid Arthritis [632-634]. Moreover, *CD226* gene may be regulated by Vitamin-D3 [635, 636] as Vitamin-D Receptor (VDR) binding sites are present in its loci [637]. We therefore investigated the expression of CD226 in the CD3/CD55 induced Tr1 cells in presence or absence of Vitamin-D3.

Naive CD-4$^+$ T-cells were stimulated with CD3/CD55 in presence or absence of Vitamin-D3 (10^{-7}M) for 72 hours and the expression of CD226 was determined by extracellular staining whereas IL-10 was determined by cytokine secretion assay (CSA). Following CD3/CD55 costimulation CD226 was seen to be present in both the IL-10 positive Tr1 population and the IL-10 negative cells, with 70% (on average, n=4) of the IL-10 positive cells also co-expressed CD226 (Figure 6.1A). Vitamin-D3 significantly increased the expression of CD226 on both IL-10$^+$ Tr1 and IL-10$^-$ cells compared to cells which were stimulated with only CD3/CD55 (Figure 6.1A and Figure 6.2). However, no difference was observed on the level of CD226 expression on IL-10$^+$ Tr1 and IL-10$^-$ stimulated with only CD3/CD55 (Figure-6.3A). In a similar way, even though Vitamin-D3 enhanced the overall expression of CD226 following CD3/CD55 stimulation, there was not any significant difference in CD226 expression between IL-10$^+$ Tr1 and IL-10$^-$ cells (Figure- 6.3 B). This supports the role of Vit-D3 in upregulating CD226 and shows that CD3/CD55 *per-se* has little effect on its expression

A)
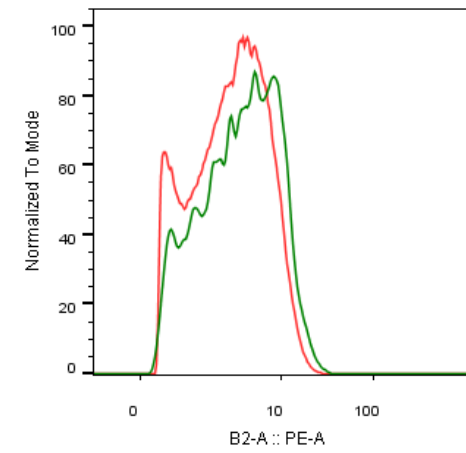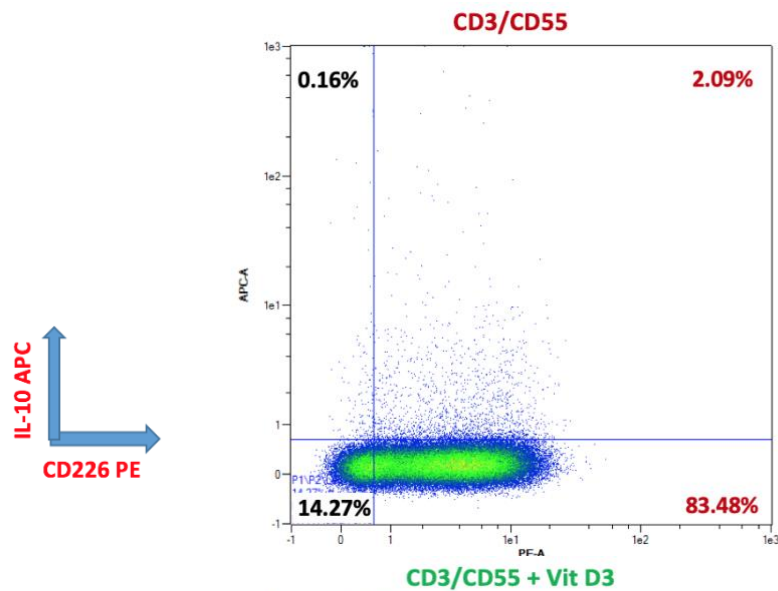
B)
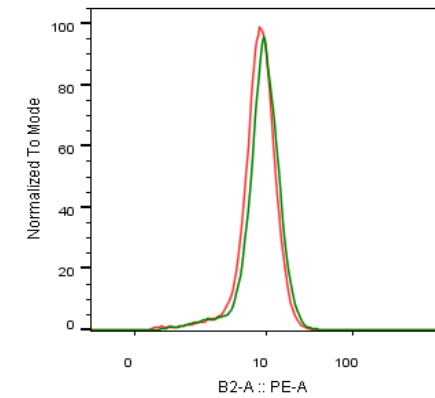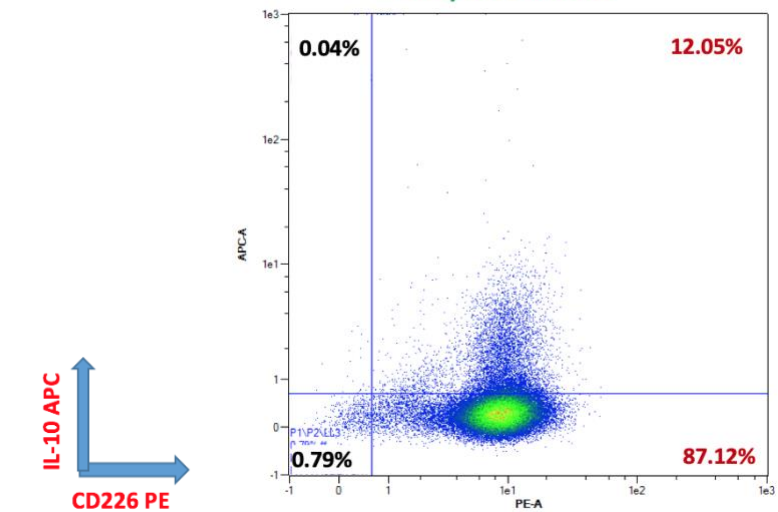
Figure 6.1: Evaluation of CD226 expression by CD3/CD55 induced IL-10$^+$ Tr1 cells and IL-10$^-$ cells in presence of Vitamin-D3. Naïve CD4$^+$ T-cells were stimulated with CD3/CD55 in absence (A) and presence of Vit-D3 (B) for 72 hours and IL-10 CSA was performed to determine the Tr1 cells while extracellular staining was performed to determine the expression of CD226. The histograms represent the expression CD226 by IL-10$^+$ (green) and IL-10$^-$ (red) in CD3/CD55 (A) and CD3/CD55+Vitamin-D3 stimulated cells. The data demonstrated here are representative of four individual experiments conducted with samples collected from healthy donors.

A)

Comparison of CD226 expression by IL-10$^+$ cell

B)

Comparison of CD226 expression by IL-10$^-$ cell
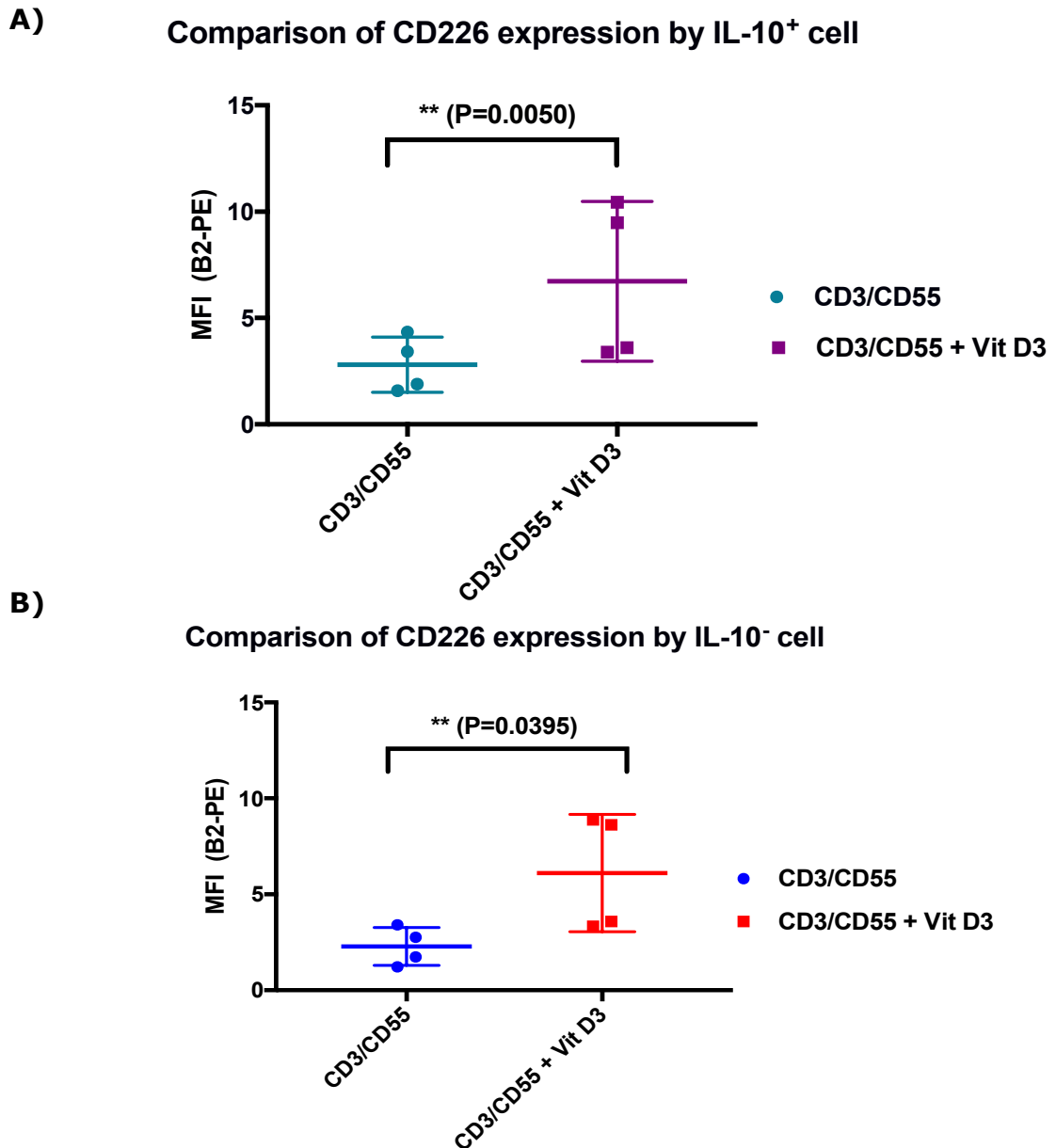
Figure 6.2: Comparison of the level of CD226 expression between IL-10$^+$ and IL-10$^-$cells induced by CD3/CD55 costimulation in presence or absence of vitamin-D3. Naïve CD4$^+$ T-cells were stimulated with CD3/CD55 in presence or absence of Vit-D3 for 72 hours and IL-10 Cytokine Secretion Assay along with extracellular staining for CD226 was performed to determine the expression of the Tr1-associated marker (n=4). The Mean Fluorescence Intensity (MFI) of CD226 expression was determined and compared between (A) IL-10$^+$ Tr1 cell and (B) IL-10$^-$ cell population in CD3/CD55 and CD3/CD55+Vit-D3 stimulated cell. For both (A) and (B), statistical significance was determined by paired t-test and n=4.

A)

MFI of CD226 Expression by cells stimulated with CD3/CD55

B) **MFI of CD226 Expression by cells stimulated with CD3/CD55 + Vit-D3**

Figure 6.3: Comparison of the level of CD226 expression between IL-10$^+$ and IL-10$^-$cells in CD3/CD55 and CD3/CD55+vitamin-D3 stimulated cells. The expression of CD226 by IL-10$^+$ Tr1 cells and IL-10$^-$ cells were determined in CD3/CD55 (A) and CD3/CD55+Vitamin D3 (B) stimulated cells. The difference in the CD226 expression between IL-10$^+$ Tr1 cells and IL-10$^-$ cells in either CD3/CD55 or CD3/CD55+Vitamin D3 stimulated cells was evaluated. Paired t-test was performed to determine the statistical significance. Data shown here is cumulative results obtained from four independent experiments (n=4).

6.2.1.2 LAG-3

Lymphocyte activation gene (LAG)-3, also known as CD223, was identified as homolog of CD4 [638]. LAG-3 is expressed by NK cells as well as T-cells and binds to MHC Class II molecules [639-641]. Recently LAG-3 has emerged as a co-inhibitory checkpoint molecule that suppresses $CD4^+$ T-cells [370, 642-644] but is required for survival of activated T-cells following antigenic stimulation [645]. It has been reported that LAG-3 expression is higher in nTreg compared to effector cells where LAG-3 is ectopically upregulated following activation [646]. It was also demonstrated that LAG-3 is needed for the suppressive function of $CD4^+CD25^+$ nTreg [646] and has been noted as a marker of immunosuppressive $IL-10^+$ Tr1 cells [431, 612, 647].

The expression of LAG-3 was assessed in Tr1 cells induced from purified naïve cells with CD3/CD55 costimulation, in presence or absence of Vitamin-D3. IL-10 Cytokine secretion assay was performed along with extra-cellular staining for LAG-3 in order to evaluate the expression of this Tr1-associated marker. LAG-3 was expressed by 90- 95% (in all donors) of the $IL-10^+$ cells after 72 hours of stimulation with CD3/CD55 (n=4, representative data shown in Figure 6.4B). The presence of immune-modulators did not alter the level of expression of LAG-3 even though the number of $IL-10^+$ Tr1 cells were elevated (Figure 6.4C). Interestingly, the CD3/CD55 induced $IL-10^+$ cells had significantly higher expression of LAG-3 compared to $IL-10^-$ cells both absence (P=0.0097, n=4) and presence (P=0.0401, n=4) of Vitamin-D3. However, Vit-D3 did not have a significant effect on the expression of LAG3 (Figure 6.5).

A)

B)

C)

Figure 6.4: Evaluation of LAG-3 expression by CD3/CD55 induced IL-10$^+$ Tr1 cells and IL-10$^-$ cells in presence of Vitamin-D3. Naïve CD4$^+$ T-cells were stimulated with CD3/CD55 in presence or absence of Vitamin-D3 (10^{-7}M) and after 72 Hours of cell activation, IL-10 CSA was preformed to detect the Tr1 cells along with extracellular staining for LAG-3. Sample stained with only detection antibody and isotype control was used as a negative control to determine the expression of IL-10 and LAG-3. Data shown here are representative of four independent experiments (n=4).

A) LAG-3 Expression by cells following CD3/CD55 stimulation

B) LAG-3 Expression by cells following CD3/CD55 + V it-D3 stimulation
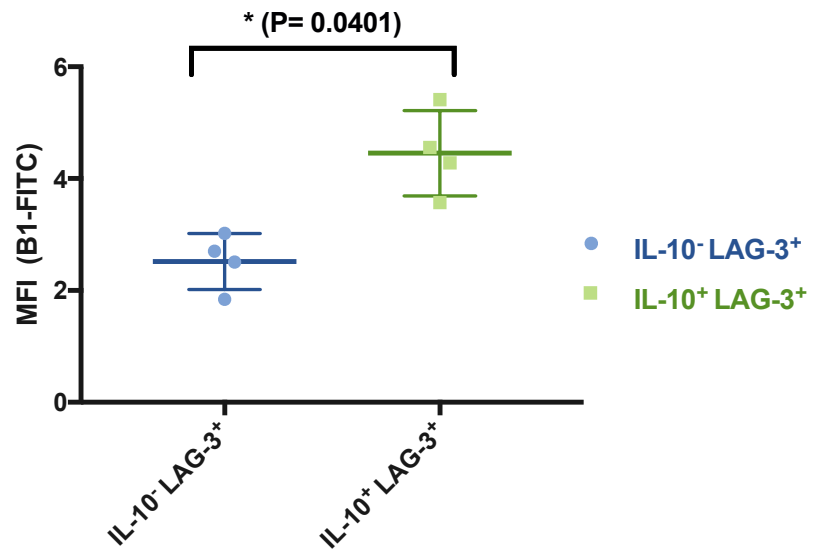
C) Comparison of LAG-3 expression by IL-10$^+$ cell
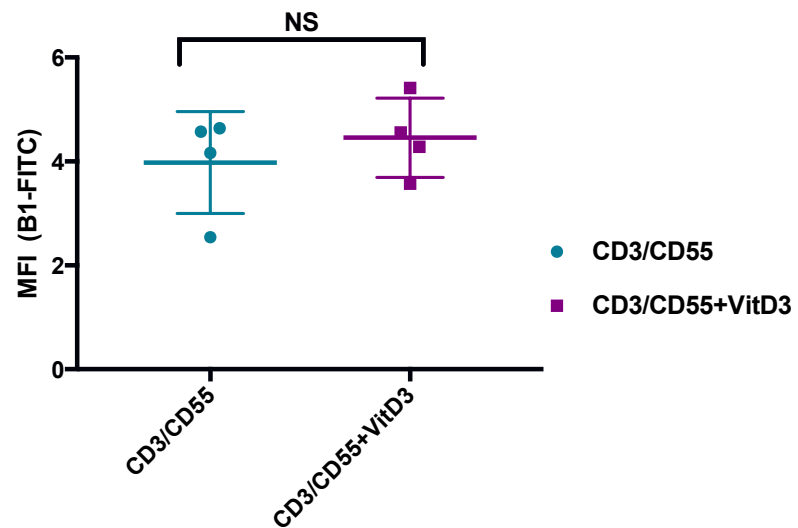
Figure 6.5: Comparison of the level of LAG-3 expression between IL-10$^+$ and IL-10$^-$cells in CD3/CD55 and CD3/CD55+vitamin-D3 stimulated cells. Naïve CD4 T-cells were stimulated with CD3/CD55 in presence or absence of Vit-D3 for 72 hours and IL-10 Cytokine Secretion Assay along with extracellular staining for LAG-3 was performed to determine the expression of the Tr1-associated marker. The Mean Fluorescence Intensity (MFI) of LAG-3 was determined for IL-10$^+$ and IL-10$^-$ populations in (A) CD3/CD55 and (B) CD3/CD55+Vit-D3 stimulated cell. The MFI of LAG-3 expression by CD3/CD55 induced IL-10$^+$ Tr1 cells in presence or absence of Vitamin-D3 was compared in (C). Statistical significance was determined by paired t-test and data shown here is cumulative results obtained from four independent experiments.

6.2.1.3 CD49b

α2β1 integrin or CD49b is one of the Tr1-associated markers which serves as a receptor for many matrix and non-matrix molecules [648]. It was reported that co-expression of CD49b and LAG-3 could be used to identify and isolate Tr1 cells in both human and mice [312]. However, similar to other cells surface molecules, the expression of CD49b is not restricted to only Tr1 cells. It has also been reported that co-engagement of IL-17 receptor and CD49b promotes IL-17 production by Th17 cells and enhance their osteoclastogenic function [649]. In another study, it was reported that there was no significant difference in the expression of CD49b by IL-10$^+$ and IL-10$^-$ cells even though CD49b expression was slightly higher in IL-10$^+$ cells on Day 3 following IL-27 mediated differentiation of Tr1 cells [613]. Our previous studies demonstrated that CD3/CD55 induced Tr1 cells also express CD49b. However, the expression of CD49b by IL-10$^+$ Tr1 cells induced by stimulation with CD3/CD55 in the presence of Vitamin D3 has not been determined yet. So, in this study, we evaluated the expression of CD49b expression by Tr1 cells following CD3/CD55 and CD3/CD55+VitaminD3 stimulation. The expression of CD49b was determined after 72 hours of cell activation. It was determined that more than 70% IL-10$^+$ cells expressed CD49b whereas 20-40% IL-10$^-$ cells expressed CD49b in both CD3/CD55 and CD3/CD55+VitaminD3 stimulated cells (Figure-6.6). Although the presence of vitamin D3 enhanced the proportion of IL-10$^+$Tr1 cells following CD3/CD55 stimulation, it did not alter the expression of CD49b. Importantly, when the level of CD49b expression was compared between IL-10$^+$ and IL-10$^-$ cells resulting from CD3/CD55 stimulation, CD49b

expression was significantly higher on the IL-10$^+$ cells in comparison to IL-10$^-$ cells (Figure-6.7A). Similarly, CD49b expression was higher in IL-10$^+$ cells than IL-10$^-$ cells upon stimulation with CD3/CD55+VitaminD3 (Figure-6.7B). There was no significant difference between the level of CD49b expression of IL-10$^+$ cells derived from CD3/CD55 and CD3/CD55+VitaminD3 stimulated cells (Figure-6.7C).

Figure 6.6: Evaluation of CD49b expression by CD3/CD55 induced IL-10⁺ Tr1 cells and IL-10⁻ cells in presence of Vitamin-D3. Naïve CD4⁺ T-cells were stimulated with CD3/CD55 in presence or absence of Vitamin-D3 (10^{-7}M) and after 72 Hours of cell activation, IL-10 CSA was preformed to detect the IL-10⁺ Tr1 cell along with extracellular staining for CD49b. The data is representative of five individual experiments.

A)

Expression of CD49b by cells stimulated with CD3/CD55

** (P=0.0147)

MFI (B1-FITC)

● IL-10⁺
■ IL-10⁻

B)

Expression of CD49b by cells stimulated with CD3/CD55+VitD3

** (P=0.0131)

MFI (B1-FITC)
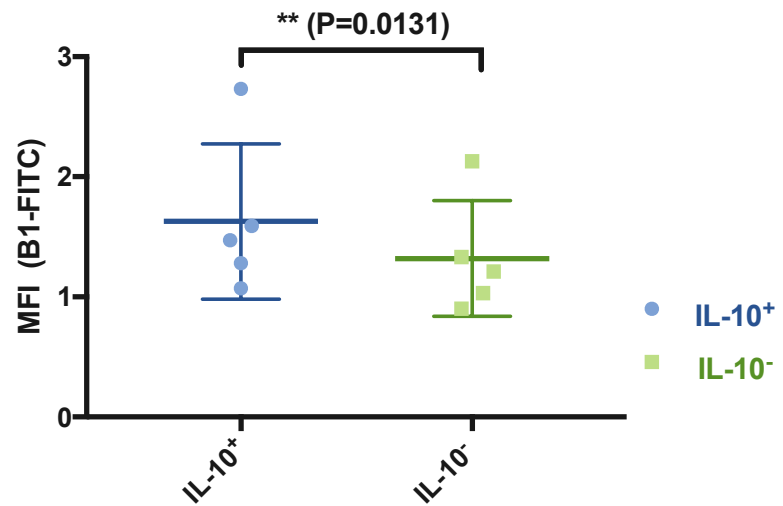
● IL-10⁺
■ IL-10⁻

C)

Expression of CD49b by IL-10⁺ cells
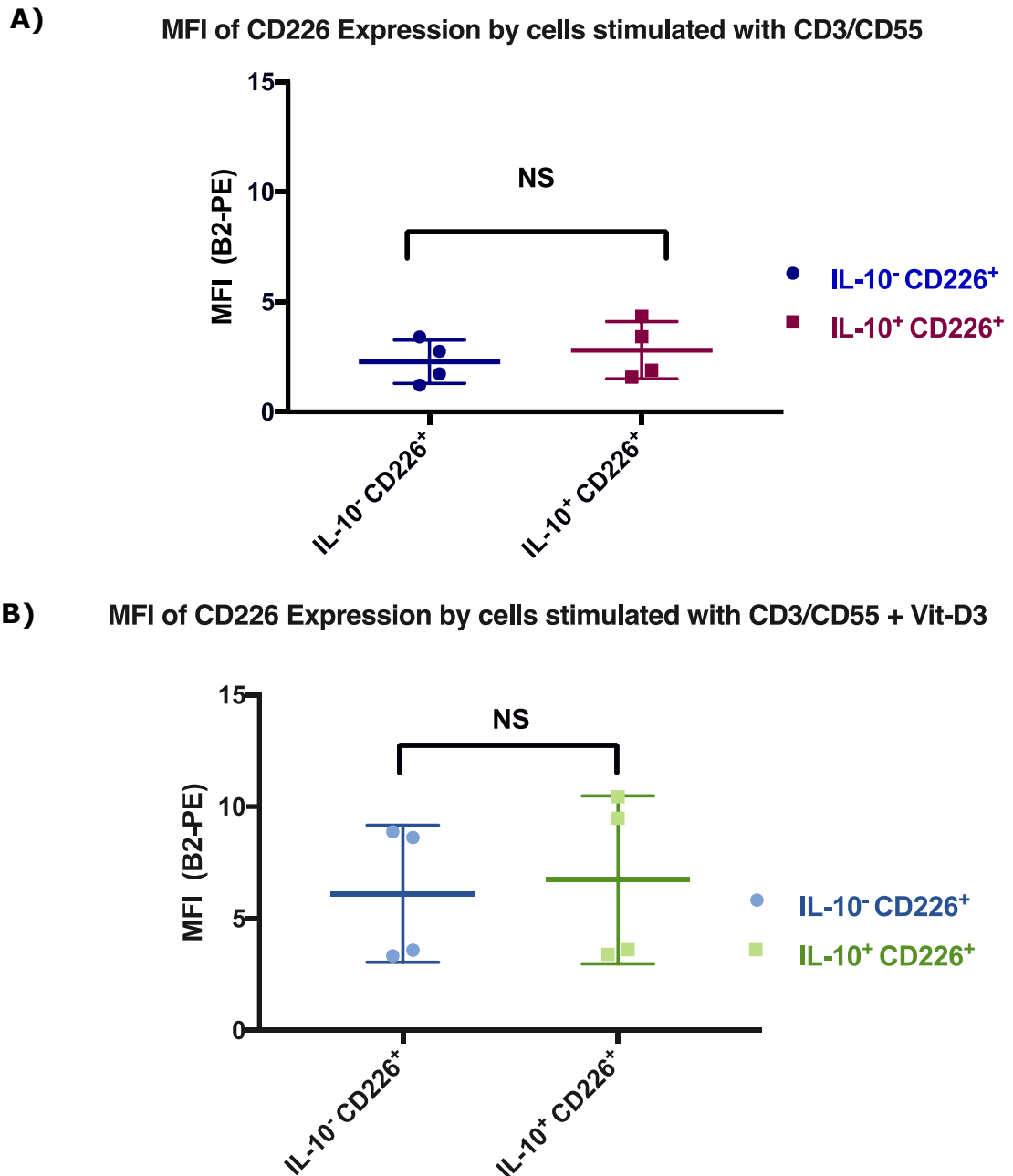

NS

MFI (B1-FITC)

● CD3/CD55
■ CD3/CD55+VitD3
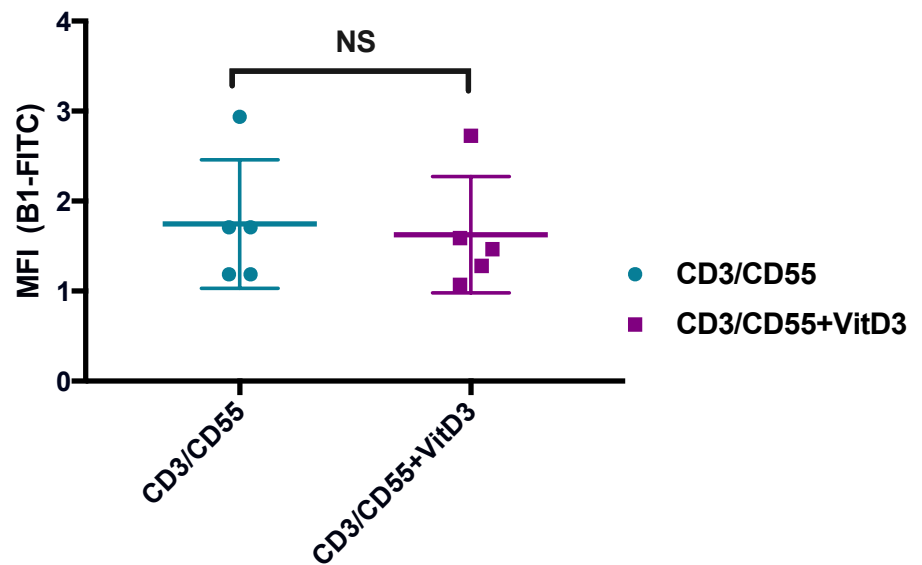
Figure 6.7: Comparison of the level of CD49b expression between IL-10$^+$ and IL-10$^-$cells in CD3/CD55 and CD3/CD55+vitamin-D3 stimulated cells. Naïve CD4$^+$ T-cells were stimulated with CD3/CD55 in presence or absence of Vit-D3 for 72 hours and IL-10 Cytokine Secretion Assay along with extracellular staining for CD49b was performed to determine the expression of the Tr1-associated marker. The Mean Fluorescence Intensity (MFI) of CD49b (B1-FITC) expression was determined for IL-10$^+$ and IL-10$^-$ population and compared in (A) CD3/CD55 and (B) CD3/CD55+Vit-D3 stimulated cells. The IL-10$^+$cells expressed significantly higher CD49b compared to IL-10$^-$ regardless of the absence or presence of Vitamin-D3 upon CD3/CD55 stimulation (**P=0.0147 and ** P=0.0131 for A and B respectively). The expression of CD49b by IL-10$^+$ Tr1 cells induced following CD3/CD55 and CD3/CD55+VitD3 was also compared (C). Statistical significance was determined by paired t-test. Data shown here is cumulative results obtained from five independent experiments (n=5).

6.2.1.4 LAP

Latency Associated Peptide (LAP) is the N-terminal pro-peptide precursor of TGF-β which non-covalently binds to TGF-β, forming a latent TGF-β complex and facilitates release of TGF-β1 into the extracellular matrix. It has been reported that LAP is expressed by both FoxP3^{+} nTreg and IL-10^{+} inducible regulatory T-cells [650-654]. It was also reported that tumour infiltrating CD4^{+}LAP^{+} cells were ~50% more potent than nTreg to confer immune-suppression [7]. Considering the important role of LAP in immune regulation, we studied the expression of LAP in CD3/CD55 induced IL-10^{+} Tr1 cells in presence and absence of VitaminD3. Naïve CD4 T-cell were stimulated with CD3/CD55 and CD3/CD55+VitaminD3 for 72 hours before IL-10 secretion assay was performed along with extracellular staining for LAP. It was determined that ~60% IL-10^{+} cells did not express LAP while more than 80% IL-10^{+} did not express LAP following stimulation with CD3/CD55 and CD3/CD55+vitaminD3 respectively (Figure 6.8, n=4). The was no significant difference in the level of LAP expression between IL-10^{+} and IL-10^{-} cells in either CD3/CD55 or CD3/CD55+vitaminD3 stimulated cells (data not shown).
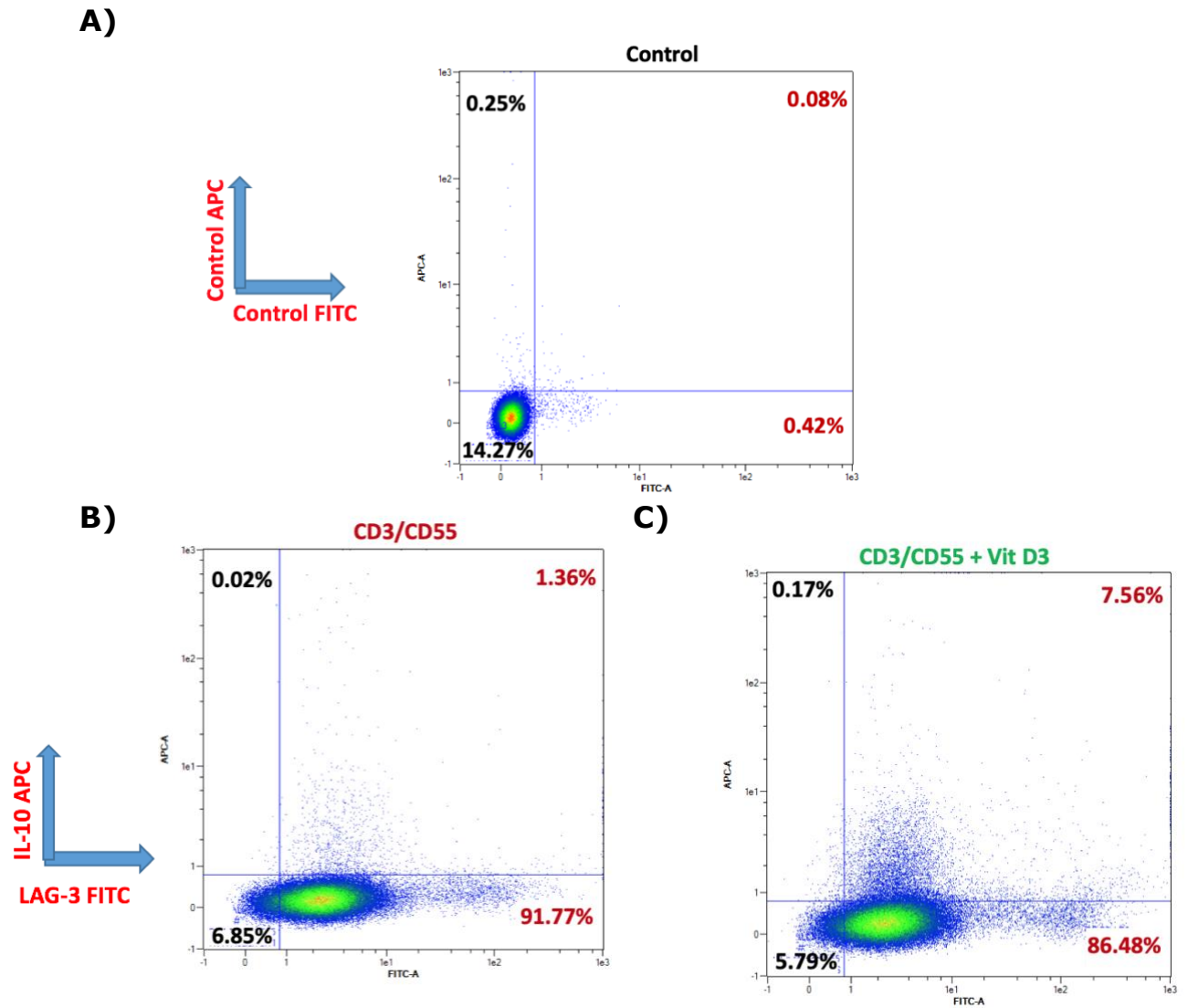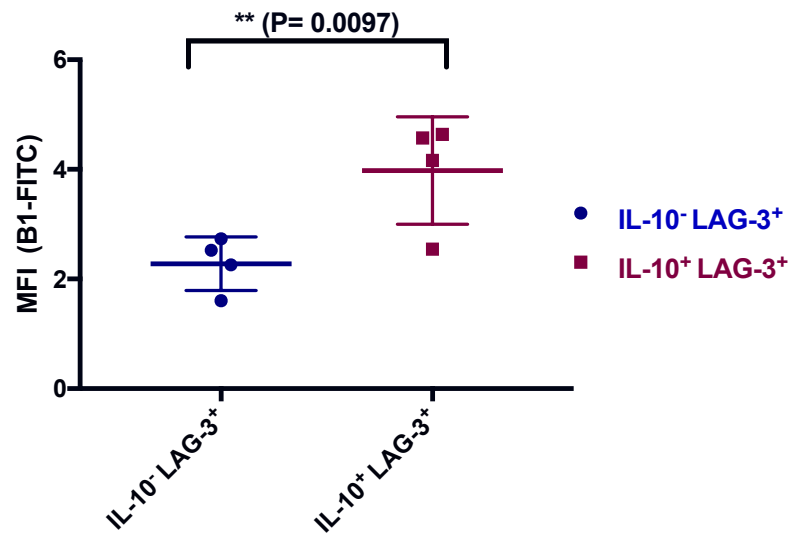
Figure 6.8: Evaluation of LAP expression by CD3/CD55 induced IL-10⁺ Tr1 cells and IL-10⁻ cells in presence of Vitamin-D3. Naïve CD4⁺ T-cells were stimulated with CD3/CD55 in presence or absence of Vitamin-D3 (10^{-7}M). The expression of LAP by IL-10⁺Tr1 cells was determined by performing IL-10 CSA along with extracellular staining for LAP after 72 Hours of cell activation The data is representative of four independent experiments.

6.2.1.5   TIM-3

T cell immunoglobulin and mucin-domain containing-3 (Tim-3) is a type I trans-membrane protein which was initially identified as a marker expressed IFN-γ^+ Th1 cells [655] and led to the discovery of the gene family of T-cell immunoglobulin mucin (TIM) proteins [656, 657]. TIM-3 is also expressed by CD8+ T cytotoxic 1 (Tc1), nTreg [658-660] **,** IL-10$^+$ Tr1 cells [613], Dendritic cells [661, 662] and NK cells .

TIM-3 has been described as a co-inhibitory receptor as blocking TIM-3 can enhance IFN-γ production and restored Th1 CD4 and Cytotoxic CD8 T-cell function [663, 664]. It has been also demonstrated that TIM-3 is upregulated by tumour infiltrating cells (TILs) in Colorectal cancer [665] as well as Head and Neck cancer [666]. TIM-3 has also been associated with exhausted and dysfunctional phenotype of CD4 T-cells in chronic infection such as HIV [667, 668], Hepatitis-C [669]. Interestingly, it has been reported that TIM-3 is a key inhibitory molecule expressed by IL-27 mediated IL-10 producing cells. IL-27 induces nuclear factor NFIL3 which in turn enhances TIM-3 expression by chromatin remodelling and promotes IL-10 production, leading to T-cell dysfunction [670]. However, the expression of TIM-3 upon CD3/CD55 co-stimulation has not been previously studied and we determined TIM-3 expression in CD3/CD55 induced Tr1 cells in presence or absence of Vitamin-D3.

Naïve CD4 T-cells were stimulated with CD3/CD55 in presence or absence of Vitamin-D3 for 72 hours before IL-10 CSA along with extracellular staining TIM-3 was performed to determine the expression

of this co-inhibitory receptor by Tr1 cells. 50%-60% IL-10$^+$ cells expressed TIM-3 whereas only 30% IL-10$^-$ cells expressed TIM-3 when cells were stimulated with only CD3/CD55 (n=4, representative data in Figure 6.9A). In CD3/CD55+ Vitamin-D3 stimulated cells, TIM-3 expression was downregulated in both IL-10$^+$ and IL-10$^-$ Cells (Figure 6.9B). Moreover, IL-10$^+$ Tr1 cells expressed significantly higher (P=0.0206) amount of TIM-3 compared to IL-10$^-$ cells (Figure 6.10A) in CD3/CD55 stimulated cells. Although the overall expression of TIM-3 was reduced following CD3/CD55 stimulation in presence of Vitamin-D3, the mean fluorescence intensity for TIM-3 expression was still significantly higher (P=0.0367) in IL-10$^+$ Tr1 cells compared to IL-10$^-$ cells (Figure 6.10B). The expression of Tim-3 was significantly reduced in both IL-10$^+$ Tr1 cells (Figure 6.11A) and IL-10$^-$ cells (Figure 6.11B) in the Vitamin-D3 treated CD3/CD55 stimulated cells in comparison only CD3/CD55 stimulated cells.
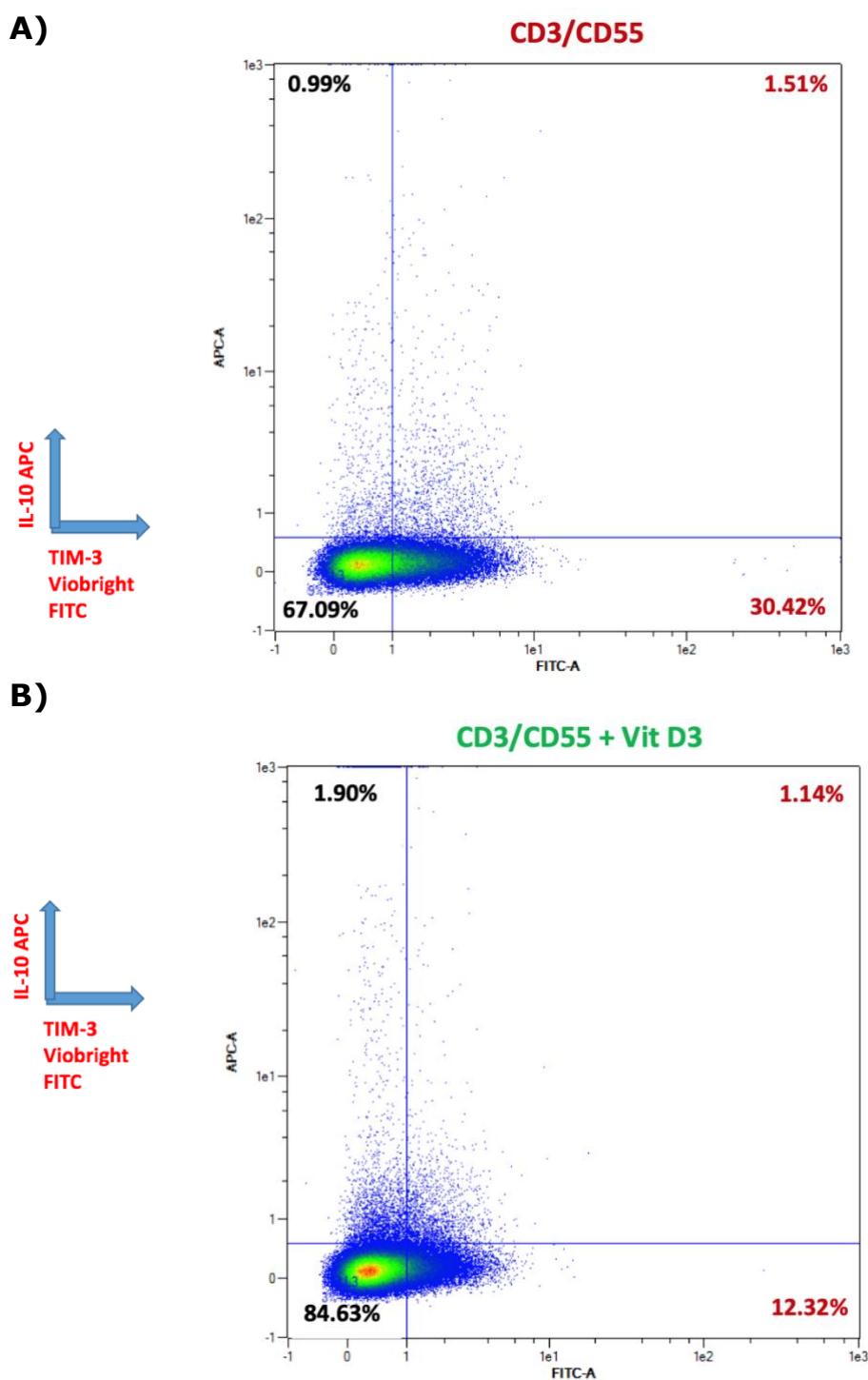
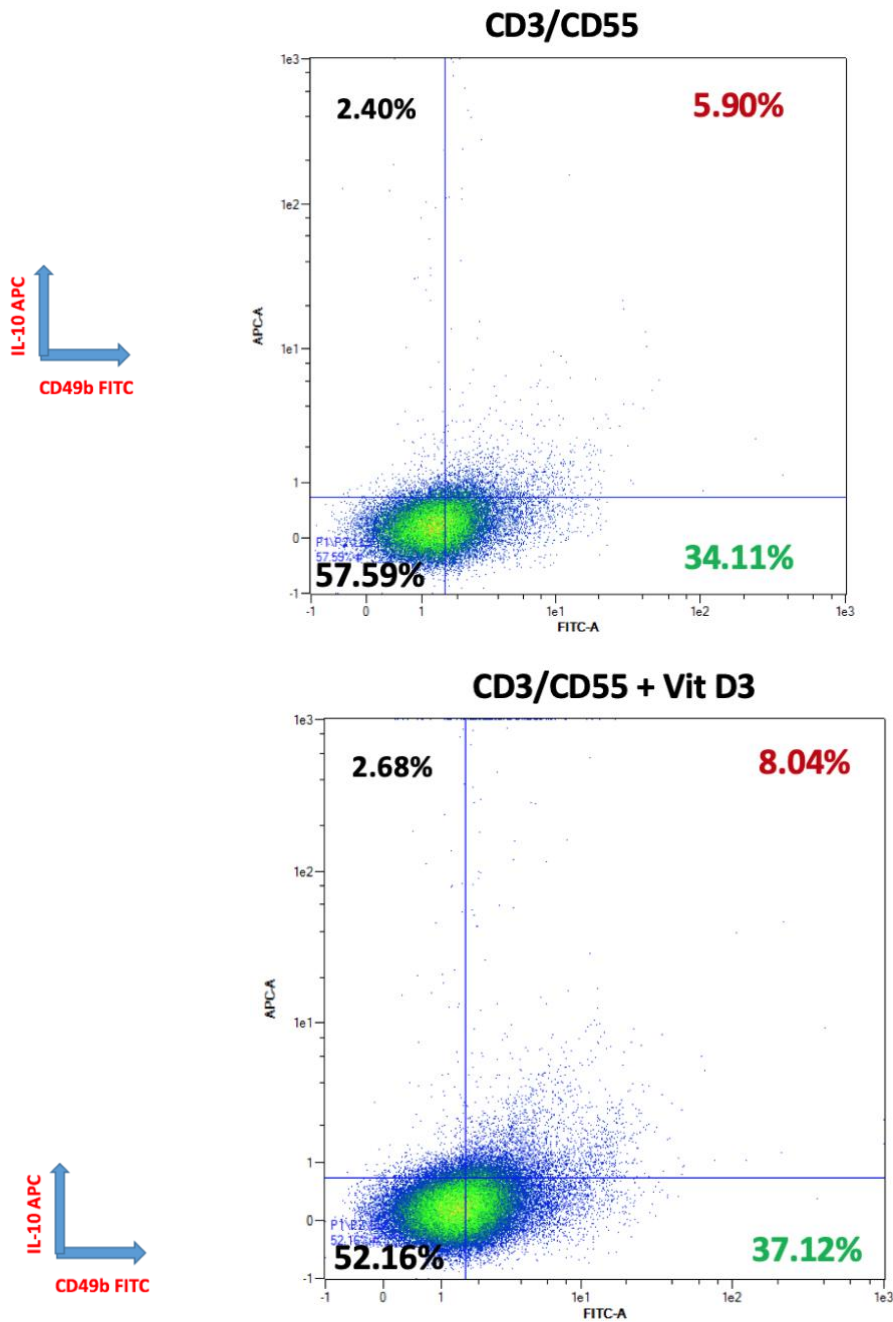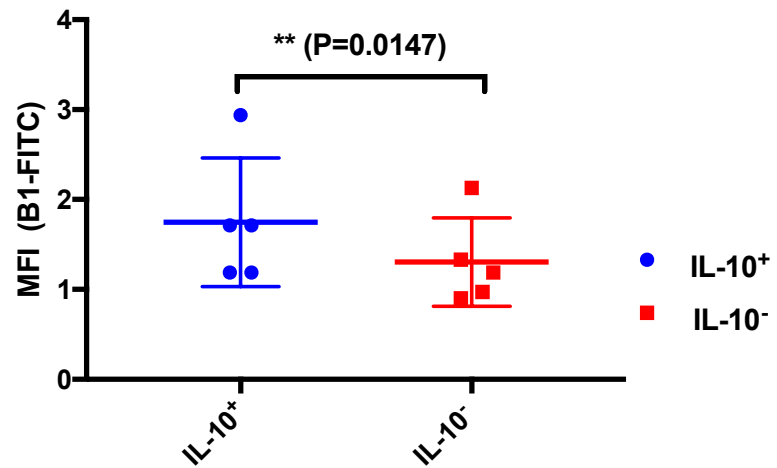**Figure 6.9: Evaluation of TIM-3 expression by CD3/CD55 induced IL-10⁺ Tr1 cells and IL-10⁻ cells in presence of Vitamin-D3.** Naïve CD4⁺ T-cells were stimulated with CD3/CD55 in presence or absence of Vitamin-D3 (10^{-7}M) and after 72 Hours of cell activation, IL-10 CSA was preformed to detect the Tr1 cell along with extracellular staining for TIM-3. The data is representative of four individual experiments.

A)

Expression of TIM-3 by cells stimulated with CD3/CD55
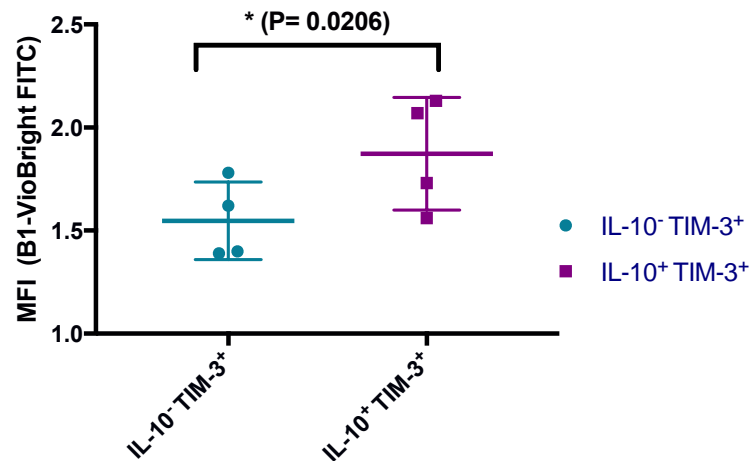
B)

Expression of TIM-3 by cells stimulated with CD3/CD55+Vit D3
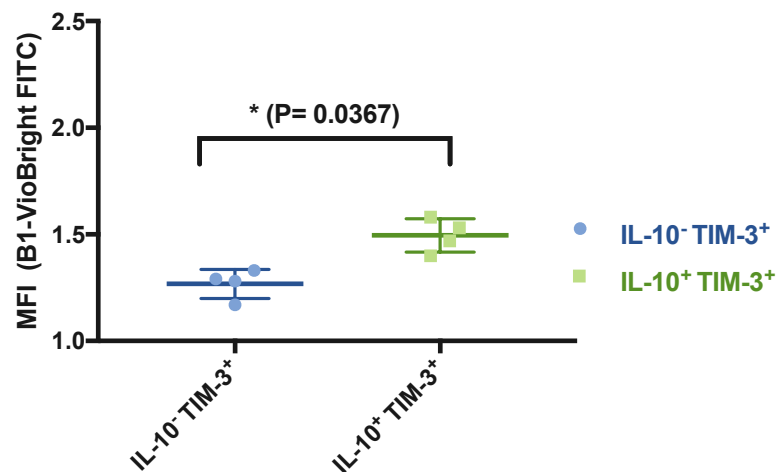
Figure 6.10: Comparison of the level of TIM-3 expression between IL-10$^+$ and IL-10$^-$cells in CD3/CD55 and CD3/CD55+vitamin-D3 stimulated cells. Naïve CD4$^+$ T-cells were stimulated with CD3/CD55 in presence or absence of Vit-D3 for 72 hours and IL-10 Cytokine Secretion Assay along with extracellular staining for TIM-3 was performed to determine the expression of the Tr1-associated marker. The Mean Fluorescence Intensity (MFI) of TIM-3 (B1-FITC) expression was determined for IL-10$^+$ and IL-10$^-$ population in (A) CD3/CD55 and (B) CD3/CD55+Vit-D3 stimulated cell. Statistical significance was determined by paired t-test (P= 0.0206 and P=0.0367 for A and B respectively; n=4).

A) Comparison of TIM-3 expression by IL-10$^+$ cell
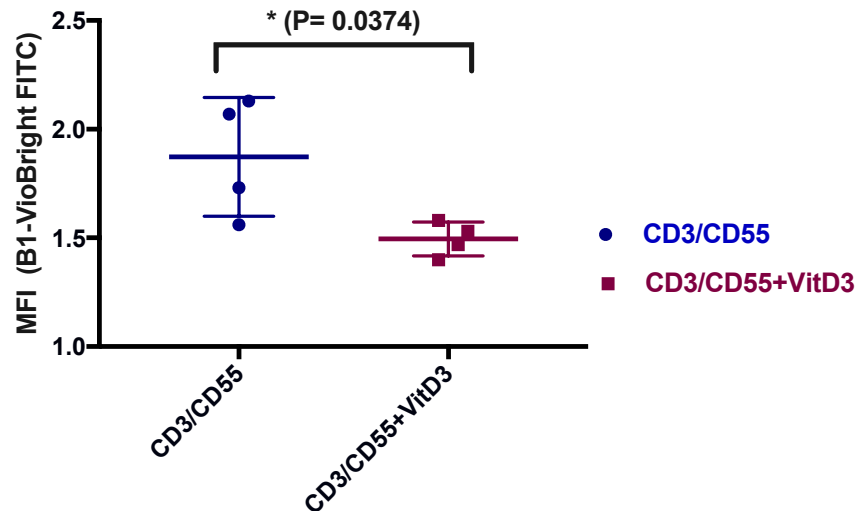
B) Comparison of TIM-3 expression by IL-10$^-$ cell
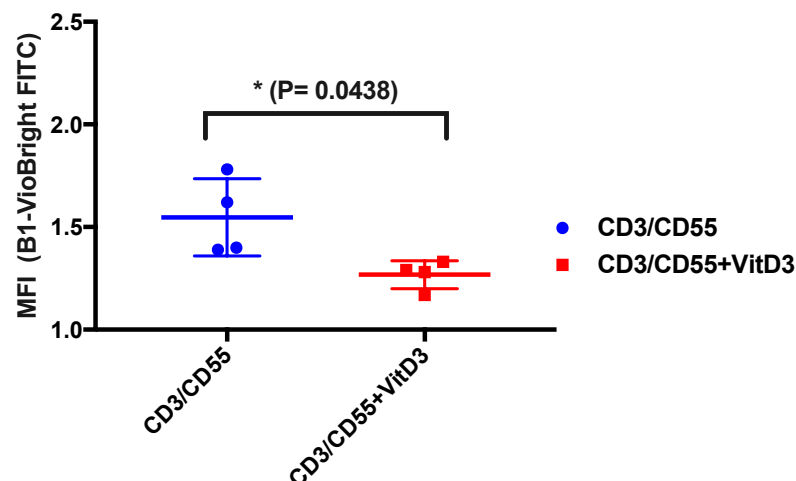
Figure 6.11: Comparison of the level of TIM-3 expression between IL-10$^+$ and IL-10$^-$cells induced by CD3/CD55 costimulation in presence or absence of vitamin-D3. Naïve CD4$^+$ T-cells were stimulated with CD3/CD55 in presence or absence of Vit-D3 for 72 hours and IL-10 Cytokine Secretion Assay along with extracellular staining for TIM-3 was performed to determine the expression of the Tr1-associated marker. The Mean Fluorescence Intensity (MFI) of TIM-3 expression was determined for (A) IL-10$^+$ and (B) IL-10$^-$ population in CD3/CD55 and CD3/CD55+Vit-D3 stimulated cell. Data shown here are cumulative summary from four independent experiment and statistical significance was determined by paired t test.

6.2.1.6 CTLA-4

Cytotoxic T lymphocyte antigen-4 (CTLA-4), a CD28 homologue, is widely recognized coinhibitory check point molecule which negatively regulates the function of conventional effector T-cells [671-673]. However, the ability of CTLA-4 to impart its immunosuppressive effect has been partially attributed to its function via regulatory T-cells. It has been demonstrated that CD4$^+$CD25$^+$ regulatory T-cells constitutively express CTLA-4 [673-675]. nTreg specific deficiency of CTLA-4 results in spontaneous development of systemic lymphoproliferation, fatal T cell–mediated autoimmune disease, and hyperproduction of immunoglobulin E in mice while simultaneously promoting T-cell immunity [676]. Moreover, it was also demonstrated that CTLA-4 expression by nTreg controls the inappropriate activation and expansion of naïve T-cells in order to maintain tolerance [677]. The regulatory role of CTLA-4 is not only restricted to nTreg and it has been reported that CTLA-4 is also expressed by Type 1 regulatory T-cells [678, 679]. It was demonstrated that blocking CTLA-4 reduces the suppressive ability of Tr1 cells [330]. It was also demonstrated that immature dendritic cell driven conversion of Tr1 cells from antigen specific anergic T-cells were dependent on CTLA-4 [679]. Considering the important role of CTLA-4 in immune tolerance, we studied the expression of CTLA-4 in CD3/CD55 induced Tr1 cells. We also evaluated the expression of CTLA-4 in the Tr1 cells resulting from the activation of naïve CD4 T-cells with CD3/CD55 in the presence of Vitamin D3.

CTLA-4 expression was determined after 72 hours of naïve cell stimulation with CD3/CD55 in presence or absence of vitamin-D3 and IL-10 CSA was performed in order to evaluate CTLA-4 expression by Tr1 cells (n=5). Both CD3/CD55 and CD3/CD55+Vitamin-D3 stimulation resulted in IL-10$^+$ Tr1 cells and ~60-75% IL-10$^+$ cells expressed CTLA-4 under both experimental conditions (Figure- 6.12). Interestingly, CTLA-4 was also expressed by 30-50% of IL-10$^-$ cells. The level of CTLA-4 expression by IL-10$^+$ and IL-10$^-$ cells was determined by evaluating mean fluorescence intensity (MFI). The level of CTLA-4 expression was significantly higher (*P=0.0127 and *P=0.186 for CD3/CD55 and CD3/CD55+Vitamin-D3 stimulated cells respectively) in IL-10$^+$ Tr1 cells compared to IL-10$^-$ cells (Figure 6.13 and 6.14 A, B). However, no significant difference was noted between the level of expression of CTLA-4 by IL-10$^+$ Tr1 resulting from CD3/CD55 and CD3/CD55+Vitamin-D3 stimulation (Figure-6.14 C).
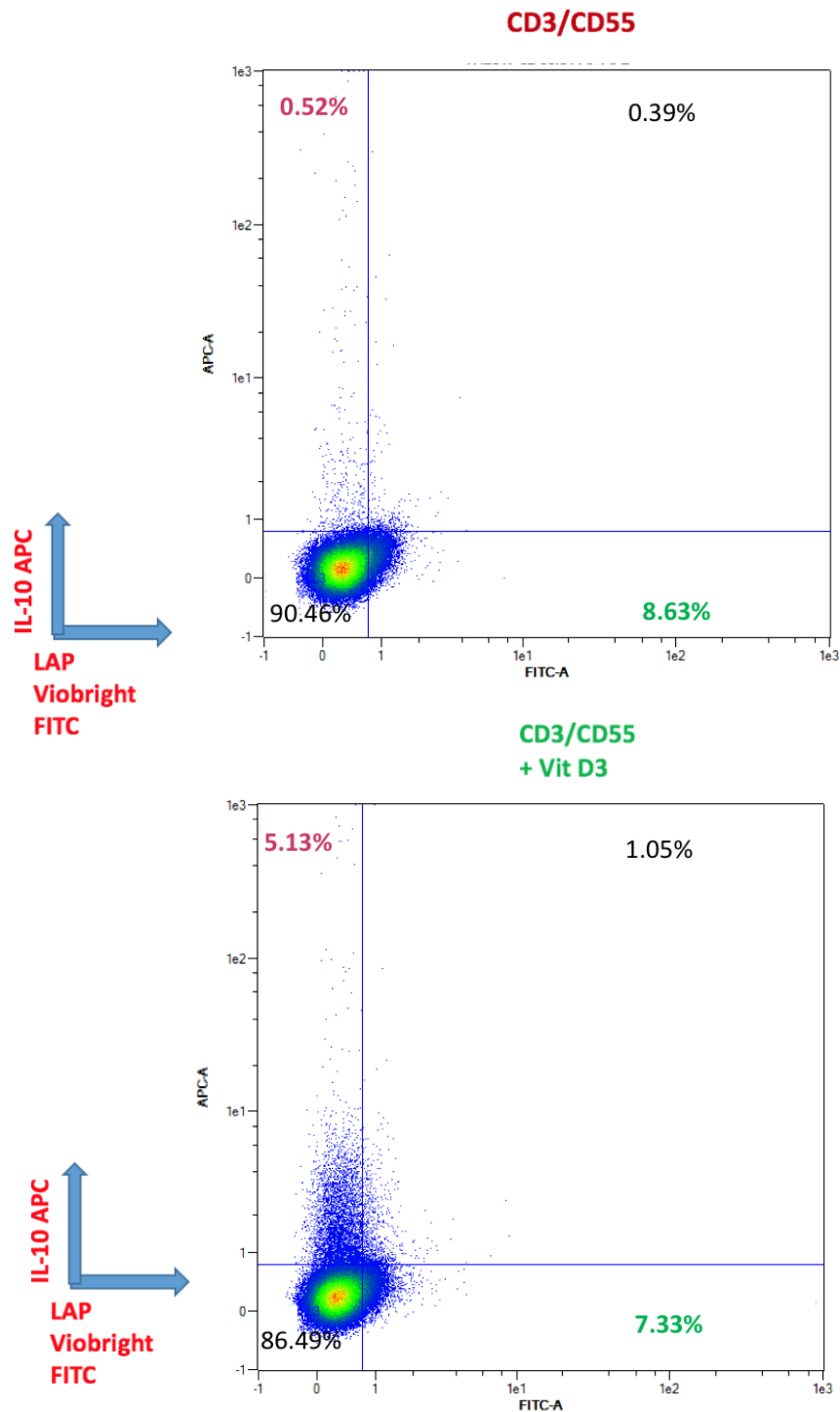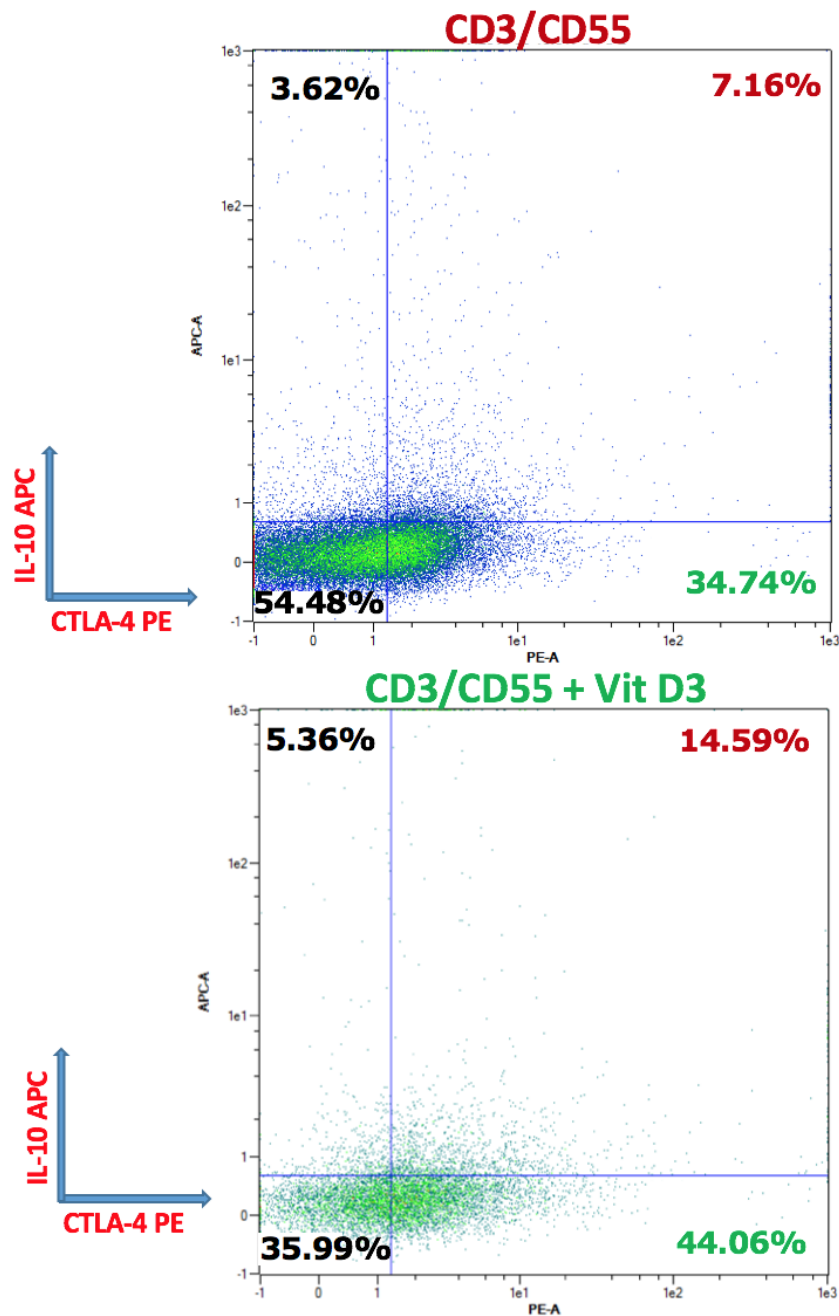
Figure 6.12: Evaluation of CTLA-4 expression by CD3/CD55 induced IL-10^{+} Tr1 cells and IL-10^{-} cells in presence of Vitamin-D3. Naïve CD4^{+} T-cells were stimulated with CD3/CD55 in presence or absence of Vitamin-D3 (10^{-7}M) and after 72 Hours of cell activation, IL-10 CSA was preformed to detect the Tr1 cell along with extracellular staining for CTLA-4. The expression of IL-10 and CTLA-4 was determined on the basis of negative control (only detection antibody + isotype control, data not shown). Data demonstrated here are representative of five independent experiments.

A)

CD3/CD55

		Sample Name	Subset Name	Median : B2-A
IL-10+ CTLA-4+	□	TM2018-11-09.027.mq	Q2: PE-A+ , APC-A+	10.3
IL-10- CTLA-4+	□	TM2018-11-09.027.mq	Q3: PE-A+ , APC-A-	3.25

B)

CD3/CD55 + Vit D3

		Sample Name	Subset Name	Median : B2-A
IL-10+ CTLA-4+	□	TM2018-11-09.028.mq	Q2: PE-A+ , APC-A+	9.29
IL-10- CTLA-4+	□	TM2018-11-09.028.mq	Q3: PE-A+ , APC-A-	3.50
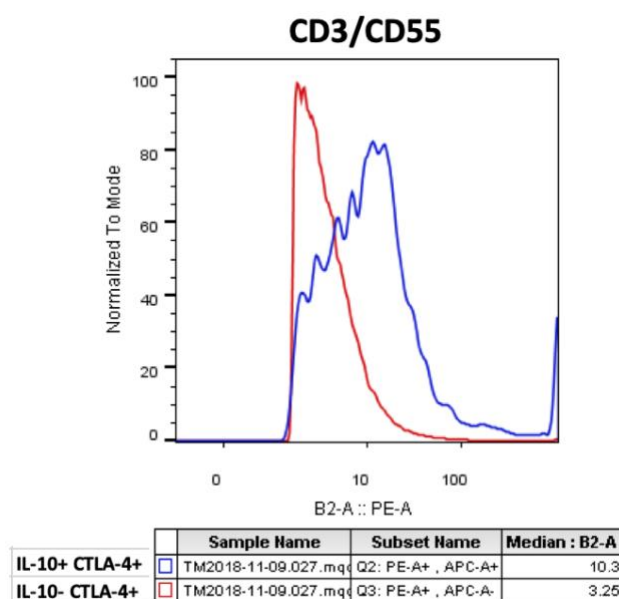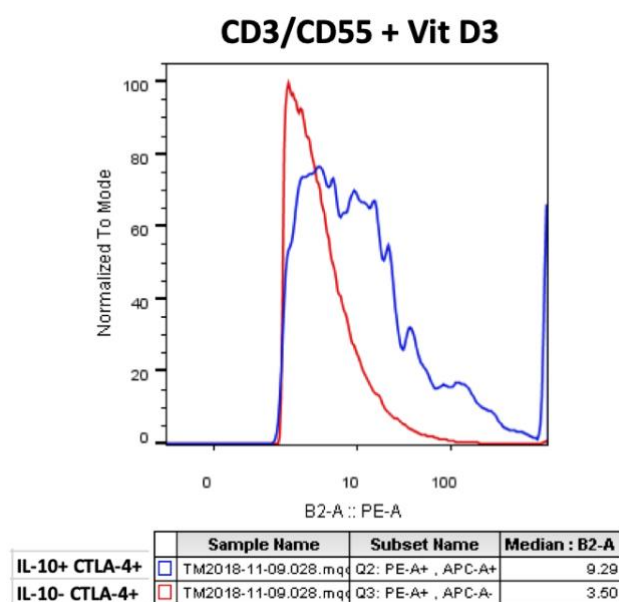
Figure 6.13: Comparison of the level of CTLA-4 expression between IL-10$^+$ and IL-10$^-$cells in CD3/CD55 and CD3/CD55 + vitamin-D3 stimulated cells. Naïve CD4$^+$ T-cells were stimulated with CD3/CD55 in presence or absence of Vit-D3 for 72 hours and IL-10 Cytokine Secretion Assay along with extracellular staining for CTLA-4 was performed to determine the expression of CTLA-4 by IL-10$^+$ and IL-10$^-$ cells. The histograms represent the expression CTLA-4 by IL-10$^+$ (blue) and IL-10$^-$ (red) in CD3/CD55 (A) and CD3/CD55+Vitamin-D3 stimulated cells. Data shown here are representative of five independent experiments.

A)

Expression of CTLA-4 by cells stimulated with CD3/CD55
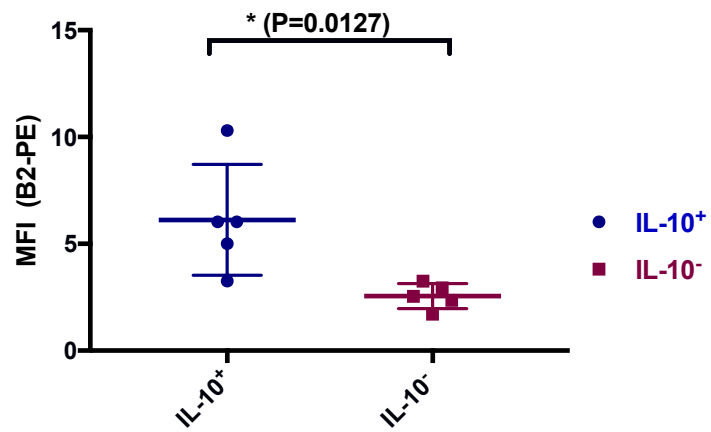
B)

Expression of CTLA-4 by cells stimulated with CD3/CD55 + V it D3
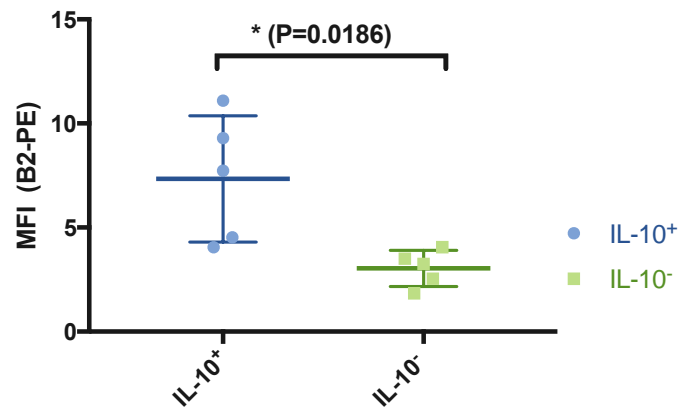
Comparison of CTLA-4 expression by IL-10$^+$ cell

C)
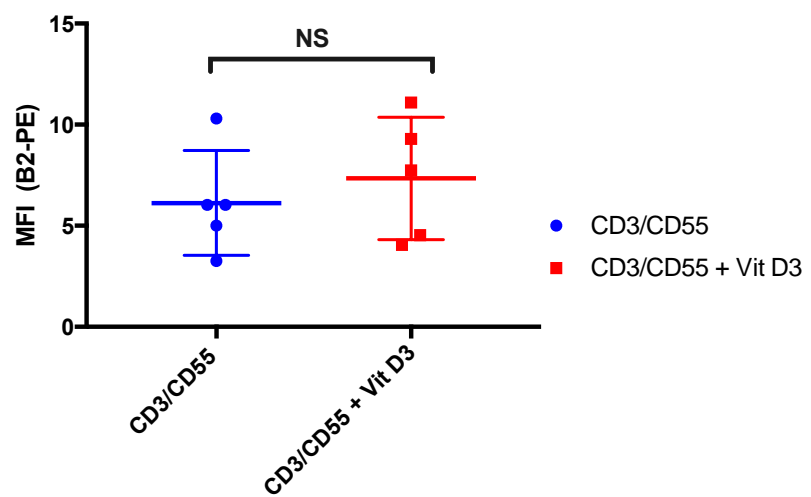
Figure 6.14: Comparison of the level of CTLA-4 expression between IL-10$^+$ and IL-10$^-$cells induced by CD3/CD55 costimulation in presence or absence of vitamin-D3. Naïve CD4$^+$ T-cells were stimulated with CD3/CD55 in presence or absence of Vit-D3 for 72 hours and IL-10 Cytokine Secretion Assay along with extracellular staining for CTLA-4 was performed to determine the expression of the Tr1-associated marker. The Mean Fluorescence Intensity (MFI) of CTLA-4 expression was determined and compared for IL-10$^+$ and IL-10$^-$ population in (A) CD3/CD55 and (B) CD3/CD55+Vit-D3 stimulated cell. (C) The level of CTLA-4 expression by IL-10$^+$ Tr1 cells obtained from stimulating cells with CD3/CD55 and CD3/CD55+Vitamin-D3 were also compared. Data shown here are cumulative summary from five independent experiment and statistical significance was determined by paired T-test.

6.2.1.7 PD-1

Programme cell death-1 (PD-1) is member of CD28 superfamily and PD-1 is one of the coinhibitory molecules which plays critical role in induction and maintenance of immune tolerance by regulating autoreactive T-cells [680]. It has been demonstrated that lack of PD-1 leads to development of autoimmune disease and augmented function of T-cells in mice [681]. PD-1 expression is also associated with impaired the function of tumour infiltrating T-cells [682-684]. Importantly, it has been demonstrated that PD-1 is a mediator of nTreg as it negatively regulates the activation and expansion of nTreg, impairs the suppressive function but prevents apoptosis via Fas and Bcl-2 [685-687]. It was reported that PD-1 expression by nTreg was higher in MS patients compared to healthy donors [687]. Moreover, it was determined that PD-1 expression was significantly higher in IL-10$^+$ Tr1 cells compared to IL-10$^-$ cells when it was determined after short period of cell stimulation (16 hours) but no notable difference was noted at later time points [688]. It was also reported that allergen-specific IL-10$^+$ Tr1 cells expressed high level of PD-1 [330]. As the expression of PD-1 by CD3/CD55 induced IL-10$^+$ Tr1 cells has not been studied previously, we determined the expression of PD-1 in Tr1 cells following CD3/CD55 stimulation in presence and absence of Vitamin-D3.

Naïve CD4 T-cells were stimulated with CD3/CD55 and CD3/CD55+Vitamin-D3 for 72 hours before IL-10 cytokine secretion assay was performed along with extracellular staining for PD-1. The level of PD-1 expression was determined by evaluating MFI. More than 95%

IL-10$^+$ Tr1 cells expressed PD-1 and the presence of vitamin D3 enhanced the induction of Tr1 cells without altering the expression of PD-1 (more than 95% IL-10$^+$ cells expressed PD-1 following CD3/CD55+Vit D3 stimulation) (Figure-6.15). However, PD-1 was also expressed by more than 90% and 80% IL-10$^-$ cells upon stimulation with CD3/CD55 and CD3/CD55+VitaminD3 respectively. When the level of PD-1 expression by IL-10$^+$ and IL-10$^-$ cells were compared, the expression of significantly higher in IL-10$^+$ cells than negative cells in both CD3/CD55 and CD3/CD55+VitaminD3 activated cells (Figure- 6.16; 6.17 A,B). In fact, on average, the PD-1 was two-fold higher in IL-10$^+$ Tr1 cells than IL-10$^-$ cells (Figure- 6.16; 6.17). Also, both IL-10$^+$ and IL-10$^-$ cells stimulated with CD3/CD55 in the presence of vitamin-D3 had reduced PD-1 compared to cells stimulated with only CD3/CD55. However, the reduction in the PD-1 expression in the presence of vitamin D3 was not statistically significant (Figure-6.17 C).
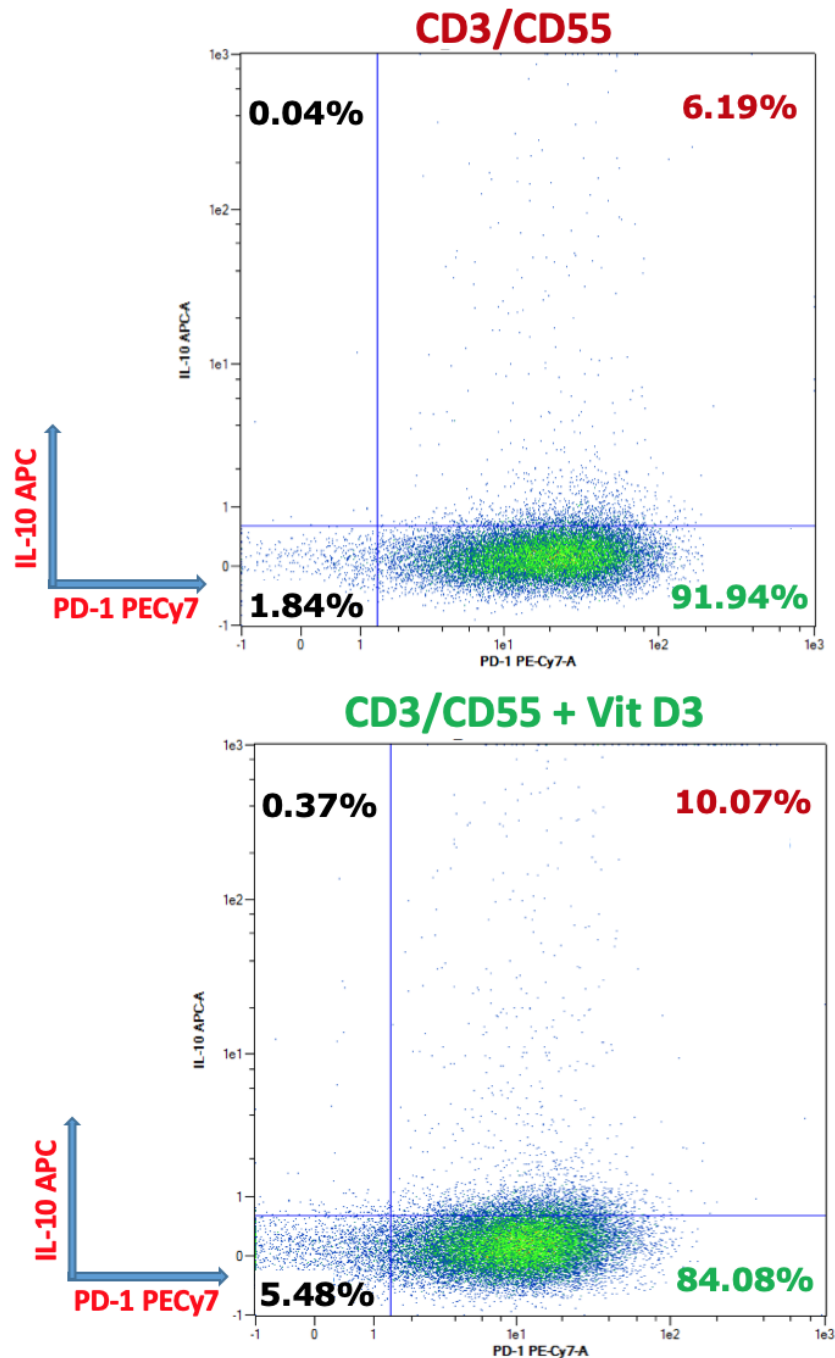
Figure 6.15: Evaluation of PD-1 expression by CD3/CD55 induced IL-10$^+$ Tr1 cells and IL-10$^-$ cells in presence of Vitamin-D3. Naïve CD4$^+$ T-cells were stimulated with CD3/CD55 in presence or absence of Vitamin-D3 (10^{-7}M) and after 72 Hours of cell activation, IL-10 CSA was preformed to detect the Tr1 cell along with extracellular staining for PD-1. The expression of IL-10 and CTLA-4 was determined on the basis of negative control (only detection antibody + isotype control, data not shown). Data demonstrated here are representative of five independent experiments.

A)

	Sample Name	Subset Name	Median : B4-A
IL-10+ PD-1+	TM2018-05-18.021.mqc	Q2: PD-1 PE-Cy7-A+ , IL-10 APC-A+	37.7
IL-10- PD-1+	TM2018-05-18.021.mqc	Q3: PD-1 PE-Cy7-A+ , IL-10 APC-A-	13.8

B)

	Sample Name	Subset Name	Median : B4-A
IL-10+ PD-1+	TM2018-05-18.022.mqc	Q2: PD-1 PE-Cy7-A+ , IL-10 APC-A+	32.5
IL-10- PD-1+	TM2018-05-18.022.mqc	Q3: PD-1 PE-Cy7-A+ , IL-10 APC-A-	12.4
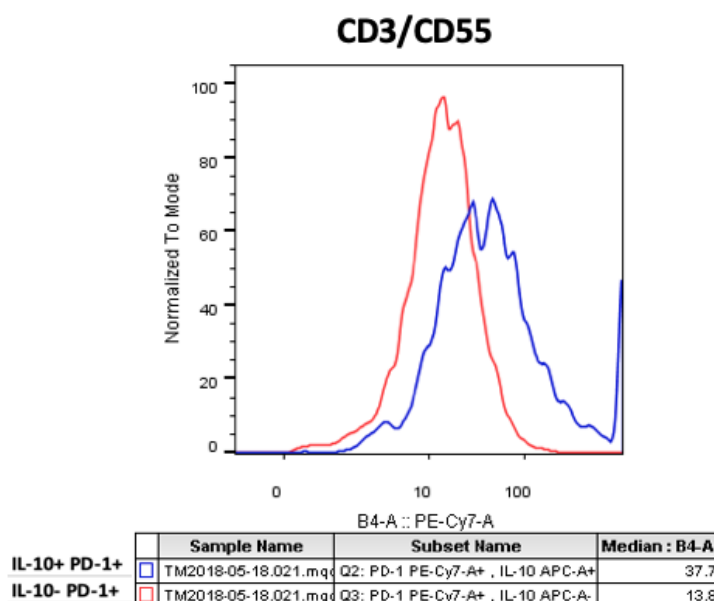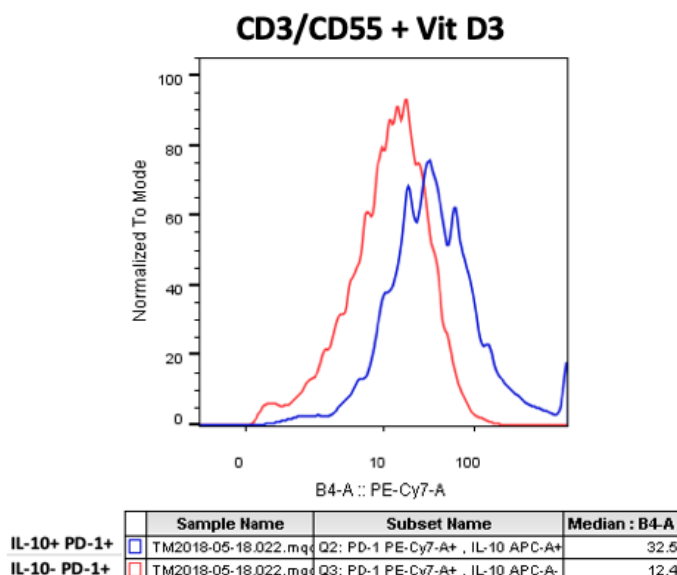
Figure 6.16: Comparison of the level of PD-1 expression between IL-10$^+$ and IL-10$^-$ cells in CD3/CD55 and CD3/CD55+vitamin-D3 stimulated cells. Naïve CD4$^+$ T-cells were stimulated with CD3/CD55 in presence or absence of Vit-D3 for 72 hours and IL-10 Cytokine Secretion Assay along with extracellular staining for PD-1 was performed to determine the expression of PD-1 by IL-10$^+$ and IL-10$^-$ cells. The histograms represent the expression CTLA-4 by IL-10$^+$ (blue) and IL-10$^-$ (red) in CD3/CD55 (A) and CD3/CD55+Vitamin-D3 stimulated cells. Data shown here are representative of five independent experiments.

A) Expression of PD-1 by cells stimulated with CD3/CD55
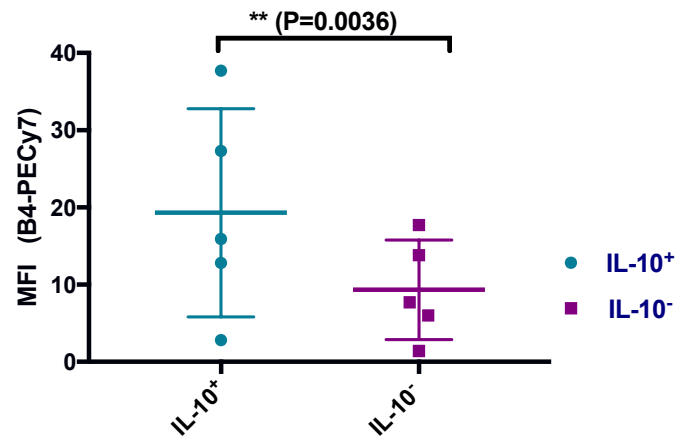
B) Expression of PD-1 by cells stimulated with CD3/CD55 + Vit D3
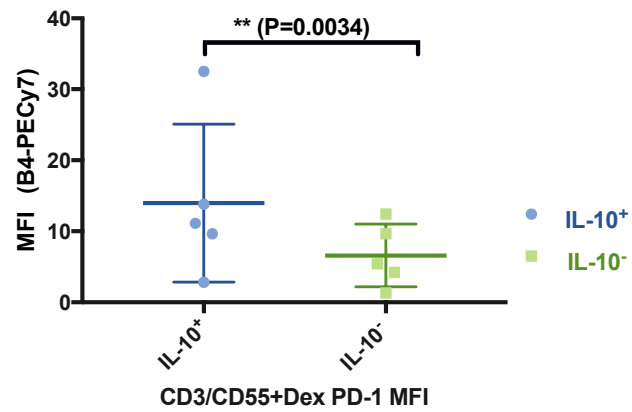
C) Comparison of PD-1 expression by IL-10$^+$ cell
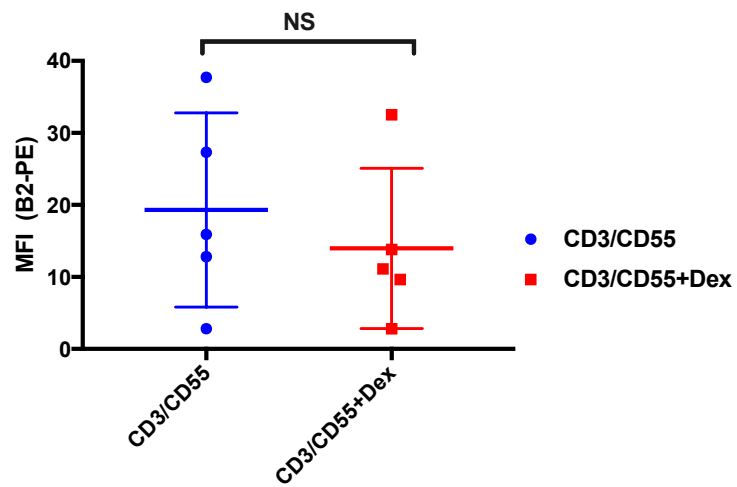
Figure 6.17: Comparison of the level of PD-1 expression between IL-10$^+$ and IL-10$^-$cells induced by CD3/CD55 costimulation in presence or absence of vitamin-D3. Naïve CD4$^+$ T-cells were stimulated with CD3/CD55 in presence or absence of Vit-D3 for 72 hours and IL-10 Cytokine Secretion Assay along with extracellular staining for PD-1 was performed. The Mean Fluorescence Intensity (MFI) of PD-1 expression was determined for IL-10$^+$ and IL-10$^-$ population in (A) CD3/CD55 and (B) CD3/CD55+Vit-D3 stimulated cell. (C) The level of PD-1 expression by IL-10$^+$ Tr1 obtained from stimulating cells with CD3/CD55 and CD3/CD55+Vitamin-D3 were also compared. Data shown here are cumulative summary from five independent experiment and statistical significance was determined by paired T-test.

6.2.2 Transcription Factor expression by CD3/CD55 induced IL-10$^+$-like cells

Transcription factors profile is not only essential to characterise the cells, they are also important to determine the functional stability of the cell in long term. The master-regulator of IL-10 producing CD3/CD55 induced Tr1 cells is yet to be determined. So, in this study, we have investigated the expression of six transcription factors in CD3/CD55 Tr1 cells in presence or absence of immune-modulator Dexamethasone and compared with CD3/CD28 stimulated cells.

6.2.2.1 FoxP3

The transcription factor FoxP3 plays a critical role in the development of thymus-derived natural regulatory T-cells (nTreg) [689, 690] and maintaining immune-homeostasis. Mutation in *FOXP3* gene is associated with immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) in human while mutation in the ortholog gene in mice causes scurfy syndrome in mice [691]. FoxP3 can be transiently expressed by activated nonregulatory T-cells following TCR ligation [692]. It has also been demonstrated that naïve T-cells are stimulated in presence of TGF-β and IL-2 express FoxP3 and the additional signal via CD28 contributes to the induction of FoxP3 only by enhancing endogenous IL-2 production [693]. However, the transiently FoxP3 expressing cells do not retain the immune-suppressive function, produce inflammatory cytokine and accumulate in inflamed tissue in auto-immune condition [694]. Stable FoxP3 expression is one of the factors that

differentiates nTreg from inducible Treg generated in the periphery and it indicates different mechanism of action by which they impart they immuno-suppressive function. So, we determined the expression of FoxP3 in the CD3/CD55 induced Tr1 cells in presence or absence of immune-modulator Dexamethasone and compared it to the CD3/CD28 stimulated cells.

Naïve CD4 T-cells were stimulated with either CD3/CD55 or CD3/CD28 in presence or absence of Dexamethasone (5×10^{-8} M) for 72 hours. Then IL-10^+ Tr1 cells were detected by performing IL-10 CSA and the expression of FoxP3 was determined intracellular staining (Figure 6.18). Upon stimulation with CD3/CD55, higher amount of IL-10^+ Tr1-cells cells were detected compared to cells which were stimulated with CD3/CD28 (Figure 6.17, n=3). Moreover, CD3/CD55 induced IL-10^+ were further enhanced in the presence of Dex whereas it did not increase in the CD3/CD28 stimulated cells. More than 95% IL-10^+ Tr1 cells did not express FoxP3 when cells were stimulated with only CD3/CD55 (Figure 6.18). While the presence of Dexamethasone (Dex) induced more IL-10^+ Tr1 cells following CD3/CD55, it did not induce FoxP3 expression in those cells. Similarly, IL-10^+ cells stimulated with CD3/CD28 did not express FoxP3 regardless of the presence or absence of Dex.

Figure 6.18: Evaluation of FoxP3 expression by CD3/CD55 and CD3/CD28 stimulated cells in presence or absence of dexamethasone. Naïve CD4 T-cells were stimulated with either CD3/CD55 or CD3/CD28, in presence or absence of Dexamethasone ($5X10^{-8}$ M). After 72 hours of cell stimulation, IL-10 CSA was performed along with intracellular staining for FoxP3 to determine the expression of the transcription factor by the IL-10^{+} Tr1 cells. Data shown here are representative of three independent experiments.

6.2.2.2 T-bet

T-bet, a member of the T-box transcription factor family, is pivotal for the development of Th1 cells from Naïve CD4 T-cells and has been described as the linage defining transcription factor of Th1 cells [695-699]. It has been reported that T-bet is rapidly induced by IFN-γ which works as a positive feedback loop [695, 697]. The expression of T-bet was assessed in the CD3/CD55 induced Tr1 cells in presence or absence of Dex. The expression of T-bet was also determined in cells stimulated with CD3/CD28 for comparison as it has been reported to induce T-bet expression in Th1 cells [700]. Naïve cells were stimulated with CD3/CD55 and T-bet expression was detected in more than 75% of cells after 72 hours of cell activation. The presence of Dex enhanced the number of IL-10$^+$ Tr1 cells but it did not alter the percentage of T-bet$^+$ following the CD3/CD55 stimulation (Figure 6.19, n=3). ~85% of the CD3/CD55 induced IL-10$^+$ Tr1 cells co-expressed T-bet whereas more than 70% of CD3/CD55+Dex stimulated cells expressed T-bet. CD3/CD28 stimulation also induced the expression of T-bet in more than 55% of the cells but gave rise to a smaller fraction of IL-10$^+$T-bet$^+$ (0.27%) cells compared to CD3/CD55 stimulated cells (4.17%). The presence of Dex did not increase the IL-10$^+$T-bet$^+$ cells when cells were stimulated with CD3/CD28.
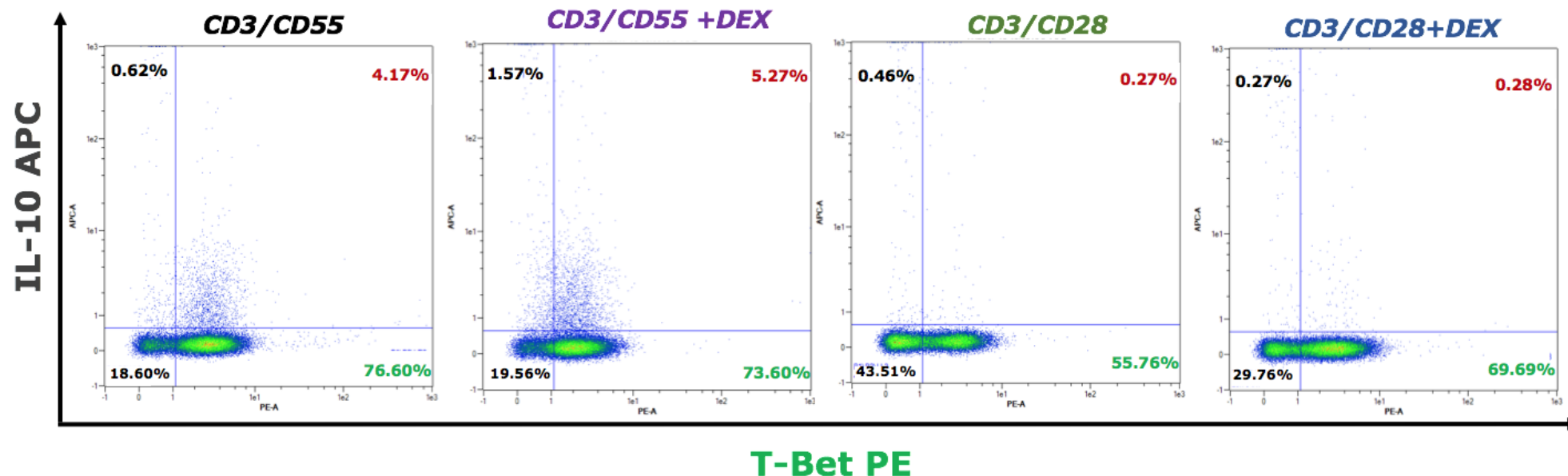
Figure 6.19: Determining the expression of T-bet by CD3/CD55 and CD3/CD28 stimulated in presence or absence of dexamethasone. Naïve CD4 T-cells were stimulated with either CD3/CD55 or CD3/CD28, in presence or absence of Dexamethasone (5×10^{-8} M). After 72 hours of cell stimulation, IL-10 CSA was performed along with intracellular staining for T-bet (PE) to determine the expression of the transcription factor by the IL-10$^+$ Tr1 cells. The T-bet expression was determined on the basis of negative isotype control in each experiment (not shown here). Data is representative of the three independent experiments.

6.2.2.3 GATA-3

GATA3, a zinc-finger transcription factor, belong to GATA (GATA-binding protein) family and it is essential for the development of Th2 cells [701]. Conditional deletion of GATA3 abrogates the differentiation to Th2 cells and maintenance of its function [702, 703]. GATA3 is not only important at the stage of Th2 development, it also plays a critical role in thymocyte development and CD4 commitment [704-707]. GATA3 is upregulated when naïve CD4 T-cells are stimulated in the presence of IL-4 and it binds to regulatory element associated with Th2 cytokine gene locus leading to production of various Th2 specific cytokines including IL-5[708] and IL-13 [709]. As IL-10 was initially identified as a cytokine inhibitory factor produced by Th2 clones [336], we investigated the expression of GATA3 by CD3/CD55 induced IL-10$^+$ Tr1 cells and compared it to the CD3/CD28 stimulated cells in presence or absence of immune-modulator Dex.

Naïve CD4 T-cell were stimulated with either CD3/CD55 or CD28 in presence or absence of Dex for 72 hour and then the expression of GATA3 was determined by intracellular staining along with cytokine secretion assay for IL-10. Almost all of the IL-10$^+$ Tr1 cells (~99%) did not express any GATA3 following CD3/CD55 stimulation both in presence and absence of Dex (Figure-6.20, data representative of three independent experiments). Moreover, none of the IL-10$^-$ cells expressed GATA3 either. Similarly, no GATA3 expression was detected in cells stimulated with CD3/CD28 in presence and absence of Dex.
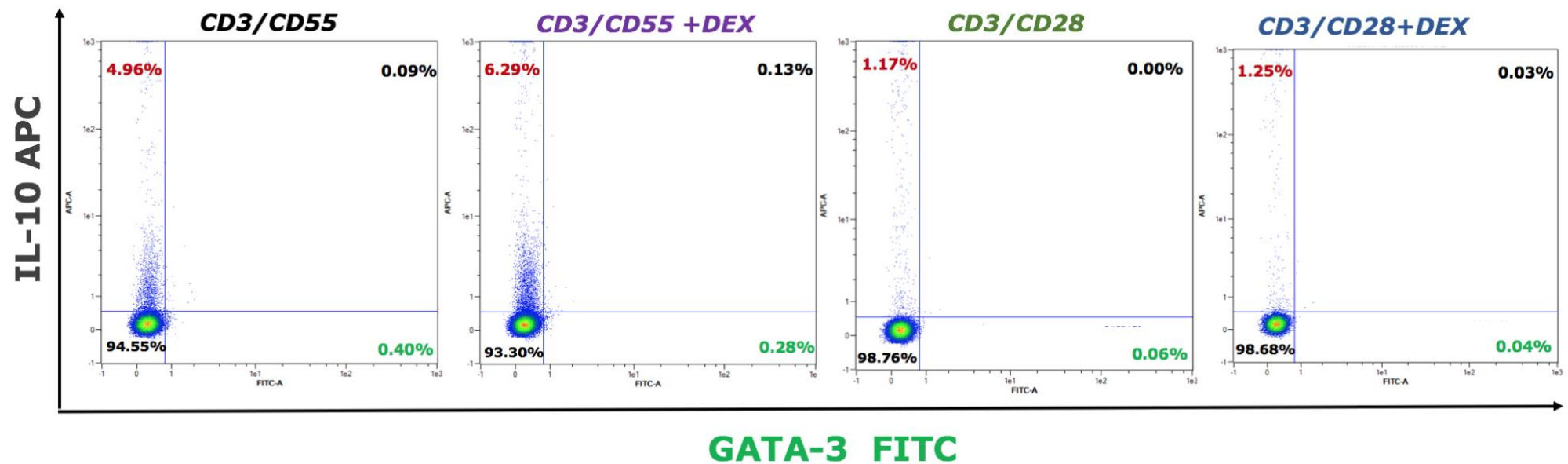
Figure 6.20: Evaluation of GATA-3 expression by cells stimulated with CD3/CD55 and CD3/CD28 in presence or absence of dexamethasone. Naïve CD4 T-cells were stimulated with either CD3/CD55 or CD3/CD28, in presence or absence of Dexamethasone (5×10^{-8} M). After 72 hours of cell stimulation, IL-10 CSA was performed along with intracellular staining for GATA3 to determine the expression of the transcription factor by the IL-10$^+$ Tr1 cells. Data is representative of three independent experiments performed with the blood sample collected from heathy individuals.

6.2.2.4 RORγt

The transcription factor RORγt belongs to the RAR-related orphan nuclear receptor (ROR) family. It is required for the differentiation pro-inflammatory IL-17 producing Th17 cell from naïve CD4^{+}T-cells under the influence of IL-1β and IL-6 [710, 711]. However, it has been reported that Th17 cells can co-express RORγt and T-bet along with IFN-γ in inflammatory environment [712, 713]. Also, it has also been reported that FoxP3^{+} regulatory T-cells express RORγt during intestinal inflammation [714]. We have determined the expression of RORγt in CD3/CD55 induced Tr1 cells in presence and absence of Dex as well as in CD3/CD28 stimulated cells in this study. When naïve cells were stimulated with CD3/CD55, more than 90% cells IL-10^{+} Tr1 cells expressed RORγt which remained consistent even in the presence of Dex (Figure 6.21, n=3). The addition of Dex to CD3/CD55 stimulation increased the IL-10^{+} Tr1 cells (7.35% vs 4.86% in only CD3/CD55) and more than 85% of IL-10^{+} Tr1 cells co-expressed RORγt. In the IL-10^{-} population, more than 80% cells expressed RORγt following CD3/CD55 whereas 75% cells expressed RORγt following CD3/CD28 stimulation. The presence of Dex did not induce IL-10^{+} Tr1 cells in the CD3/CD28 stimulated cells and it also did not alter the expression of RORγt.
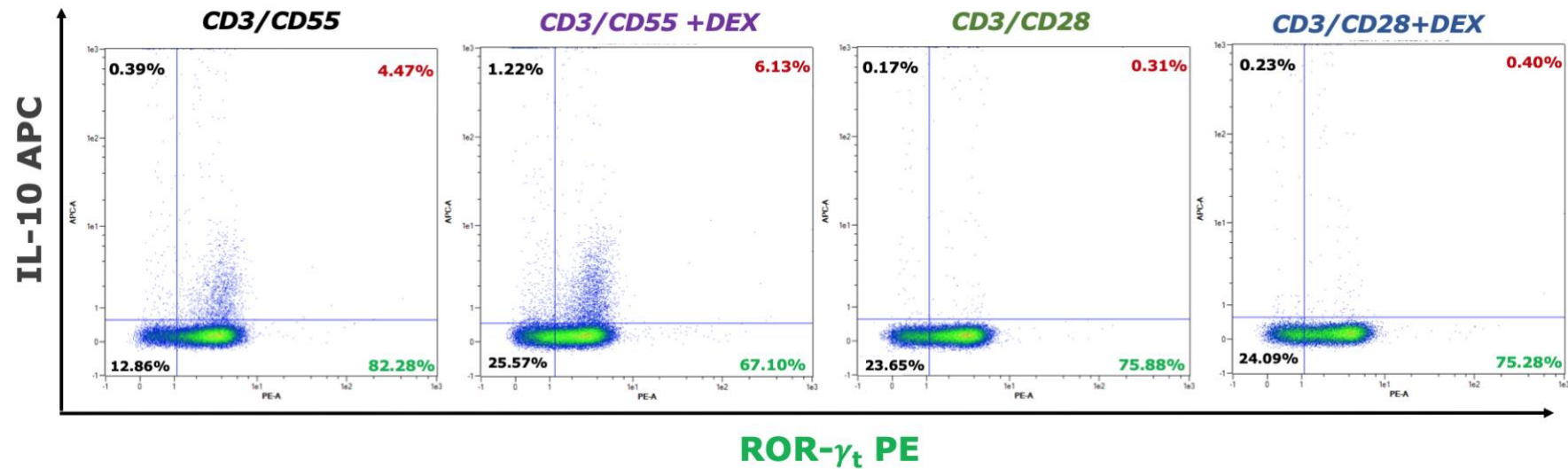
Figure 6.21: Evaluation of RORγt expression by CD3/CD55 and CD3/CD28 stimulated cells in presence or absence of dexamethasone. Naïve CD4 T-cells were stimulated with either CD3/CD55 or CD3/CD28, in presence or absence of Dexamethasone ($5X10^{-8}$ M). After 72 hours of cell stimulation, IL-10 CSA was performed along with intracellular staining for RORγt to determine the expression of the transcription factor by the IL-10$^+$ Tr1 cells. The expression of RORγt was determined on the basis of isotype control in each experiment (not shown here). Data is representative of three independent experiments.

6.2.2.5 HELIOS

HELIOS, a member of the Ikaros transcription factor family, has been reported to be expressed by a subset of FoxP3$^+$ nTreg in both human and mice [715, 716]. Recently it has been demonstrated that HELIOS deficiency partially impairs the suppressive function of effector nTreg in vivo [717]. However, it has also been reported that HELIOS is upregulated during cell activation and proliferation in CD4$^+$ and CD8$^+$ T-cells. Thus, it could not be considered as transcription factor associated with only regulatory T-cells [718]. We have investigated the expression of HELIOS by CD3/CD55 induced IL-10$^+$ Tr1 cells and CD3/CD28 stimulated cells in presence and absence of Dexamethasone (Dex) (Figure 6.22, n=3). More than 98% CD3/CD55 induced IL-10$^+$ Tr1 cells did not express HELIOS and the presence of Dex did not induce the expression of HELIOS in these cells. Similarly, the IL-10$^+$ cells in CD3/CD28 stimulated cells did not express HELIOS and its expression remain consistent even with the addition of Dex to the CD3/CD28 stimulated cells. Moreover, the expression of HELIOS was low in the IL-10$^-$ population in both CD3/CD55 and CD3/CD28 stimulated cells ($\sim$1-2%) regardless of treatment with Dex.
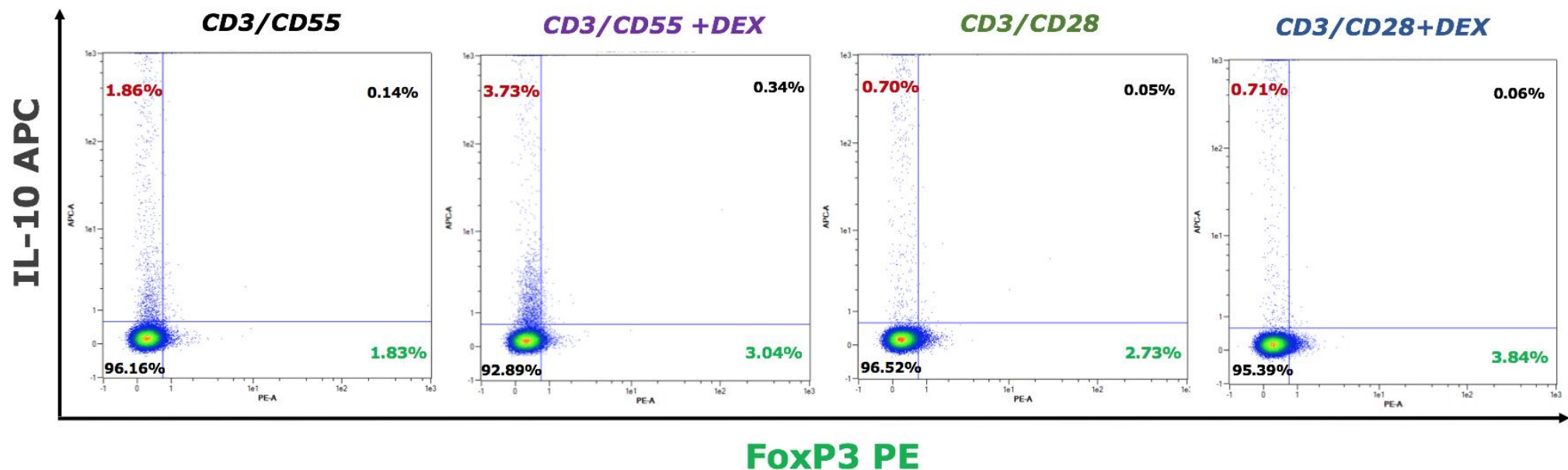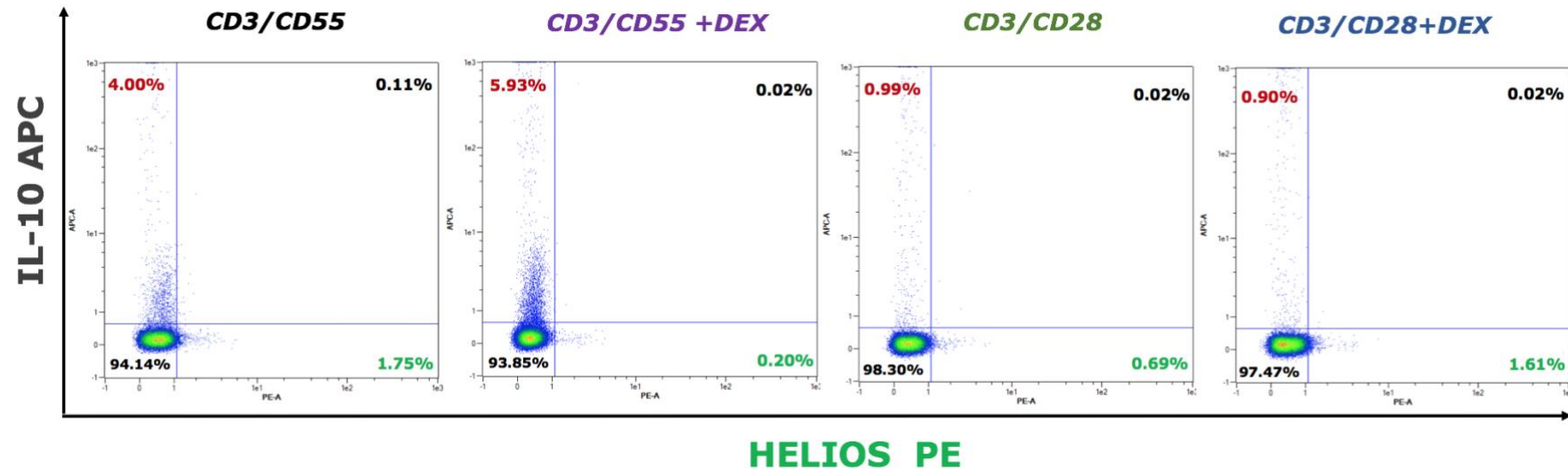
Figure 6.22: Evaluation of HELIOS expression by CD3/CD55 and CD3/CD28 stimulated cells in presence or absence of dexamethasone. Naïve CD4 T-cells were stimulated with either CD3/CD55 or CD3/CD28, in presence or absence of Dexamethasone ($5X10^{-8}$ M). After 72 hours of cell stimulation, IL-10 CSA was performed along with intracellular staining for HELIOS to determine the expression of the transcription factor by the IL-10^{+} Tr1 cells. Most of CD3/CD55 induced IL-10^{+} cells did not express HELIOS and the presence of Dex increased the IL-10^{+} Tr1 cells without altering the expression of HELIOS. The cells stimulated with CD3/CD28 did not express HELIOS both in presence and absence of Dex but demonstrated markedly reduced amount of IL-10^{+} cells compared to CD/CD55 stimulated cells. Data is representative of three independent experiments.

6.2.2.6   c-MAF

The transcription factor c-MAF, a member of the AP-1 family of basic region/leucine zipper factor, emerged as a Th2 specific transcription factor as it was reported to bind to the c-MAF Response Element (MARE) on the proximal promoter of IL-4 and promoted cells differentiating to Th2 phenotype [719-721]. It was also demonstrated that c-MAF impaired the production of IFN-g by Th1 cells in IL-4 independent manner [720]. However, the role of c-MAF is not restricted to only Th2 cells and it was later described to be essential for the development and cytokine production of Follicular helper T cells (Tfh) [722-724] and Tr1 cells [725]. Importantly, c-MAF has also been associated with IL-10 production by Th17 [726-729], Th1 [730], Tr1 [725, 731, 732] and macrophages [733]. It has been reported that c-MAF binds to the proximal promoter as well as around a 6-kb site in *il-10* locus in T-cells [729]. Moreover, it was determined that IL-10, IL-4 and RORγ were direct targets of c-MAF whereas FoxP3 and Tbx21 were indirect targets. Interestingly, c-MAF plays a crucial role in the IL-27 mediated differentiation Tr1 cells by transactivating IL-21 which acts as an autocrine factor and promotes further expansion of IL-10$^+$ Tr1 cells [725]. Thus, c-MAF positively regulates IL-10 production via direct and indirect manner depending on the cell type. Considering the role of c-MAF on IL-10 regulation, we studied the expression of c-MAF in CD3/CD55 induced Tr1 cells and CD3/CD28 stimulated Th1 cells in presence or absence of Dex.

Naïve CD4$^+$T-cells were stimulated with either CD3/CD55 or CD3/CD28 in presence or absence of immune-modulator Dexamethasone for 72 hours before the expression of c-MAF by IL-10$^+$ Tr1 cells was determined by

performing IL-10 cytokine secretion assay and intracellular staining for c-MAF. CD3/CD55 stimulation induced IL-10$^+$ Tr1 cells and more than 70% of the IL-10$^+$ cells expressed c-MAF (n=5). The expression of c-MAF remained consistent when the cells were stimulated with CD3/CD55 in presence of Dex (Figure 6.23 A). CD3/CD28 stimulation resulted in lower percentage of IL-10$^+$ cells and most of those cells did not express c-MAF. The presence of Dex did not alter the expression of either IL-10 or c-MAF in CD3/CD28 stimulated cells. Interestingly, CD3/CD55 stimulation resulted in significantly higher (**P=0.0035, n=4) overall expression (combined expression in total IL-10$^+$ and IL-10$^-$ cells) of c-MAF compared to CD3/CD28 stimulation (Figure 6.23 B). The presence of Dex did not modulate the overall expression of c-MAF in CD3/CD55 stimulated cells and percentage of c-MAF expressing cells were still significantly higher (***P=0.0007,n=4) following CD3/CD55+Dex stimulation in comparison to CD3/CD28 stimulated cells. But no significant difference was observed between c-MAF expression in CD3/CD55 and CD3/CD55+Dex stimulated cells. Moreover, the expression of c-MAF, determined by Mean Fluorescence Intensity (MFI), was higher in IL-10$^+$ cells than IL-10$^-$cells following cell stimulation with both CD3/CD55 and CD3/CD55+Dex (Figure 6.24).

A)

B)
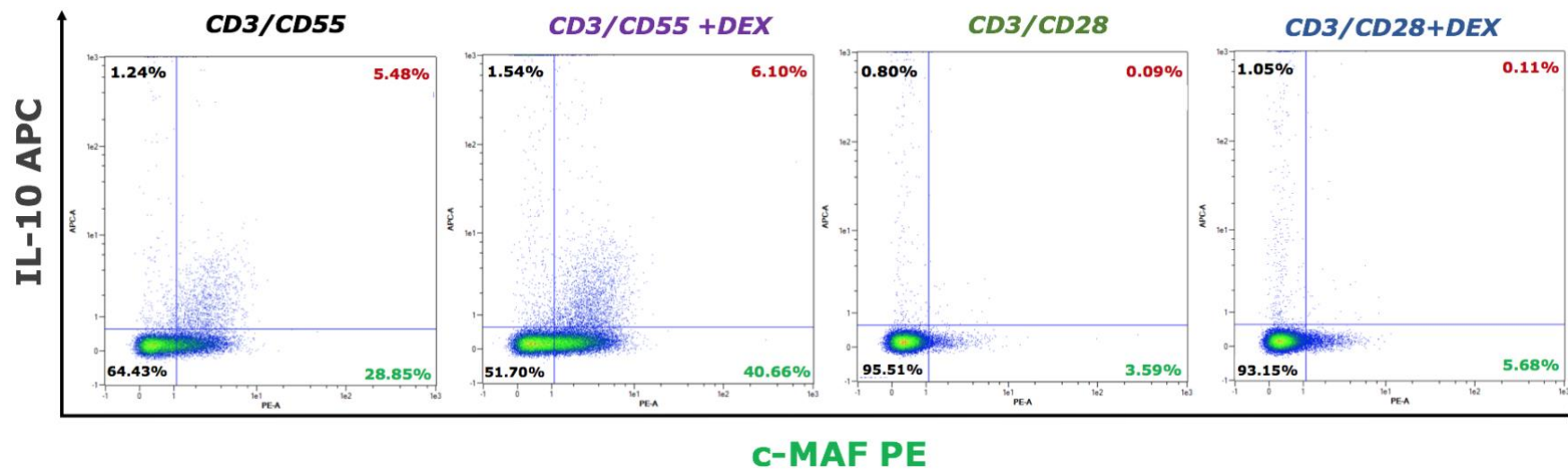
The expression of c-MAF after 72 hours of cell stimulation

Figure 6.23: Determining the expression of c-MAF by CD3/CD55 and CD3/CD28 stimulated cells in presence or absence of dexamethasone. A) Naïve CD4 T-cells were stimulated with either CD3/CD55 or CD3/CD28, in presence or absence of Dexamethasone (5×10^{-8} M). After 72 hours of cell stimulation, IL-10 CSA was performed along with intracellular staining for c-MAF to determine the expression of the transcription factor by the IL-10^+ Tr1 cells. Data is representative of four independent experiment. **B)** The overall expression of c-MAF in CD3/CD55 stimulated cells was compared to CD3/CD28 stimulated cells both in presence and absence of Dex. Statistical significance was determined by two-way ANOVA with data collected from four independent experiments (n=4).

CD3/CD55

A)

		Sample Name	Subset Name	Median : B2-A
IL-10+ c-MAF+	☐	TM2018-09-21.014.mqd	Q2: PE-A+ , APC-A+	8.32
IL-10- c-MAF+	☐	TM2018-09-21.014.mqd	Q3: PE-A+ , APC-A-	2.94

CD3/CD55 + Dex

B)

		Sample Name	Subset Name	Median : B2-A
IL-10+ c-MAF+	☐	TM2018-09-21.015.mqd	Q2: PE-A+ , APC-A+	8.02
IL-10- c-MAF+	☐	TM2018-09-21.015.mqd	Q3: PE-A+ , APC-A-	3.25
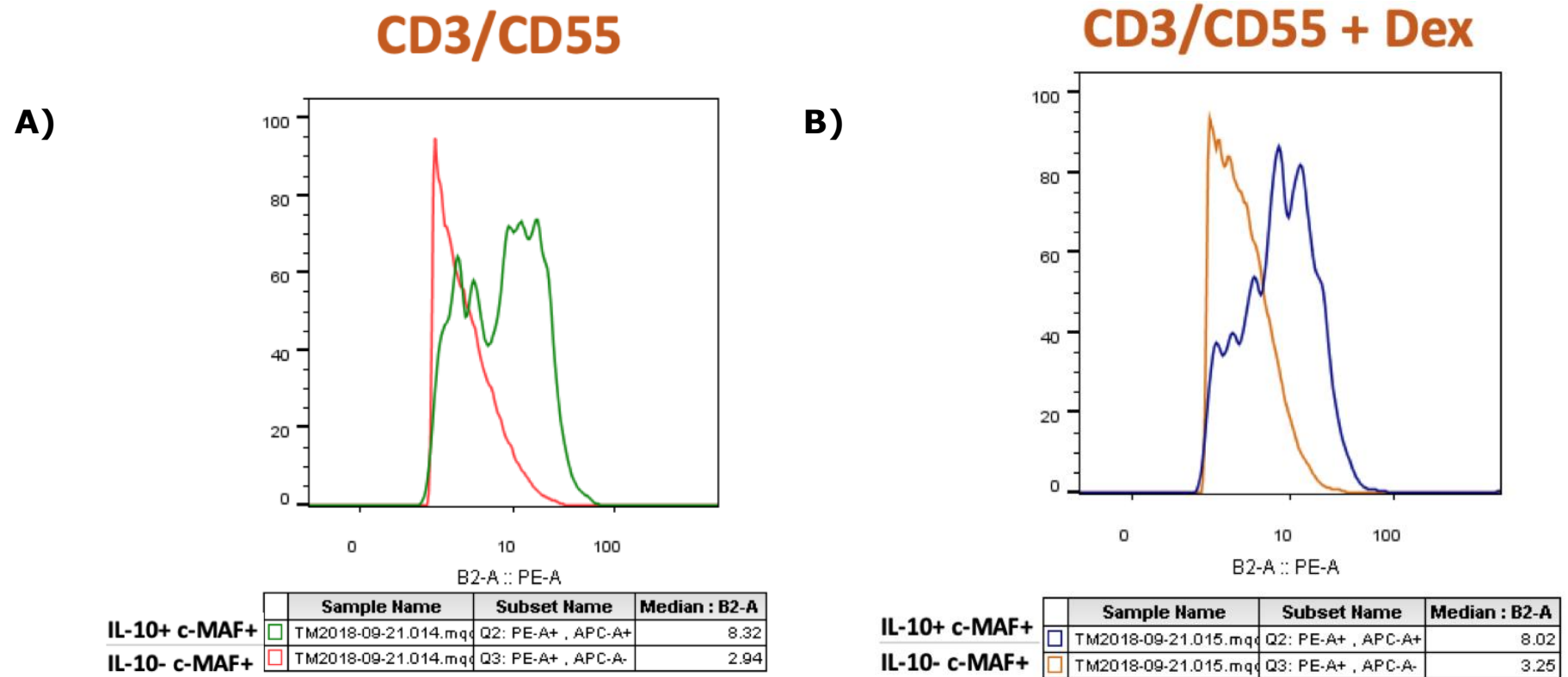
Figure 6.24: Comparison of the level of c-MAF expression between IL-10$^+$ and IL-10$^-$cells in CD3/CD55 and CD3/CD55+Dexamethasone stimulated cells. Naïve CD4 T-cells were stimulated with CD3/CD55 (A) and CD3/CD55+Dex (B) for 72 hours and the expression of c-MAF by IL-10$^+$ and IL-10$^-$ cells was determined by performing IL-10 CSA and intracellular staining for c-MAF. The cells were gated for IL-10 expression before the MFI for c-MAF (B2- PE) was determined for IL-10$^+$ and IL-10$^-$ cells. IL-10$^+$ cells resulting from CD3/CD55 stimulation expressed significantly higher amount (P=0.0172) of c-MAF compared to IL-10$^-$ cells. The data is representative of five independent experiments.

6.2.2.7 c-MAF expression on primary and secondary restimulation with CD3/CD55 and during cell divisions

c-MAF expression by CD3/CD55 induced Tr1 cells following the primary stimulation indicated that it plays an important role in CD3/CD55 mediated IL-10 production. In the next stage, we investigated whether the expression of c-MAF is consistent in all IL-10$^+$ (Figure 6.25) cells and if its expression is retained upon restimulation. Also, we investigated if c-MAF is only induced by CD3/CD55 but not CD3/CD28 by performing cross-restimulation experiment (Figure 6.26). Proliferation dye Cell Trace Violet labelled Naïve CD4$^+$ T-cells were stimulated with CD3/CD55 in presence or absence of Dex in order to determine if the c-MAF expression is restricted only to the cells which recently proliferated as it has been reported that c-MAF required for IL-10 production by Th1 during resolution phase [734]. It was determined that c-MAF expression is consistent in cells which went through multiple rounds of cells division (3-4 cells division in 72 hours) and c-MAF was also detected in cells which stopped proliferating after one or two rounds of cell division (Figure 6.24). Both IL-10$^+$ and IL-10$^-$ cells demonstrated similar proliferation (3-4 cell divisions) but c-MAF expression was higher in the IL-10$^+$ cells (MFI 6.77) compared to IL-10$^-$ cells (MFI 1.86). The c-MAF expression was similar in CD3/CD55+dex stimulated cells where c-MAF expression was consistent in most of the IL-10$^+$ cells regardless of how many times they divided and c-MAF expression was not restricted to only the most recently proliferated active cells.

CD3/CD55
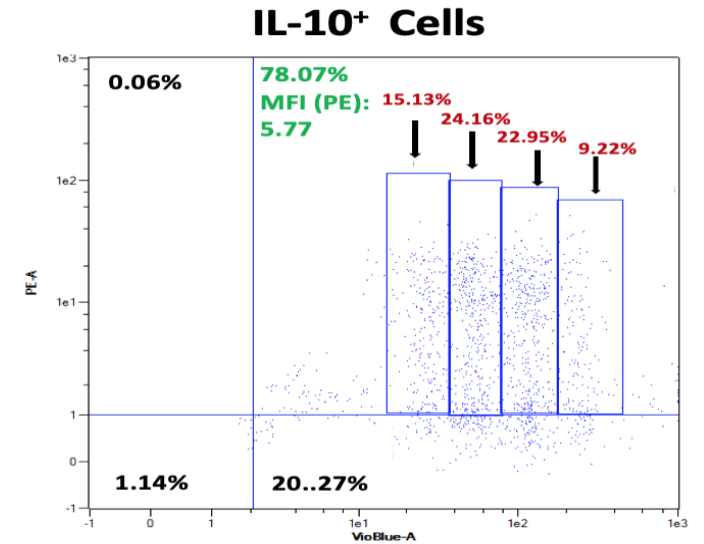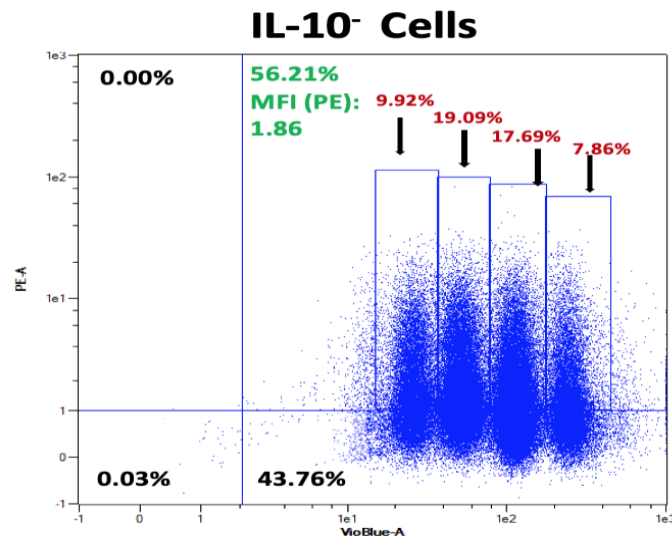
CD3/CD55 + Dex
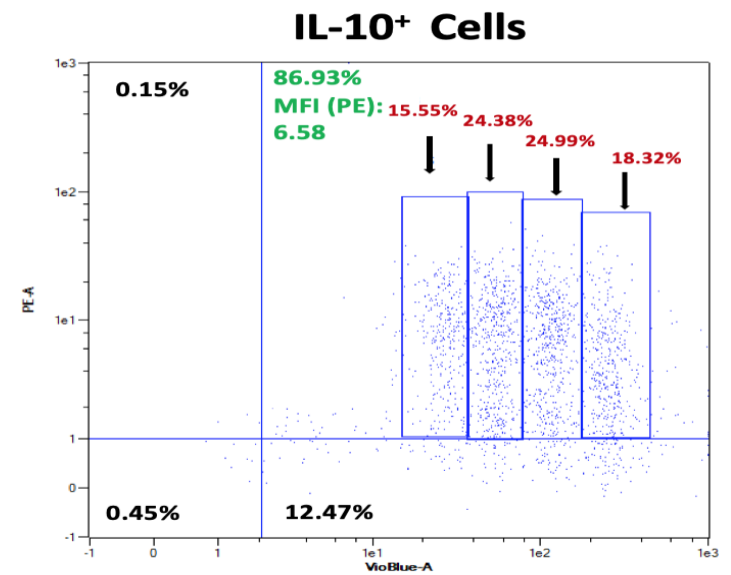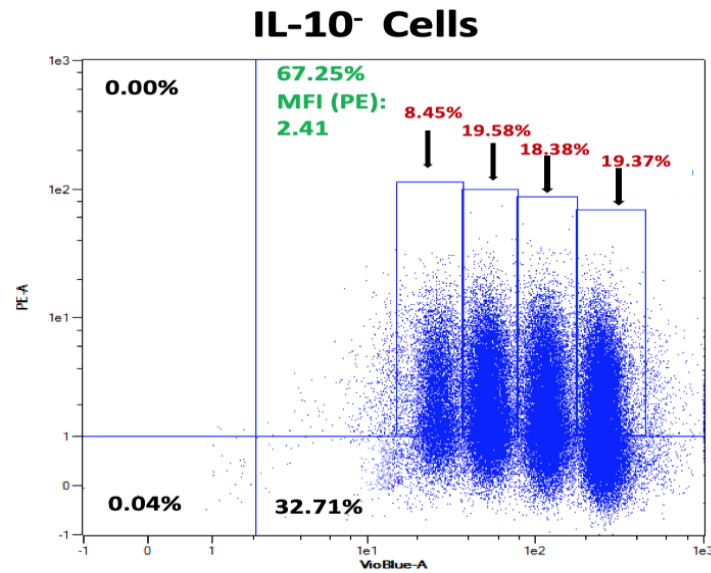
Figure 6.25: Determination of c-MAF expression by the proliferating IL-10$^+$ Tr1 cells and IL-10$^-$ cells activated by CD3/CD55 in presence or absence of dexamethasone. Naïve CD4 T-cells were labelled with cell proliferation dye Cell trace violet and stimulated with either CD3/CD55 or CD3/CD55+Dex for 72 Hours. Then the expression of c-MAF was evaluated by intracellular staining along with IL-10 production by performing IL-10 CSA. The overall expression of c-MAF was determined in IL-10$^+$ and IL-10$^-$ cells by evaluating MFI in both CD3/CD33 and CD3/CD55+ Dex stimulated cells. Data is representative of three independent experiments.

We studied the expression of c-MAF upon restimulation in order to determine if c-MAF expression is retained after reactivation and if c-MAF was only induced by CD3/CD55 signalling. Naïve cells were stimulated with CD3/CD55 for 3 days for primary stimulation and then cells were rested for another 7 days before they were restimulated with CD3/CD55, CD3/CD28 and non-specific stimuli PMA/Ionomycin. For both Primary and secondary stimulation of cells, expression of both T-bet and c-MAF was determined by intracellular staining (Figure 6.26). In the primary stimulation of cells, CD3/CD55 stimulation induced expression of T-bet in most of the cells (~90%) whereas a smaller fraction of cells expressed both T-bet and c-MAF (~15%). When CD3/CD55 primed cells were restimulated, c-MAF expression was retained upon restimulation with CD3/CD56. Less T-bet$^+$ c-MAF$^+$ cells were detected when cells were restimulated with CD3/CD28 (9.32%) compared to CD3/CD55 (21.03%) restimulated cells. Interestingly, PMA/Ionomycin failed to induce c-MAF expression in CD3/CD55 primed cells upon restimulation even though the non-specific stimuli induced expression of T-bet. These findings indicate that c-MAF expression is retained upon restimulation and its expression restricted to CD3/CD55 stimulation in CD3/CD55 primed cells.
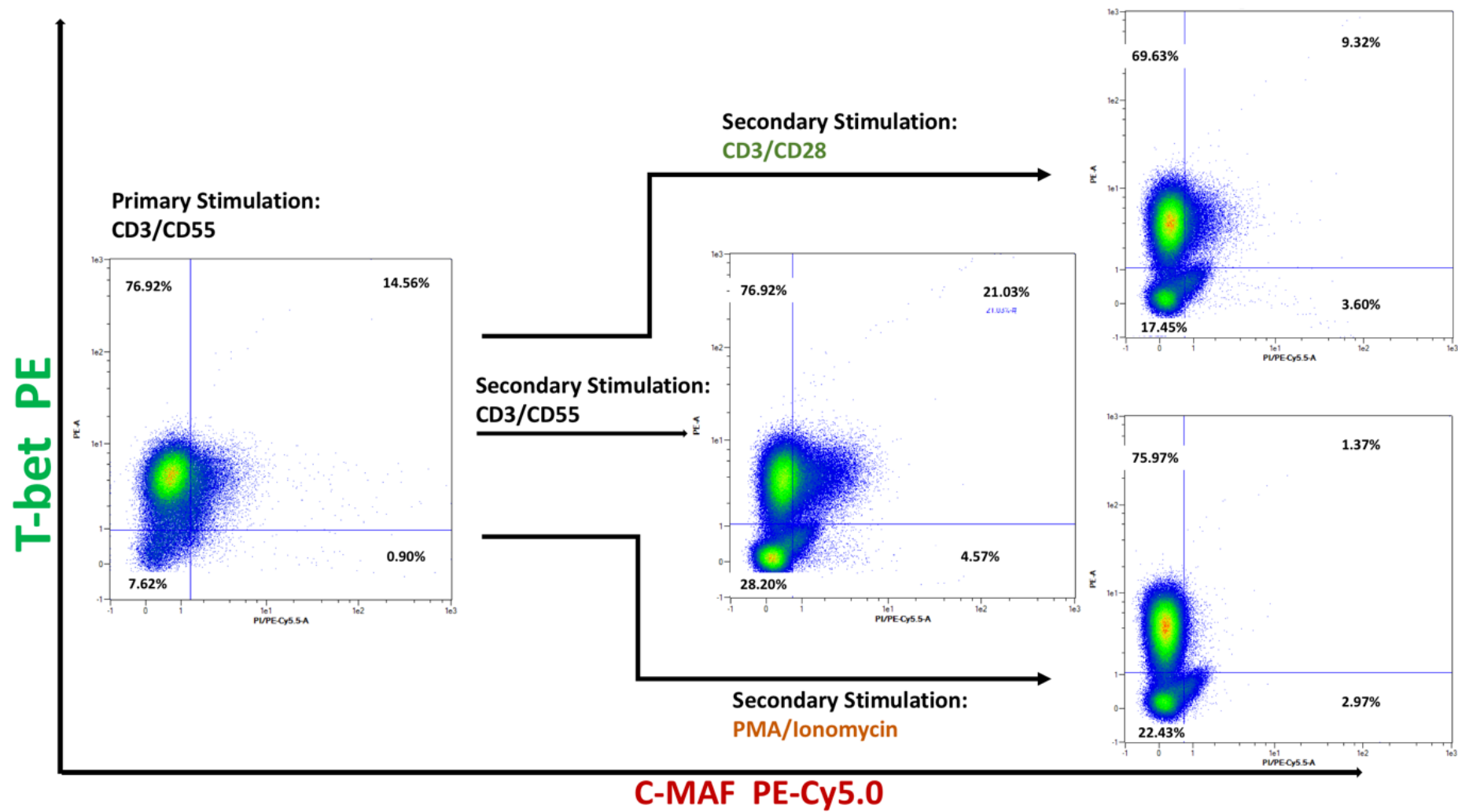
Figure 6.26: Evaluation of c-MAF and T-bet expression by CD3/CD55 primed cells upon secondary restimulation with various stimuli. Naïve CD4 T-cells were stimulated with CD3/CD55 and the expression of T-bet and c-MAF was determined by intracellular staining after 72 hours of cell stimulation. Then cells were rested for 7 days before they were restimulated with CD3/CD55, CD3/CD28 and PMA/Ionomycin for 36 hours before the expression of T-bet and c-MAF were evaluated. Data is representative of three independent experiments.

6.2.3 The evaluation of cytotoxicity potential of CD3/CD55 induced IL-10+ Tr1 cells

6.2.3.1 Granzyme-B

IL-10$^+$ Tr1 cells have been reported to have cytotoxic properties and it has been demonstrated that they confer immune-suppression by killing myeloid cells in a Granzyme-B and perforin dependent manner [332, 735]. IL-10$^+$ Tr1 cells generated by using different methods demonstrated upregulation of granzyme-B. For instance, IL-10$^+$ cells resulting from stimulating naïve CD4$^+$ T-cells with CD3/CD46 had higher expression of granzyme-B compared to the cells stimulated with CD3/CD28 and the CD3/CD46 stimulated CD4 T-cells demonstrated higher killing potential [333, 736]. Engineered IL-10$^+$ Tr1 cells generated by lentiviral vector (LV)-mediated human IL-10 gene transfer were capable of lysing myeloid target cells by granzyme-B in a HLA-class I-dependent but antigen-independent manner [735]. It was also reported that primary Tr1 cells from metastatic melanoma patients demonstrated anti-tumour effect by eliminating tumour promoting macrophages [334]. In contrast, Tr1 cells from gastric cancer patients, with Helicobacter pylori (H. pylori) infection, were impaired compared to heathy individual as they produced less IL-10 and secreted lower amount of granzyme-B and perforin [737]. In this study, we investigated the cytotoxic potential of CD3/CD55 induced IL-10$^+$ Tr1 cells in presence or absence of dexamethasone and compared to the CD3/CD28 stimulated Th1 cells. CD3/CD55 stimulated IL-10$^+$ Tr1 cells were evaluated for granzyme-B expression after 72 hours of cell stimulation. More than 90% of CD3/CD55 induced IL-10$^+$ Tr1 cells expressed granzyme-B whereas more

than 80% cells expressed granzyme-B when cells were stimulated with CD3/CD55 in presence of Dex (figure 6.26). No significant difference in the granzyme-b expression by IL-10$^+$ Tr1 cells was observed between CD3/CD55 and CD3/CD55+dex stimulated cells (data not shown here). Cells stimulated with CD3/CD28 expressed significantly lower amount of IL-10$^+$ cells and only ~60% of those cells expressed granzyme-B. Treatment with Dexamethasone along with CD3/CD28 stimulation did not alter the expression of granzyme-B in IL-10$^+$ cells. Both CD3/CD55 (77.80%) and CD3/CD28 (64.49%) stimulation induced granzyme-B even in the IL-10$^-$ cells population (figure 6.27). The presence of Dex reduced the expression of granzyme-B in the IL-10$^-$ cells following both CD3/CD55 (77.80% vs 56.92%) and CD3/CD28 s (64.49% vs 36.46%) stimulation but it was nor significantly different compared to the cells which were not treated with Dex (n=4, data not shown).
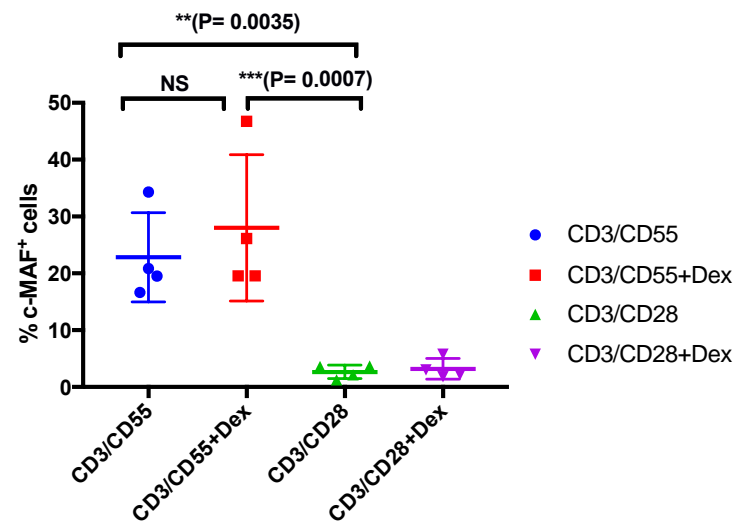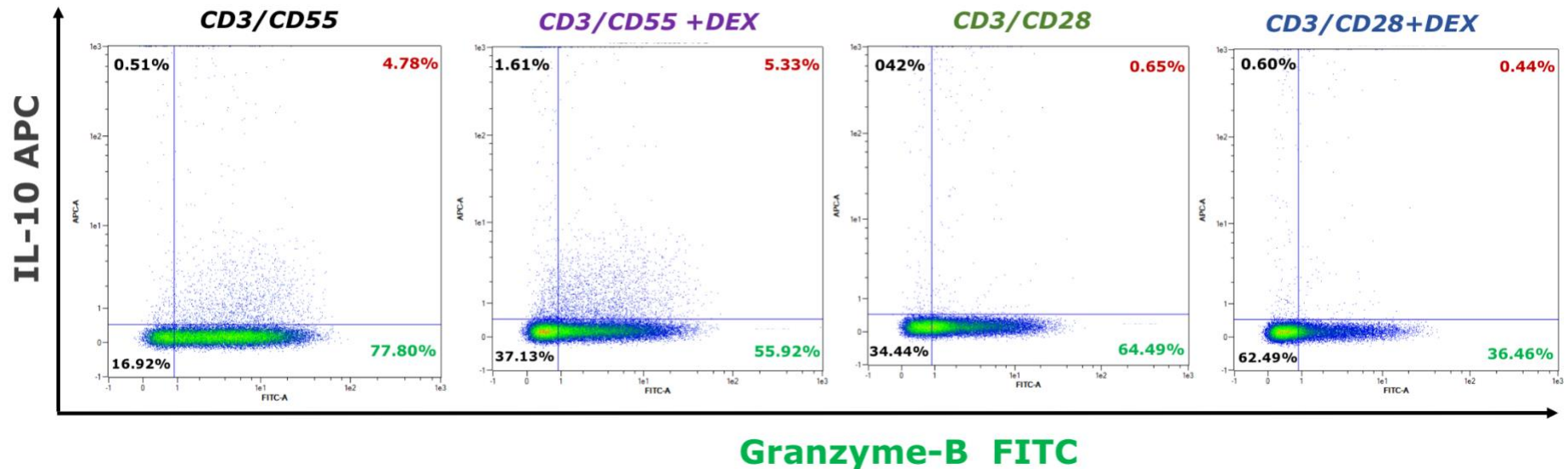
Figure 6.27: Evaluation of granzyme-B expression by CD3/CD55 and CD3/CD28 stimulated cells in presence or absence of dexamethasone. Naïve CD4 T-cells were stimulated with either CD3/CD55 or CD3/CD28, in presence or absence of Dexamethasone (5X10^{-8} M). After 72 hours of cell stimulation, IL-10 CSA was performed along with intracellular staining for Granzyme-B (FITC) by the IL-10$^+$ Tr1 cells. Data is representative of three independent experiment.

6.2.3.2 Perforin

The cytotoxic activity of Tr1 cells, mediated by secretion of granzyme-B and perforin, has been described in several studies (mentioned in 6.2.3.1). As the CD3/CD55 induced IL-10$^+$ cells expressed granzyme-B, we investigated the expression of perforin by these cells. After 72 hours of cell stimulation with CD3/CD55 and CD3/CD55+ Dex, the expression of perforin was determined by intracellular staining. More than 90% of the IL-10$^+$ Tr1 cells did not express perforin following CD3/CD55 stimulation (Figure 6.28). The presence of Dex did not change the expression of perforin in CD3/CD55+ Dex activated cells. Similarly, the most of the IL-10$^+$ cells did not express perforin in response to CD3/CD28 and CD3/CD28+Dex stimulation. CD3/CD55 stimulation resulted in lower expression of perforin even in the IL-10$^-$ cells than CD3/CD28 stimulated cells (6.95% and 25.01% respectively). Treatment with Dex reduced the expression of perforin in IL-10$^-$ cells following CD3/CD28 stimulation but is did not alter percentage of IL-10$^+$ cells and the expression of perforin by these cells.
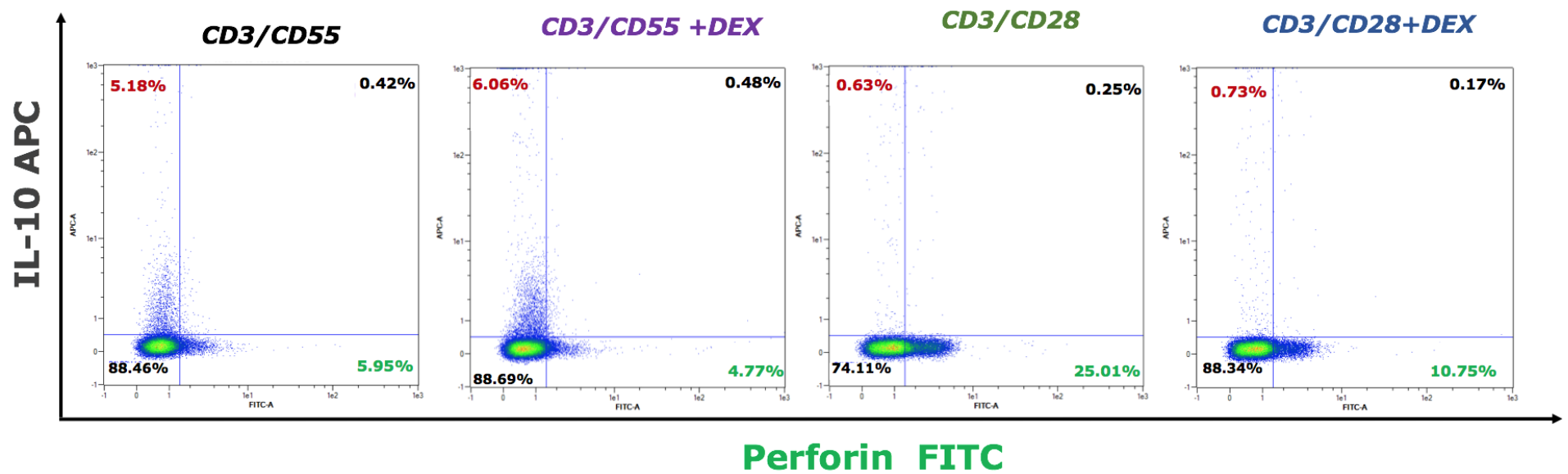
Figure 6.28: Evaluation of perforin expression by CD3/CD55 and CD3/CD28 stimulated cells in presence or absence of dexamethasone. Naïve CD4 T-cells were stimulated with either CD3/CD55 or CD3/CD28, in presence or absence of Dexamethasone ($5X10^{-8}$ M). After 72 hours of cell stimulation, IL-10 CSA was performed along with intracellular staining for perforin by the IL-10$^+$ Tr1 cells. Data is representative of three independent experiment.

6.3 Discussion:

The expression of various cell surface molecules and transcription factors by distinct immune cell populations help to elucidate their activation state as well as their potential role in immune system. The ambiguity regarding the phenotypic profile of Tr1 cells reflects the complexity of identifying this rare cell population. It also highlights the context dependent role of these cells, reported in different studies, which varies depending on the selected experimental settings from a wide range of methods for generating Tr1 cells [729]. In this study, we assessed the phenotype of IL-10$^+$ Tr1 cells differentiated from naïve CD4 T-cells in response to CD3/CD55 costimulation *in vitro*. The CD3/CD55 induced Tr1 cells expressed multiple Tr1-associated markers including LAG-3, CD49b, CD226, TIM-3, CTLA-4 and PD-1 in different proportion. While none of these markers were exclusive expressed by IL-10$^+$ Tr1 cells and were also detected in the IL-10$^-$ cells, the level of certain markers (LAG-3, TIM-3, CTLA-4 and PD-1) were significantly higher in IL-10$^+$ cells compared to IL-10$^-$ cells. However, it still remains unclear how these molecules modulate the function of Tr1 cells and contribute to the regulation of immune homeostasis. Studies on nTreg cells indicate that expression of these inhibitory receptors (IRs) can contribute to the immunosuppressive function and mediate the expansion of the regulatory T-cells [685]. It would be intriguing to determine if the phenomenon of "regulating the regulators" is true for Tr1 cells and Tr1-associated markers where the expression of IRs modulate the induction and function of CD3/CD55 induced IL-10$^+$ Tr1 cells. Nonetheless, it should be noted that the expression of the Tr1-markers was evaluated at only one time

point (after 72 hours of cells stimulation) and the expression of these molecules could change over time. In a recent study, it was demonstrated that the expression of PD-1 was significantly higher in IL-10^+ Tr1 cells following a short stimulation but no significant difference in PD-1 expression between IL-10^+ and IL-10^- cells was determined following a secondary restimulation [613]. So further research is required to determine if the expression as well as the level of expression of Tr1-associated markers on CD3/CD55 induced Tr1 cells are consistent over a long period of time and upon restimulation. It should also be investigated if expression of important check point molecule such as PD-1 facilitates immune-suppression by promoting the function of Tr1 cells or if it negatively the regulates Tr1 cells to restrict the uncontrolled expansion of these cells in order to maintain immune homeostasis. The potential role of PD1 and other IRs on the function of Tr1 cells could have critical implication for immunotherapies targeting these molecules. Moreover, in our study, it was determined that the presence of immune-modulators did not significantly alter the expression of Tr1-associated markers by CD3/CD55 induced Tr1 cells except for CD226 and TIM-3. The expression of CD226 was upregulated in both IL-10^+ and IL-10^- cells in the presence of VitaminD3. The higher expression of CD226 following CD3/CD55+VitaminD3 stimulation could potentially be attributed to the VDR binding site in the *CD226* gene loci which could have promoted the CD226 expression [637].

Table 6.1: The expression of various Tr1-associated cell surface markers by CD3/CD55 induced IL-10$^+$ Tr1 cells and IL-10$^-$ cells (Summarized data from section 6.2.1). [+ , ++ and – denote moderate expression, higher expression and no expression respectively]

Cell surface marker	Expression by IL-10$^+$ Tr1 cells	Expression by IL-10$^-$ cells
CD226	++	++
LAG-3	++	+
CD49b	++	+
TIM-3	++	+
LAP	-	-
CTLA-4	++	+
PD-1	++	+

The transcription factor expression by different T-helper cell populations following differentiation from naïve CD4$^+$ T-cells by co-stimulatory signal and various cytokine has been studied extensively and provided us with an insight about the key transcription factors, termed as "master regulator", which determine the functional characteristics of the cells. In our study, it was determined that CD3/CD55 induced IL-10$^+$ cells were as well as CD3/CD28 stimulated cells express multiple transcription factors (Table 6.2).

Table 6.2: The expression of the transcription factor by cells after 72 hours of stimulation with CD3/CD55 and CD3/CD28 (Summarized data from section 6.2.2). [+ and – denote moderate expression, and no expression respectively]

Transcription Factor	Expression by CD3/CD55 stimulated cells	Expression by CD3/CD28 stimulated cells
FoxP3	-	-
T-bet	+	+
GATA-3	-	-
ROR-γ_c	+	+
HELIOS	-	-
c-MAF	+	-

The fate of Naïve CD4 T-cells had been restricted to the symmetric model of differentiation to either Th1 or Th2 T-cells until last decade. With the discovery of Th17 [738-741], Th9 [216, 742] and inducible Treg (iTreg) as well as the reported plasticity of nTreg resulting in IL-17 [740, 743] , IFN-γ [297] production and T-bet expression [744], the adaptive nature or plasticity of T-cells has been brought to attention. Global mapping of epigenetic modification revealed co-localization of H3K4me3 and H3K27me3 on lineage specific "master regulator" transcription factors such as *Tbx21*(T-bet) and *Gata3* genes in Naïve, Th1, Th2,Th17, iTreg and nTreg [297]. It indicated bivalent domains which has been associated with transcription factors poised for expression while keeping their expression restrained in pluripotent embryonic stem cells [745,

746]. The bivalent domain indicates the dynamic co-expression of multiple master-regulator transcription factor and the potential alternate fate of linage specific T-helper cells. It also emphasises on the potential impact of transcription factors on T-cell heterogeneity under the influence of different immunological cues [747]. Indeed, it has been demonstrated that in Th1 environment induced by the *T. gondii* infection resulted in T-bet expression and IFN-γ production by nTreg in mice [748]. Similarly, it has also been demonstrated that IFN-γ producing cells with Th1-like phenotype cells derived from cells with Th17 origin under the influence auto-immune disease condition EAE in mice [749] and IL-17$^+$IFN-γ^+ cells were found to be elevated in the blood of patients with chronic inflammatory disorders [750]. Whether these observations indicate only cellular plasticity or also represent population plasticity is not well understood yet [751]. In line with these observations, it was also reported that the cellular phenotype regulated by a specific transcription factor can be further enhance or suppressed by the expression of other transcription factors. For instance, follicular helper T-cell differentiation is dependent on the expression Bcl6 but the interplay among other transcription factor such as c-MAF, BATF, IRF-4 positively or negatively influence this process [752]. Similarly, it could be possible that interplay of c-MAF, T-bet and ROR-γ_t determines the function of CD3/CD55 induced IL-10$^+$ Tr1 cells. Considering the role of c-MAF in the regulation of IL-10 in various T-cell subsets (Th1, TH2, Th17 etc.), it could also be possible that c-MAF drives the differentiation of Tr1 cells upon CD3/CD55 stimulation, but its function is modulated by other transcription factors. The higher expression of c-MAF by IL-10$^+$ cells helped us to establish a

link between CD3/CD55 signaling and the transcriptional control of IL-10 production by the Tr1 cells for the first time. However, the underlying molecular pathway which induces c-MAF expression following CD3/CD55 costimulation still requires to be elucidated. Also, further studies are needed to determine the level of plasticity in CD3/CD55 induced Tr1 cells.

The low frequency of Tr1 cells has been a major limitation for its clinical application for the treatment of auto-immune diseases. As the presence of immune-modulators enhance the induction of CD3/CD55 mediated IL-10^+ Tr1 cells without altering most of the phenotypic characteristics, its potential to be utilized for immunotherapy by adaptive Tr1 cell transfer should be explored in future.

7 Chapter 7: Discussion and future work

The fate of naïve T-cell differentiation is partially affected by the costimulatory signal influencing TCR signaling and the effector function is reflective of all the signaling cascades incited by specific costimulatory signal at the time of T-cell priming. In this study, we have demonstrated that costimulatory receptor (CD55) mediates induction of IL-10$^+$ Tr1 cells which depends on the competitive signaling strength of CD55 and CD28. We have also demonstrated that signaling via CD55 results in specific phenotypic characteristics which are retained upon restimulation. Importantly, we are reporting for the first time that immune-modulators dexamethasone and Vitamin D3 have differential effect on CD3/CD55 induced Tr1 cells in comparison to CD28 stimulated cells, specifically in their ability to secret IL-10 and IFN-γ. The identification of enhanced IL-10 production by CD3/CD55 induced Tr1 cells could be one of the potential mechanisms of action of these immuno-suppressive therapies used for the treatment of several auto-immune diseases including MS.

The differential effect of costimulation on naïve cells, in terms of generating different ratio of IFN-γ and IL-10 producing cells and notable predominance of IL-10$^+$ cell and IFN-γ^+ cells in response to CD55 and CD28 respectively, raises the question about how these signals result in different phenotypes. Assessment of our data in context of the findings and observations reported by others provide us with several potential explanations which could help us to understand the underlying mechanisms leading to alternate fate.

The heterogeneity of the naïve T-cell population could contribute to the differential response to CD55 and CD28 costimulation. Recent studies suggest that naïve CD4 T-cells population is not as developmentally synchronized as it was previously assumed to be and it consists of multiple populations which could be identified depending on expression of cell surface molecules such as CD31, PTK7 etc. [34]. The Recent Thymic Emigrant (RTE) naïve CD4^{+} T-cells, representing cells recently egressed from thymus, differ in function from the older non-RTE naïve cells (mature naïve cells) which have matured in secondary lymphoid organs [753]. While RTE naïve cells are more responsive to IL-7 and contain more TCR excision circles (TREC), they are produce less IL-2, IFN-γ and proliferate less in response to CD3/CD28 stimulation compared to mature naïve cells [754, 755]. Though the effect of CD3/CD55 stimulation on RTE and mature naïve T-cells are still unknown, variation in response to CD3/CD28 stimulation within the naïve T-cells compartment indicates that the diversity in naïve T-cell population could also contribute to differential response to costimulation.

It has been reported that CD28 and CD55 are localized in two different kind of microdomains that can result in different functional outcomes [756]. CD28 is located in ganglioside GM1 enriched microdomain and several other studies confirmed it accumulates with TCR in the central supramolecular activation complex (cSMAC) where it initiates and stabilizes the immune synapse formation [472, 756, 757]. Interestingly, recruitment of other molecules in the microdomain such as Fas led to alternative outcome – recruitment of Fas with CD28 led to apoptosis but

recruitment with CD55 inhibited cell death [756]. As membrane compartmentation is required for efficient T-cell activity [758], these observations indicate that different pathways could be activated by CD55 and CD28 depending on the type of domain they are localized in. Also, CD28 -mediated redistribution of microdomains at the TCR site has been linked to amplified / sustained TCR signaling and exclusion of other regulatory components of the synapse, such as CD45 [759, 760]. This might provide an explanation to the dominance of CD28 signaling over that of CD55 . It is still not known if, like CD45, CD55 is excluded from the cSMAC during activation or where it localizes in the synapse over the time course of T-cell activation. Similarly, it is not clear if the synapse makeup of Tr1 cells and Th1 cells are comparable.

Another salient potential explanation for the differential induction of Th1 and Tr1 cells is based on the observations reported on the thymic selection and development of nTreg which indicates the possible role of TCR specificity in the induction of Tr1 cells. It has been suggested that the development of nTreg in the thymus depends on the TCR self-reactivity which requires to be below the threshold for negative selection to prevent deletion but above the threshold for positive selection to ensure cell survival. It has also been demonstrated that the low affinity of TCR for its cognate antigen did not support the differentiation of nTreg in the thymus and it has been suggested that TCR avidity might not always compensate for TCR affinity in order to generate appropriate TCR interaction for the induction of nTreg in the thymus. Importantly, studies with TCR transgenic mice has demonstrated that intraclonal competition

between nTreg and conventional T-cells for the limited resources (such as thymic APC, antigen) affect the development these cells [259]. Unlike conventional T-cells, maintenance of nTreg in the periphery depends on the presence of self-antigen and interaction with the cognate antigen resulted in activation as well as prolonged persistence in the tissue even when the antigen expression became undetectable [761, 762]. Interestingly, it is not only nTreg which has been reported to be self-reactive but both naïve and memory responses to self-antigen (e.g. citrunillated peptide, myelin basic protein) has also been observed in both heathy donors and autoimmune disease patients [763, 764]. The existence of self-reactive nTreg as well other conventional T-cells which react to self-peptides in heathy individuals raises the possibility that some self-reactive conventional T-cells might escape negative selection in thymus and serve as precursor for generation of Tr1 cells via alternative costimulatory pathways in the peripheral immune system to control immune response to self-peptide while cells specific for foreign antigens respond to costimulation by obtaining pro-inflammatory Th1 phenotype. In our study, the induction of Tr1 cells following co-culture of CD4 T-cells with dendritic cells without any exogenous foreign peptide indicates that Tr1 cells could be generated even when only self-peptide is presented by antigen presenting cells. Further study is required to determine the TCR specificity of Tr1 cells induced by CD55-CD97 interaction.

For natural Treg only basal CD28 is required for Treg generation in thymus and higher CD28 signalling, via its ligand (CD86), restricts induction of Treg in peripheral immune system. This CD28 mediated Lck-

signaling suppresses induction of inducible Treg (CD4$^+$CD25$^+$FoxP3$^+$)
from naïve T-cells (CD4$^+$CD25$^-$) following TCR activation in an IL-2
independent manner [765]. In a similar way CD28 signaling may prevent
induction Tr1 cells in favor of the Th1 phenotype. In contrast, no limiting
effect of CD55 signaling has been reported on the induction of either Th1
or Tr1 cells. It has been demonstrated that cross-linking of CD55 and
TCR leads to TCR-zeta and ZAP-70 tyrosine phosphorylation and IL-2
secretion [518]. However, the downstream signaling leading to induction
of IL-10$^+$ Tr1 by CD55 is not well understood and warrants further
investigation, as gaining control over Tr1 cells would be beneficial in both
autoimmunity and cancer.

Lastly, TCR ligand density, affinity and duration of TCR signaling also
have been reported to be involved in the induction of regulatory T-cells
[766]. It has been demonstrated that disruption of TCR : pMHC complex
or short interaction between T-cells and dendritic cells favors induction of
regulatory function while stable interaction between T-cell : DC promote
effector function [525]. The impact of CD28 costimulation on stabilizing
and prolonging TCR signaling could help to promote Th1 induction and if
the impact of CD55 costimulation is not similar to CD28, it could
potentially assist in the induction of regulatory Tr1 phenotype.

Tr1 cells were initially discovered in Human SCID patients and they were
defined as the induced immunosuppressive cells that maintain peripheral
tolerance by producing IL-10. However, unlike nTreg cells which can be
identified by FoxP3 and CD25 expression, Tr1 could not be determined

any specific transcription factor or markers which has been consistently expressed by these cells. The definition of Tr1 cells has evolved in the last decade with more studies focusing on generation and characterization of these cells. Many Tr1-associated markers were combined with the functionality to determine these cells in mouse models as well as human. Nevertheless, due to the disparities among the methods of generating IL-10$^+$ Tr1 cells, it has been difficult to deduce if the same cell population has been studied or if multiple different populations were described which were similar in function. While some studies reported the differentiation of Tr1 from naïve cells by co-stimulatory signals (CD55, CD2 etc.), others reported the differentiation under the influence of cytokine (IL-10, IL-27, combination of IL-10 and IL-4) and recurrent exposure to antigens. Moreover, IL-10 production by Th1 cells raises the question if Tr1 cells represent the state of Th1 cells during immune-resolution [339]. CD46 and IL-2 mediated "Switch from IFN-γ^+ Th1 to IL-10$^+$ Tr1" has also been proposed as a way of induction for Tr1 cells [324, 767]. This hypothesis supports the definition of Tr1 as a phenotype and does not recognize these cells to be bona fide regulatory cells with specific lineage. However, in our current work as well as previous studies [315, 386], we have demonstrated the differentiation of naïve T-cells into IL-10$^+$ cells in response to CD55 costimulation by meeting differentiation criteria such as proliferation, IL-2 production etc., which contradicts the notion that Tr1 cells derive from Th1 cells and it supports the possibility that costimulatory signal induced IL-10$^+$ represent a novel subset of inducible regulatory T-cells. To further complicate the quest for the definition of Tr1 cells, our findings

demonstrated that the presence of immunomodulators enhance the induction of IL-10$^+$ cells following CD3/CD55 which raised the question including- i) if immune-modulators amplify CD55 mediated signals to promote Tr1 cells, ii) if they regulate other IL-10 modulating pathways which synergizes with CD55 signaling. As the answer to these questions are still unknown, we addressed the CD55 induced IL-10$^+$ cells as "Tr1" cells in this study.

The expression of multiple transcription factors by CD3/CD55 induced IL-10$^+$ Tr1 cells which have been previously ascribed to determine different phenotypes as "master regulators" contest the idea of Tr1 cells belonging to a specific lineage rather than being a manifested phenotype of exhausted, immune-resolution phase Th1 cells. However, there are examples of multiple transcription factor expression by cells with specific lineage. For example, follicular helper T-cell differentiation is dependent on the expression Bcl6 but the interplay among other transcription factor such as c-MAF, BATF, IRF-4 positively or negatively influence this process [752]. Similarly, it has also been demonstrated that Aryl hydro carbon receptor (AhR) mediates the differentiation of Th22 cells which further promoted by the expression of T-bet. Interestingly, the expression of transcription factors with opposing role is also well established as a way of regulating generation of cells with specific lineage and to prevent the exacerbated function of one subset of cells during immune response. For instance, it has been reported that T-bet expression is not required for induction and differentiation of IL-23 driven Th17 cells but it was necessary to restrain the function of Th17 in intestinal inflammation and

modify the colitogenic response [768]. Considering the role of c-MAF is the induction of IL-10^{+} cells and reported plasticity in lineage fate of different subsets of CD4 T-cells population [297], it could be possible that the expression of T-bet and ROR-γt modifies the responses of these regulatory cells. Indeed, it was determined that T-bet was involved in the induction of tolerance upon repeated antigen stimulation where it was not associated with Th1 differentiation, rather promoted transcription program of regulatory cell in collaboration Erg-2 and reduced IL-2 production [769]. In line with these observations, the proposed "tide model" of cell surface signaling molecules suggests that diverse expression of co-signaling molecules- both costimulatory and coinhibitory, allows differential and dynamic control of T-cell responses in accordance to immunological cues [770]. This revised T-cell signaling and activation model suggests the primary signal through the TCR recognition and costimulation serves as the "initiator" but the other cell surface molecules, which are upregulated in response to the initiator and might be expressed in time-dependent manner, determine the direction and magnitude of the cell responses by acting as "modifier" and preferentially participating in one or more functional aspects of immune cell function. Although no exclusive cell surface molecule has been identified which could potentially govern the function of Tr1 cell, the expression of a myriad of cell surface molecules by IL-10^{+} Tr1 cells (such as LAG-3, CD226, CTLA-4, PD-1 etc.) might contribute to modifying and regulating the function of these cells. Following our work in CD3/CD55 induced IL-10^{+} Tr1 cells, we propose several suggestions for future studies which might help to reduce the ambiguity regarding this regulatory cell

population. Careful contemplation of experimental data from our studies as well as reports from other studies, it could be extrapolated that Tr1 cells should be defined based on both functional aspects as well as phenotype. We suggest that Tr1 cells are not only defined by combined expression of various marker, they should also be described in terms of the level of expression. For example, nTreg cells are defined as FoxP3$^+$CD25^{High}CD127^{Low}. In a similar way, based on the data from this study, Tr1 cells could be defined as IL-10$^+$IFN-γ^-LAG-3^{High}PD-1^{High}c-MAF^{High}. However, further investigations are required to discern which markers will be appropriate to define Tr1 cells generated by various methods.

In our study, significant difference was noted in the CD3/CD55 induced IL-10$^+$ cells between Multiple sclerosis patients and healthy individual. It is still unclear if the reduced induction of Tr1 cells stems from any defect in the CD55 mediated IL-10 pathway including genetic mutation and alteration of molecular signaling. As the CD55 regulated IL-10 pathway is still not well characterized, it is difficult to understand the underlying mechanisms which suppress the induction Tr1 cells in MS. Moreover, it has also been reported that the expression of CD55 is diminished in autoimmune disease patients (e.g. Systemic Lupus Erythematosus, SLE) compared to heathy individuals [771]. The expression of CD55 by naïve CD4 T-cells was not assessed in this study and it would be interesting to determine if there is any variation in expression of CD55 between MS patients and healthy donors.

One of the obstacles in the way of studying the role of CD3/CD55 induced Tr1 cells *in vivo* is that mouse models could not be used as CD55 costimulation does not result in Tr1 induction in mice (unpublished data). It could probably reflect evolutionary difference between human and mice. Several other studies with CD55$^{-/-}$ knock-out mice demonstrated that CD55 might suppress T-cell immunity and play in a critical role in regulation of autoimmune responses [771-774]. However, most of these studies attributed the effect of CD55 deletion on immune response to its complement regulatory function and did not investigate the role of CD55 as a costimulatory molecule in those experimental settings. For instance, it was reported that the deletion of DAF (CD55) in resulted in exacerbated auto-immune disease development in MRL/lpr mice - a model for human systemic lupus erythematosus [775]. It was also demonstrated in the EAE model of CD55$^{-/-}$ knock-out mice , the absence of CD55 (DAF) leads to enhanced IFN-γ production which is accompanied with reduced IL-10 secretion compared to the CD55$^{+/+}$ wildtype mice [772]. In the same study, it was also determined that the effect of CD55 deletion could be reversed in double knock-out CD55$^{-/-}$ C3$^{-/-}$ mice with the exception of IL-10 production which remained low in the absence of CD55 and despite the absence of C3, which would suggest that effect of CD55 in immune response is not restricted to only its function as a complement regulator protein. Considering the information provided by previous studies and difficulties associated with studying the role of CD55 in animal model, in order to further explore the role of CD55 costimulation, we would recommend studying CD3/CD55 mediated induction of Tr1 cells in Clinically isolated syndrome (CIS) patients and

early stage Multiple sclerosis patients who have not received any disease modifying treatment (DMTs). As it has been noted that CIS patients often develop MS, it would be intriguing to investigate CD3/CD55 induced Tr1 cells through different stages of disease progression. Also, the role of CD55 mediated IL-10 production should be studied in cancer patients in future as it has been reported that Tr1 cells are more prevalent in the tumour infiltrating lymphocytes [569, 776]. It would be interesting to determine if CD55 contributes to Tr1 induction in cancer patients and if it could be targeted for immunotherapy in order to control immune-suppression in the tumour microenvironment to enhance the efficacy of the other treatments.

8 References:

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