GENERATION OF HEMATOPOIETIC STEM CELL DERIVED TOLEROGENIC DCs WITH VITD3

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ABSTRACT

GENERATION OF HEMATOPOIETIC STEM CELL DERIVED TOLEROGENIC DCs WITH VITD3

Dendritic cells (DCs) are main activator and regulator cells in immune response due to their bridging function between the innate and adaptive immune system. Upon ingestion of antigens from peripheral tissues in response the triggering of an immune response, DCs migrate to lymph node to activate antigen specific T cells. It is a well-known fact that DCs are the most important antigen presenting cells for the T cell stimulation, a phenomenon that allows them to be utilised as therapeutic agents against cancer and chronic infections. Besides that DCs have been used for immune suppression against autoimmune diseases, including multiple sclerosis (MS), atherosclerosis, psoriasis etc. DCs are considered as a heterogeneous cell population mainly due to their cell surface receptors such as classical DCs (cDCs), plasmacytoid DCs (pDCs) and monocyte derived DCs (moDCs). Each DC has different properties under inflammation conditions depending on their surface receptors and cytokine secretions. moDCs have been used as a therapeutic vaccine to suppress autoimmune reactions after treatment with immune suppressive molecules such as, corticosteroids, cyclosporine (CsA), prostaglandin E2, VitD3, rapamycin etc. Other DCs especially cDCs may be used as antigen specific immune suppressive DC vaccine based on their T cell interaction and stimulation capacity which is known to be better than that for moDCs.

In this thesis, tolerogenic DCs (tol-DC) were differentiated from hematopoietic stem cells using stimulatory agents such as Flt-3L, GM-CSF, SCF, IL-4, and VitD3. Then tol-DCs were co-cultured with T lymphocytes to examine their tolerogenic properties on cell mediated immune system. To our knowledge this study is one of the first examples to demonstrate VitD3 induced tol-DCs generation from HSC while negatively affecting DC generation. Although, tol-DCs have decreased HLA-DR expression which means that the presentation capacity of DCs are also decreased, they present an increase CD86 expression that may causes inhibition of T lymphocytes based on the interaction between CTLA-4 rather than CD28. Strikingly, their T cell stimulation capacity and induction of anti-tumor response are lower than control DCs. Overall findings of this study suggest that HSC-derived tol-DCs may be an alternative way to prepare tol-DC vaccines.

ÖZET

VITD3 ILE HEMATOPOETİK KÖK HÜCRE KAYNAKLI TOLEROJENİK DC ÜRETİMİ

Dendritik hücreler (DC'ler), doğuştan gelen ve sonradan kazanılmış bağışıklık sistemi arasında oluşturduğu köprü nedeniyle bağışıklık tepkisinde ana aktivatör ve düzenleyici hücreler olarak görev alır. . DC'ler, periferik dokulardan antijenleri aldıktan sonra antijene özgü T hücresini aktive etmek için lenf düğümüne göç eder. DC'ler, T hücre stimülasyonu için en önemli antijen sunan hücrelerdir. Ve bu özellik DC'leri kansere ve kronik enfeksiyonlara karşı terapötik bir ajan yapar. Bunun yanı sıra DC'ler, multipl skleroz (MS), ateroskleroz, sedef hastalığı vb. dahil olmak üzere otoimmün hastalıklara karşı bağışıklık sistemini bastırmak için kullanılmıştır. DC'ler, yüzey reseptörleri ve ontogeni açısından heterojen bir popülasyondur ve klasik DC'ler (cDC'ler), plazmasitoid DC'ler (pDC'ler), monosit türevli DC'ler (moDC'ler) olarak sınıflandırılırlar. Her bir DC alt tipi, enflamasyon koşulları altında farklı özelliklere sahiptir. MoDC'ler, immün baskılayıcı moleküller (TGF-β, IL-10, kortikosteroidler, siklosporin (CsA), mofetil, prostaglandin E2, VitD3, rapamisin vb.) ile tedaviden sonra otoimmün reaksiyonlarını baskılamak için terapötik bir aşı olarak kullanılmıştır. Diğer DC'ler, özellikle cDC'ler, T hücre etkileşimleri ve stimülasyon kapasiteleri moDC'lerden daha iyi olduğu için antijene özgü immün baskılayıcı DC aşısı olarak kullanılabilir.

Bu tezde, tolerojenik DC'ler (Tol-DC) hematopoietik kök hücrelerden Flt-3L, GM-CSF, SCF, IL-4 ve VitD3 varlığı ile farklılaştırıldı. Daha sonra Tol-DC'lerin T lenfositleri üzerindeki tolerojenik özelliklerini incelemek amacıyla T lenfositleri ile birlikte kültürlendi. VitD3'ün HSC'den Tol-DC oluşumunu indüklediğini ancak DC oluşumunu da olumsuz etkilediğini bulduk.

Aynı zamanda Tol-DC'ler, HLA-DR ifadesini azalttığı ve CD86 ifadesini arttırdığını gördük, eğer CD86 CD28'e bağlanmak yerine CTLA-4 ile bağlantı kuruyorsa bu durum T hücre uyarımının engellenmesine neden olmuş olabilir. VitD3'lü grupta T hücresi uyarma kapasiteleri ve anti-tümör tepkisinin indüklenmesi, kontrol DC'lerinden daha düşüktü. Genel olarak verilerimiz, HSC'den türetilen Tol-DC'lerin Tol-DC aşısının hazırlanması için alternatif bir seçenek olabileceğini göstermektedir.

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1,25 α OH_2D_3	Active form of VitD3, calcitriol
25 (OH) D_3	25-hydroxyvitamin D3, calcidiol
APC	Antigen presenting cells
BATF3	Basic leucine zipper transcriptional factor ATF –like transcription factor 3
Bcl- xl	B cell lymphoma- extra large
CADM1	Cell adhesion molecular 1
CCR5	C-C chemokine receptor 5
CCR7	C-C motif chemokine receptor 7
cDCs	Classical DCs
CDCs	Conventional DCs
CDP	Common DC precursors
CLEC9a	C- type lectin domain containing 9A
CLP	Common lymphoid progenitors
CMP	Common myeloid progenitors
CSF	Colony stimulating factors
CTLA- 4	Cytotoxic T- lymphocyte associated protein 4
CTLA-4	Cytotoxic lymphocytic antigen 4
CXCR4	CXC motif chemokine receptor 4
CYP24	1, 24, 25 VitD form by 24- hydroxylase
CYP27B1	25- hydroxyvitamin D-1α hydroxylase
DAMPs	Damage associated molecular patterns
DC	Dendritic cell
DEC- 205	Dendritic cell receptor for ecndocytosis
FasL	Fas ligand
Flt-3L	Fms like tyrosine kinase 3
FOXP3	Forkhead box P3
GM- CSF	Granulocyte- macrophage colony- stimulating factor
ICAM-1	Intercellular adhesion molecule-1
IDECs	Inflammatory epidermal dendritic cells”
IFNα/β	Interferon α/β

IL-13	Interleukin 13
IL-4	Interleukin 4
ILC	Innate lymphoid cells
ILT-3	Immunoglobulin like transcript 3
iNOS	Inducible nitric oxide synthase
IRF8	Interferon regulatory factor 8
IS	Immunological synapse
KDC	Killer dendritic cells
LNs	Lymph nodes
LRR	Leucin rich repeats
M1	Classical macrophage
M2	Alternatively activated macrophage
MDPs	Monocyte- dendritic cell progenitors
MHC	Major histocompatibility complex
MoDC	Monocyte derived DC
MP	Myeloid DC precursors
NF_KB	Nuclear factor kappa B
$NF\text{-}_KB$	Nuclear factor kappa B
NLR	Nod like receptor
NOD	Nucleotide binding oligomerization domain
PAMPs	Pathogen associated molecular patterns
pDC	Plasmacytoid DC
PDGFs	Platelet- derived growth factor
PKCθ	Protein kinase c- theta
PMA	Phorbol 12- myristate 13- acetate
pre-DC	Pre-classical DCs
Pro- VitD3	7-dihydrocholesterol
PRR	Pattern recognition receptors
RIG- 1	Retinoic acid inducible gene 1
RXR	Retinoid X receptor
SIRPα	Signal- regulatory protein alpha
TCR	T cell receptor
TGF- β	Transforming growth factor- β

TGF-α	Transforming growth factor- α
Th1	T helper-1
Th17	T helper 17
Th2	T helper 2
TLRs	Toll like receptors
TNF	Tumor necrosis factor
TPO	Thrombopoietin
TRAIL	TNF- related apoptosis- inducing ligand
Tregs	Regulatory T cells
VDRE	VitD responsive element
WASp	Wiscott aldrich syndrome protein
XCR1	X-C motif chemokine receptor 1

1. INTRODUCTION

Immune system consists of cells, tissues, and molecules that provide a defence mechanism against infection agents including parasites, viruses, bacteria, and other pathogenic agents. In addition to this vital function, it also serves a pivotal role in elimination of tumors and dead cells [1]. On the other hand, the immune response may be a cause of serious inflammatory conditions such as tissue rejection, neurodegenerative disorders, diabetes mellitus cardiovascular, and chronic kidney disease [2].

The immune response can be comprised of two main types which include innate and adaptive immunity. Innate immunity is not antigen specific, has a barrier function to prevent entry of pathogens into the body. If these pathogens pass the epithelial barrier and enter the body, they can be destroyed by the other components of the innate immune system such as natural killer cells, phagocytic cells or plasma proteins [3]. Adaptive immunity is slower, but produces antigen specific responses. The adaptive immunity has two main branches which are known as humoral and cellular immunity. B cells are effector cells of the humoral immunity and they produce antibodies. Meanwhile, T cells are effector cells in the cellular immunity, they are important in elimination of infected cells and overall immune regulation [4–6].

1.1. INNATE IMMUNE SYSTEM

The organisms have a natural defending system which is called innate immunity to protect themselves from infectious agents and have damaged or necrotic cells, which they encounter and have to deal with during their life time. The most important responses of the innate immunity include inflammation and anti-viral defending mechanisms. Inflammation is the response of the immune system to harmful stimuli such as damaged cells, radiation, pathogens, and toxic materials. This effect can annihilate the inflammation inducing agents, and, -thereby- initiate the healing process [7]. Defense mechanism provided by the mammalian innate immunity is dispersed in nearly all tissues that are barrier to external environment (especially skin and mucosal surfaces of the gastrointestinal and respiratory systems). Some kinds of cells such as specialized myeloid cells, epithelial cells, non-hematopoietic cells etc. can initiate a defense mechanism against an infection, genotoxic

stress or tissue damage. Pathogen associated molecular patterns (PAMPs) are defined as specific molecular sequences that are sourced from a diverse set of organisms excluding those found in the host. PAMPs induce production of exogenous signals so that the immune system can be alerted and a cascade of immune responses are initiated [8,9]. In addition to this, other molecules can be secreted due to organ damage or cellular stress, which are recognised as endogenous danger signals and, therefore, called damage associated molecular patterns (DAMPs). They can stimulate the innate immunity during non-infectious inflammation [10,11]. With the help of a specialized collection of receptors, which are called Pattern Recognition Receptors (PRR) [12], products of microbial origin can be recognized [13]. They are located on cell surface or endosomal compartments, [14]. PRRs are classified into four main gene families which are Toll like receptors (TLRs), the retinoic acid inducible gene 1 (RIG- 1) like receptors, C type lectin receptors, and nucleotide binding oligomerization domain (NOD) Leucin rich repeats (LRR)- containing receptors (NLR) [15]. After PPRs bind their ligand, signalling molecules are released from cells to stimulate the innate and adaptive immune system [16,17]

The first groups of cells in the innate immunity involve epithelial cells. The body organs and tissues are covered with an epithelium that serves as the main physical barrier to protect the body and also chemical barrier by producing peptide-based antibiotics against the pathogens such as defensins and cathelicidins [15].

The second group of cells includes phagocytes such as, neutrophils, monocytes, macrophages, dendritic cells (DCs). Although, neutrophils are the most intense phagocytic cell type (4,000-10,000 cell/ml), they are short lived cells, and produce a quick response in blood. Neutrophils (20,000 cell/ml) are differentiated from hematopoietic stem cells with the help of the colony stimulating factors (CSF) [18]. Neutrophils are known as the key cell type in combating bacteria and fungi infections during the inflammatory phase. They can extravasate from the blood to the infected area of the tissue, to engulf and destroy pathogens through phagocytosis [19].

Monocytes are present in the blood at fewer numbers compared to the neutrophils, however they operate similarly. Some types of monocytes can move from blood to extravascular tissues and they begin to differentiate into macrophages or DC upon encountering inflammatory or anti- inflammatory cytokines [20].

Macrophages have important roles on host defence and they secrete cytokines such as platelet- derived growth factor (PDGFs), transforming growth factor- α and β (TGF-α, TGF-β), and insulin- like growth factor (IGF), so that inflammation can be initiated. These cells can engulf and destroy the microorganisms and can clean up the infarcts and, thereby, can provide the initiation of the wound healing process. Macrophages can be differentiated into classical (M1) or alternatively activated macrophages (M2) forms under the control of physiological or pathological external factors.

M1 macrophages are generally stimulated by the transcription factors such as nuclear factor kappa B (NF-κB) and acquire high motility and destruction capacity, promote inflammation. On the other hand, M2 macrophages are generally stimulated by Interleukin 4 (IL-4) or Interleukin 13 (IL-13) cytokines. Both kinds of macrophages can control the inflammation or anti-inflammation and provide the wound healing process. The type of the present macrophage can alter the response of the host [21,22].

In summary, the immune system tries to protect the body from pathogens such as fungi, parasites, bacteria or cancer cells, toxins, and viruses. Innate immunity is the first and non-specific defense mechanism, it can only recognize the common patterns and gives the same responses every time via innate immunity dependent pathways. Thereby, the host can give a response immediately, which is not as specific or stronger as in the case of adaptive immune system. Innate immune cells do not differentiate to memory cells, while adaptive immunity operates via an antigen dependent defense mechanism, in the sense that specific antigens can be recognized, so that some adaptive immune cells can differentiate to memory cells [23].

1.1.1. Dendritic Cells

DCs are one of the important professional antigen presenting cells (APC) which plays a vital role in T cell stimulation and regulation of immune response [24-25]. DCs can generally be found in all of the tissues that allows an easier antigen detection and presentation process for T lymphocytes that execute a bridging function between the innate and adaptive immune system. Besides that DCs secrete growth factors and cytokines [26] to modify the innate immune responses and provide the interaction between other immune system components such as the innate lymphoid cells (ILC) [27] or natural killer cells [28–

30].

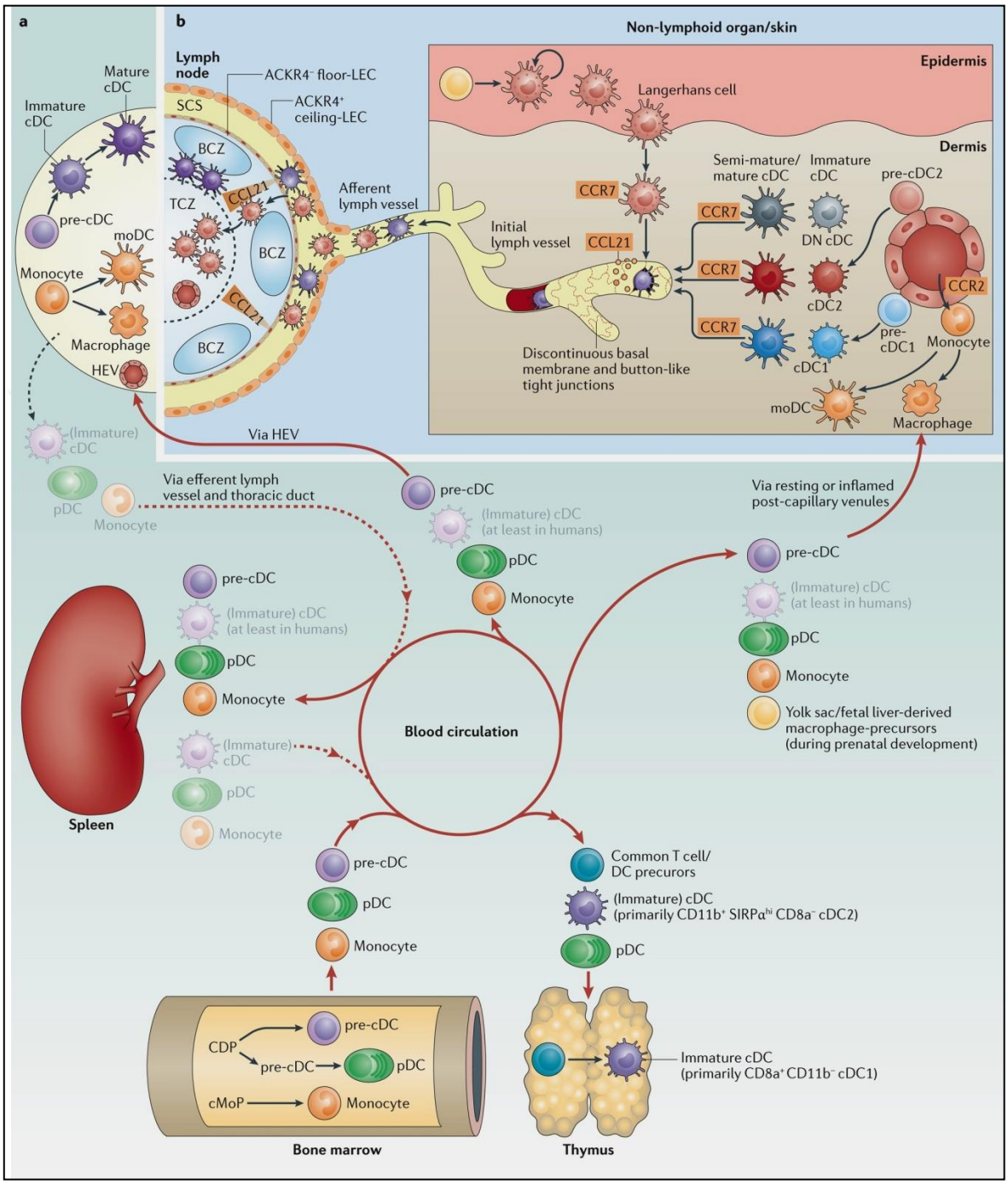

Figure 1.1. From the ‘a’ labelled area, pre-cDCs, pDCs and the monocyte precursors begin to differentiate from bone marrow than migrate to different lymphoid tissues such as thymus, spleen or lymph nodes and these can migrate to non-lymphoid organs such as lung or skin. From the ‘b’ labelled area, if immature DCs locate on a non-lymphoid organ, these undergo a spontaneous maturation process to gain a semi-mature phenotypic characteristics these are activated from PAMPs and begin to differentiate to their mature DC forms [31]

DCs can be found in mature or immature form. In the process of presenting antigens to the T cells that are located in the secondary lymphoid organs, DCs need to be in their mature form. To the presentation of the antigens to T cells in the secondary lymphoid organs, DCs have to have their mature form [32]. DC maturation is typically induced following the recognition of PAMPs or DAMPs [33,34]. During the maturation of DCs, gene transcription, metabolic and cellular pathways are activated and DCs begin to migrate from peripheral tissues to secondary lymphoid organs to induce stimulation of T lymphocytes [35–39]. After the maturation of DC their migration pathways can be observed from Figure 1.1.

With the beginning of the maturation process, adhesive molecules are lost, the arrangement of the cytoskeleton is changed, the motility and the expression of co-stimulatory molecules, the C-C motif chemokine receptor 7 (CCR7) and, MHC2 expression is increased [40–42].

Mature DCs begin to secrete cytokines which are essential for the T cell activation [43–45]. When DCs interact with $CD4^+$ cells, the fate of naïve T cells can be changed to different T helper cell types [46] such as T helper-1 (Th1) [47–49], T helper 2 (Th2) [50,51], and T helper17 (Th17) cells [52–54] . IL-12 induces Th1, IL-4 induces Th2, and IL17 is important for Th17 cell differentiation [55]. The decision on the secretion pattern for the cytokines is a complex phenomenon, which can change depending on the infection type, viral or bacterial infection [56], the location of DCs [57] or the tissue imbalance [58].

In addition, DCs have a cross presentation ability which makes them a unique APC type of immune cell [59–62]. With the help of the cross presentation property, extracellular antigens can be presented with MHC-1 molecules to $CD8^+$ T lymphocytes [63,64]. Thanks to this process, escaped antigens from professional APCs can be caught [65,66], thereby cross tolerance allows the recognition of the intracellular self- antigens [67,68]. Prior to their maturation, DCs are less effective in T lymphocyte stimulation, expression of co-stimulatory surface molecules, and chemokine secretion [69]. However, their antigen capture capacity is very high due to receptor mediated endocytosis [16,70,71] Fc, complement receptors [72], macropinocytosis [73], and lectin [74,75] receptors.

This feature provides the stimulation and induction of the immune tolerance [32]. For example, during the natural tissue turnover, apoptotic cells arise which become engulfed

by the DCs. However, this process does not stimulate an adaptive immune system dependent response due to the incomplete maturation of DCs [76,77]. Although, antigens do become presented to T cells, because the co-stimulatory molecules are not expressed at this point, the peripheral tolerance such as T cell apoptosis [78,79], anergy [80,81] or regulatory T cell (Treg) differentiation can take place [82,83]. At the same time, tolerogenic DCs decrease the expression of pro-inflammatory cytokines, co-stimulatory molecules, while increasing the expression of inhibitory molecules such as cytotoxic T-lymphocyte associated protein 4 (CTLA- 4) and programmed death ligand 1 (PD-L1) [80,84,85]. These kinds of inhibitory molecules are also essential to prevent the response of immune cells to healthy tissues [86,87]. However, this process may result a failure in contexts such as fighting against infections [56,88] or tumours [89,90] due to their roles in immune evasion pathways. As a result, in certain cases pathogens can escape being detected by the immune system.

1.1.1.1. *Human Dendritic Cell Subtypes*

Each DC type have different surface markers that depends on where the DCs are located. Typically DCs can differentiate into different subtypes and this event results in a heterogeneous DC family [91]. Classical DCs (cDCs) can be divided into resident lymphoid tissue DC and migratory nonlymphoid tissue DC, which are categorized based on their phenotypic markers and genetic profiles. Each of cDC are sourced from hematopoietic stem cells like other leucocytes. During the haematopoiesis, stem cells can give rise to common myeloid progenitors (CMP) which are sourced for granulocytes, macrophages, erythrocytes, monocytes, and megakaryocytes. Common lymphoid progenitors (CLP) are sourced for natural killer cells, B, and T lymphocytes [92,93]. It was shown that common multi lymphoid progenitors can give rise to all of the lymphoid cell types and also DCs, macrophages, and monocytes [94]. In general, most of cDCs are sourced from the CMPs under steady state conditions [95]. Myeloid DC precursors (MPs) differentiate to monocyte- dendritic cell progenitors (MDPs) that give rise to monocytes and common DC precursors (CDP). CDPs can also differentiate into plasmacytoid DCs (pDCs) and pre-classical DCs (pre-DCs). pre-DCs are the main progenitor of two main cDC subtypes which are cDC1 and cDC2, the latter will be discussed later [96]. DCs can shape their phenotypic characteristics to the microenvironment and the homeostatic state of the tissue [97]. In blood, $CD123^+$ and $CD11c^-$ DCs are called pDCs, $CD123^-$ and $CD11c^+$

cells are called classical or myeloid DCs (cDCs) [98,99]. cDC1 can be characterized based on the expression of CD141 (BDCA-3) and CLEC9A, while cDC2 can be characterized based on CD1c expression. On the other hand, plasmacytoid DCs can be characterized based on the presence of CD123, CD303 (BDCA-2), and CD304 (BDCA-4) expression.

For the cDC1 subtype is present in the blood, lymphoid or nonlymphoid tissues [100] and mainly expresses CD141, the chemokine receptor XCR1, C type lectin (CLEC9A), and cell adhesion molecule CADM1. cDCs can be generated from $CD34^+$ hematopoietic stem cells with the help of the fms-like tyrosine kinase 3 (Flt-3L) and murine bone marrow stromal cells [101] or Flt-3L and thrombopoietin (TPO) [102]. Two main transcription factors are also essential for the generation of cDC1s, these are basic leucine zipper transcriptional factor ATF-like Transcription Factor 3 (BATF3) [103] and interferon regulatory factor 8 (IRF-8) [104]. cDC1s have a cross presentation capacity via using XCR1 chemokine receptor and these are very effective in anti-tumour and anti-virus responses [105] due to their $CD8^+$ T cell activation potential.

cDC2 subtypes are present in a heterogeneous population and can be found in blood, lymphoid or nonlymphoid tissues [106] and mainly express CD172a (Signal-regulatory protein alpha, SIRPα) [99] and CD1c [106]. Expressed markers on the surface of these cells can vary depending on their location. For example, those that express CD103 are present in the gut [107], and those that express CD1a locate in dermal areas [26]. These cells also differentiate from $CD34^+$ cells like cDC1 cells. Moreover, different combinations of fibroblasts and cytokines and/or transcription factors such as FLT-3L and bone marrow stromal cells or TPO and IRF-4 are essential for this differentiation [108]. cDC2 can induce Th2 and Th17 responses [109,110].

Plasmacytoid DCs (pDCs) can be differentiated from HSC to lymphoid progenitors or these can also be differentiated from the monocytes and common DC precursors (CDP). These are characterized by $CD11c^-$, $CD11b^-$, $B220^+$, $SiglecH^+$, $CD317^+$, $CD123^+$, $CD303^+$, and $CD304^+$ markers. pDCs mainly produce IFNα/β (Interferon α/β) [111] and have a crucial role in fighting with viral infections.

Monocytes are differentiated into monocyte derived Dendritic Cells (moDCs) in the presence of GM-CSF (Granulocyte- Macrophage Colony- Stimulating Factor) and IL-4

stimulation [112]. moDCs also require IRF-4 for their differentiation like cDC2s [113] and they are phenotypically very similar to the inflammatory DCs which mainly express $CD11c^{hi}$, $MHCII^{hi}$ markers.

1.2. ADAPTIVE IMMUNE SYSTEM

The main role of the adaptive immunity is to provide neutralization and destruction of pathogens and cancer cells in an antigen specific manner. During the development of adaptive immune cells, auto reactive lymphocytes are eliminated, but some of them may escape from the central tolerance mechanism and begin to attack to the body's own cells this situation is called autoimmune disease [114]. At this point, antigen presenting cells (APC) are very significant to provide homeostatic balance. APCs engulf the antigen, process and then display them to innate or adaptive immune cells [115]. APCs can be classified as amateur and professional subsets. Endothelial cells, glial cells, keratinocytes, pancreatic beta cells, and thyroid cells can be accepted as amateur APCs and these only present antigens under specific conditions. On the other hand, the presentation of antigens is one of the main tasks of the professional APCs which can be macrophages, B cells, and Dendritic cells [116]. These APCs generally locate in the T cell dependent areas of lymphoid tissues and they also express co-stimulatory molecules that are required for the stimulation of T lymphocytes. For the presentation of the antigen to the T cells, proteins are degraded to peptides, then become loaded into the major histocompatibility complex (MHC).

1.2.1. The Interaction between DCs and T lymphocytes

Stimulation of T cells requires their interaction with DCs in the lymph nodes (LNs), spleen or Payer's patches. Therefore, DCs are very significant for the induction of T cells based on priming and tolerance. Upon this interaction between T cells and DCs, for which some requirements need to be fulfilled, T cells rapidly become activated and proliferate.

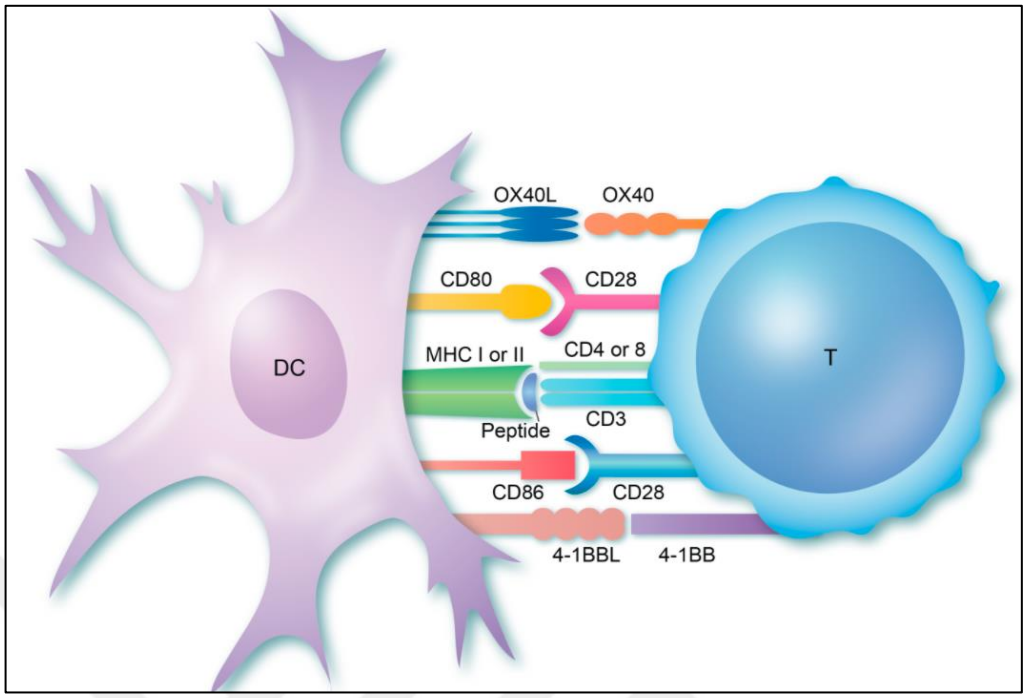

Figure 1.2. Schematic demonstration of DC-T cell interaction [117]

Firstly, MHC molecules on the DCs have to interact with T cell receptors (TCR). This interaction is known as the first signal. The second signal comes from the interaction of co-stimulatory and adhesion molecules. The most well known one occurs between CD86-CD28. Furthermore, cytokines are also secreted as the tertiary required signal (Figure 1.2.), with the help of which, naïve T cells can proliferate with clonal expansion or may differentiate to effector or memory T cells. Presentation of self antigens from DCs may change the outcome in terms of T cell type, meaning they may go under clonal deletion or unresponsiveness, nonetheless, activated DCs provide T cell priming [118] [119]. DCs may provide the proliferation of Regulatory T cells (Tregs), which suppress $CD4^+$ T cell responses [120]. If DCs interact with Tregs [121] or uptake the apoptotic cell debris [122], these may keep themselves under a tolerogenic state, indeed, DCs keep themselves tolerogenic until they become activated.

1.2.2. Dendritic Cells in Lymph Node

In peripheral tissue like skin, gastrointestinal or respiratory track, immature DCs are ready to encounter to self or foreign antigens [123]. With this, DCs (at the steady state) gain a

continuous migratory property from tissues to LNs.

DCs migrate to the LNs via the lymphatic vessels through subcapsular sinus and localize to the paracortex or T cell area. DCs derived from langerhans under steady state conditions cover the entire cortex of the LNs, whereas the latest migrated DCs localize to the cortical ridge, which is adjacent to the reticular conduits and locates near the high endothelial venules. Thereby, antigens can be recognised efficiently, increasing the probability of DC-T cell encountrance [124]. After DCs enter the lymph node, they migrate with a mean velocity of 2-6μm/min [125–128] at the same time extend and retract their dendrites which can scan the distance >60 $\mu m/min^{-1}$ [127,129]. DC-T cell interaction process occurs at three phases. During the 1st phase, cells contact with each other in <5 min. With the help of MHC presented by the DCs, DC-T cell interaction and additionally secreted signals, duration of their contact can vary. Hence, these contacts can also be called transient interactions, due to which T cells receive an activation signal and begin up-regulating their early activation markers such as CD69. Alternatively, DCs can interact with T cells via brief contacts, so that they can be activated sufficiently and can provide stabilized interaction. If the first encountering fails to provide an expected interaction, the stimulation of T cells by DCs can take approximately 1h [130,131]. This interaction also depends on the antigen amount [132]. 2nd phase involves entry of the T cells into the lymph node that may take approximately 10h. If T cells decrease their velocity in the lymph node, the interaction probability of DC-T cell is increased, therefore, the behaviour of T cells is important. During this phase, T cells up-regulate their surface activation markers [126,133]. The stability of DC-T cell interaction also depends on the number [125,134] and the quality [135] of MHC peptides present. Once, APC and T cell interact with each other, the resultant structure is called an immunological synapse (IS) which can promote the downstream signalling that is accomplished via co-stimulatory molecules and TCR. This structure is necessary to provide a T cell arrest [136,137], signal integration and asymmetrical T cell division of activated T lymphocytes [138–140]. After IS formation, naïve T cells can deliver signals for hours via their TCR [141,142]. And if the lymph nodes provide the proper environment to IS, T cell can retain its contact with the DC up to 6 hours, so the signal uptake can be maintained [143]. The 3rd phase lasts approximately 30 hours, during which most of the clusters begin to dissociate and T cells show a swarming behaviour whereby they regain their motility, migrate to the localized area, then they begin

to proliferate.

Cytotoxic lymphocytic antigen 4 (CTLA-4/CD152) can be mentioned as a negative regulator of T cells. When CTLA-4 expression is increased, basal T cell motility is also increased, which overrides antigen specific T lymphocyte arrest on DCs [144]. CTLA-4 is an inhibitory co-receptor, therefore it can nullify the co-stimulation of T cells by APC proteins such as CD80 and CD86. When the CTLA-4 molecule is blocked *in vivo,* tolerated T cell motility remains unchanged and the stability of DC-T cell interaction is not provided. PD-L1 is another molecule which like a brake for the action of CTLA-4 and, thereby, body's immune response can be kept under control. When the PD-L1 is blocked, T cell motility is changed, DC-T cell interaction is increased and autoimmune development is provided. The last product of T cell depends on the DC-T cell interaction duration [145].

DC cytoskeleton is an important key player for the stabilization of DC-T cell interaction. To provide this stabilization, DCs control Rho, GTPases, Rac1, and Rac2 genes [129]. For example, a study showed that WASp deficient DCs alter the T cell cytoskeleton such that the conjunction between DCs and naïve $CD8^+$ T lymphocytes becomes reduced [146]. At the same time, adhesion molecules also impact the cytoskeleton arrangements. When intercellular adhesion molecule-1 (ICAM-1) interact with TCR ligands, it provides T cell arrest and polarization [147]. Upregulation of ICAM-1 molecule on DC surface also increases the duration period of DC-T cell interaction [148]. ICAM-1 deficient mice showed that DC-T cell interaction takes place as brief contacts. In this mouse model, [149] induction of IFN-γ cannot be provided by the activated T cells, resulting in their failure differentiate to memory T cells [148]. Absence of ICAM-1 may create problems in receptor polarization and effector cytokine secretion into the intercellular space. After a stable DC-T cell interaction is established, T cells detach from DCs, and become more dynamic and undergo brief interactions with DCs. For this situation to occur, T cells may induce the down-modulation of MHC proteins on DCs [150]. If an external antibody is presented to DCs, the interaction becomes abrogated [143] and as a result T cells may detach from DCs. Once a T cell become motile and the stability of DC-T cell is lost, T cell cannot re-engage in a contact with DCs [145]. However, if DC-T cell interaction happens to be restored, the T cell effector function may be enhanced [151] or impaired [152]. Whereas, during the late phase of a stable DC-T cell interaction, secreted chemokine environment mediates the go signal to T cells when the interaction is finalized.

1.3. AUTOIMMUNE THERAPEUTIC VACCINES

Immune tolerance ensures that self antigens are ignored, so that the body is protected from destructive responses to its own tissues and cells. Both in regulation of innate and adaptive immune system and immune based therapies, tolerance mechanisms are in charge of promoting and maintaining immune homeostasis like in the example of B and T cell depletion [153]. with using CD86- CTLA-4 interaction, T cell stimulation can be blocked [154,155] or cytokines can be neutralized by TNF-α or anti IL-6 etc. [156–158]. However, both CTLA-4 and TNF-α blunting therapies are generally not long lasting treatment options because treatment in question has to maintain suppression of autoimmunity [159]. At the same time, these are general immunosuppressant agents which means that many effector immune cells can also be suppressed, thus critical and life threating consequences can occur. To avoid this, more antigen specific and targeted therapies with lower toxicity and prolonged prognosis offer long term solutions. Therefore, DCs, the safety of which , these proved, are one of the most efficient and powerful option in the treatment of autoimmune diseases and cancer [160].

Human sourced tumour cells express some proteins which can be recognized by T lymphocytes. In order for these Tumor Specific Antigens to be used in therapeutic applications, tumor cells or antigens are engulfed by DCs and these presents to T lymphocytes, this mechanism provides therapeutic and protective anti tumour immunity. Although, moDC vaccines were commonly used in recent years, their deficiency in robustness remains as a major limitation to be overcome. cDCs are desirable candidates for empowering antitumor immunity, because of their effectiveness in the presentation of antigens to active natural killer cells and cDCs can prime antigen specific cytotoxic T lymphocytes [160].

DCs also play a crucial role in the initiation and progression of autoimmune responses. These cells provide the production of pro-inflammatory cytokines such as TNF-α, which induces the infiltration of collagen reactive T cell in the joint cartilage [161]. At the same time, DCs can modulate the autoimmunity via presentation of self antigens to T cells [162]. The safety of therapeutic DC-based vaccines were also tested in the clinical trials of MS, TID, arthritis, and Chron’s diseases [163].

1.3.1. Dynamics of DC- T Cell Interactions during T Cell Tolerance

Central tolerance is an essential requirement to keep autoreactive T cells under control and DCs can be considered as the main pillars of this control mechanism. Autoreactive T cells can escape from the negative selection from thymus. That being the case, central tolerance mechanism plays a vital role in preventing auto-immune reactions by ensuring that these fugitive autoreactive T cells are captured by the antigen-targeting immature DCs, which are resident to the lymph nodes. When these immature DCs encounter the autoreactive T cells (Figure 1.2.) the contact made between the two, initiates a signaling cascade which ends up turning on DC-specific antibodies [164] or DC specific promoters [165,166]. Overall, induction of tolerance to the corresponding antigens presented by T cells become established. If DCs are activated from the targeted antigens under the same conditions, the approach of DC-T cell interaction can change the outcome of lymphocyte profile which can either be primed or to be tolerated. If long-lasting DC-T cell interaction does take place, tendency for effective T cell priming is stronger than that for tolerance. However, if these interactions are short-lived, the possibility for tolerance, rather than the priming, to occur becomes higher [167]. Consequently, transiently activated $CD8^+$ T lymphocytes can be rendered clonally deleted as a result of the developed T cell tolerance against antigens [118,119,168]. If DCs are activated with LPS or CD40 antibodies, DC- $CD8^+$ T lymphocyte interaction can be prolonged (<5h.) and effective T cell priming is observed [145]. $CD4^+$ T cells can also be tolerated similar to that seen for $CD8^+$ T cells, this provides quicker motility behavior (18h) than priming (24h.) [130].Larger DC-T cell clusters correlate with longer lasting DC-T cell contacts [169]. If the number of Tregs are too high, that also decreases the stability of DC- $CD4^+$ T lymphocyte interaction. DC-T cell interaction can also be indicative of a T cell tolerance that is one of the example of the avidity of TCR peptides for MHC complexes which are expressed by DCs [135]. Were different antigen concentrations given to T cells, both an arrest of T cells and long lasting DC-T cell interaction would be observed. Consequently, brief signals, which are delivered from different DC types, result in the induction of tolerance. Secreted antigens are sourced from mature DCs, which are sufficient for the events of the induction. Tolerogenic DCs are required for the presentation of antigens from high numbers of steady state DCs. In other words, if self-antigens are presented from a high number of DC pool, T lymphocytes can show tolerance to this stimulation. T cells have a dendritic-cell scanning ability [125,132],

meaning these can detect rare mature DCs, so DCs have to be productive to induce efficient T cell priming [170].

It appears that, DCs have a huge role in the development and pathology of autoimmune diseases [171–173]. When the immune response of the body begin to fight against its own antigens, a cascade of events are initiated by the loss of tolerance to self-determinants from autoreactive lymphocytes or antibodies, impacting neighboring cells, tissues, and ultimately organs. As a consequence of this intolerance, organ systems may suffer detrimental damage. Some kinds of DCs are considered as potential therapeutic agents to provide tolerance against autoimmune disorders, these DCs are called tolerogenic DCs [174]. To provide semi–mature DCs endogenous immunosupressors (glucocorticoids) synthetic drugs (asprine, rapamycin) or natural products (resveratrol, curcumin), and carticostreoids or active form of VitD3 can be used [51,175–177]. In this thesis, the suppressive effects of VitD3 is investigated.

1.4. VITAMIN D3

Vitamin D is a kind of a fat-soluble prohormone discovered by Adolf Otto Reinhold Windaus, who won the Nobel Prize in Chemistry in 1928. VitD3 has some pivotal role in the body, one of which is absorbing calcium in the small intestines and the other being in the regulation of re-absorption of calcium by the bone. The latter function provides bone-calcium homeostasis and stimulates osteoclast differentiation. It can also promote mineralization on a collagen matrix of bone and also provide the autocrine signalling. Almost each immune cell type has VitD3 receptors inside the cell, attributing a modulatory role for VitD3 in regulation of the immune system. VitD has two main sources, ergocalciferol (VitD2 form) is a natural form and can be taken from the yeast or mushrooms and cholecalciferol (VitD3 form) can be taken from the UVB radiation via skin. Melanin absorbs UVB radiation and becomes synthesized to 7-dihydrocholesterol (Pro-VitD3). Next, Pro-VitD3 is hydroxylated in the liver under the catalysis of 25-hydroxylase. The resulting product of 25-hydroxylase-dependent reaction becomes converted to 25-hydroxyvitamin D3 (25 (OH) D3) (calcidiol), which is still an inactive, but measureable form of Vitamin D3. Calcidiol then translocates to the kidneys through proximal tube, where it becomes hydroxylased, generating the active form of VitD3

(caltiriol) by the enzyme 25- hydroxyvitamin D-1α hydroxylase (CYP27B1) that is stimulated by parathyroid hormone. Synthesized calcitriol becomes released into the blood stream. With the help of 1,25D form of VitD3, calcium absorption is promoted in the small intestine via this osteoblast differentiation and matrix calcification is provided. Then calcitriol can also be converted to 1,24,25 VitD3 form by the 24- hydroxylase (CYP24) enzyme. 1,25 D imposes a negative feedback type mechanism to regulate the levels of active form of VitD3. If the levels are too high, 1-α hydroxylase enzyme is inhibited, while 24- hydroxylase becomes activated, so that the excessive amount of VitD3 can be prevented [178]. To exert its effect, active form of VitD3 binds to the VitD3 receptors of the relevant cell. The bound VitD3-VitD3 receptor complex dimerizes with retinoid X receptor (RXR) and 1,25D-VDR-RXR heterodimer is formed. The resultant holo-complex translocates to the nucleus and binds to the promoter region of VitD responsive element (VDRE) resulting in an upregulating effect on VitD responsive gene expression. For example, when TLR is stimulated, both the expression of 1-α hydroxylase and VDR are increased. The heterodimer binds to the cathelocidin and β- defensin, promoting transcription of these genes. VitD3 has some effects on general immune system. 1,25-OH_2D_3 changes the phenotypic properties of DCs based on morphology (more adherent, spindle shaped), cytokine production, and also alteration in cell surface markers [179–181]. Because of this tolerogenic phenotype, the expression of MHC- II, CD80, CD86 co-stimulatory molecules, and CD54 adhesion molecules can be decreased and the expression of CCR5 chemokine receptor, DEC205 antigen uptake receptor, F4/80 macrophage marker, and CD40 can be increased [182,183]. At the same time, VitD3 upregulates the expression of programmed death ligand 1 (PD-L1), immunoglobulin like transcript 3 (ILT-3), and promotes Treg differentiation [184]. DCs also secrete TNF that provide the production of antigen specific suppressive T lymphocytes [185]. If naïve T lymphocytes are stimulated by the mature DCs, immune response can be increased. However, if presentation is promoted by the immature DCs that ease the tolerance. For instance, in the physiological cell death environment, lots of antigens are present, but because their presentation are promoted by the immature DCs, immune system can maintain the tolerogenic property to their self-antigens.

VitD3 also affects T lymphocytes directly or indirectly. Active form of VitD3 can inhibit the secretion of Th1 (IL-2, IFN- γ), Th17 (IL-17, IL-21) and Th9 (IL-9) cytokines [186–

189]. Tregs promote IL-10 production in response to VitD3-driven gene expression fired from the FoxP3 (Forkhead Box P3) promoter region that induces CTLA-4, which is essential for Treg suppression [189,190]. Upregulated CTLA-4 can enhance the tolerogenic character towards the T lymphocytes [188].

VitD3 also affects B lymphocytes. It may induce the apoptosis of B cells, impedes the proliferation of plasma cells because of CD40 and NF_KB (Nuclear factor kappa B) modulation [191,192]. Secretion of IL-10 can also be achieved by the B lymphocytes that can result in a regulatory effect [193]. At the same time, T lymphocyte activation may be decreased with the help of B lymphocytes through downregulation of CD86 and upregulation of CD74 [194].

In this study, the effects of VitD3 on cDCs differentiation and function were investigated. Firstly, human $CD34^+$ hematopoietic stem cells were differentiated to cDCs with cytokines and VitD3 under *in vitro* condition. Then cDCs differentiation ratio and expression of their surface molecules (HLA-DR, CD86, CD11c, PD-L1, and CLEC- 9A) were determined. Following that, functional assays were performed. Firstly, DCs were co-cultured with naïve and PMA (Phorbol 12- myristate 13- acetate)-Ionomycin induced $CD3^+$, $CD8^+$ T cells and percentage, geometric mean intensity of TNF-α and INF- γ cytokines were determined. Secondly, cDCs were loaded with A549 tumour cell lysate and co-cultured with $CD8^+$ T cells. Five days following the start of the co-culture, $CD8^+$ T cells were combined with A549 cells. Effector functions of $CD8^+$ T cells were determined with analyses of apoptotic cell percentages in A549 cells.

2. METHODS

2.1. HEMATOPOIETIC STEM CELL (HSC) ISOLATION

$CD34^+$ HSCs were isolated from PBMCs (ATCC, Cat# PCS-800-011) with EasySep Human CD34 Positive Selection Kit II, (Cat#17856 Stemcell Technologies) according to manufacturer's instructions. Briefly, $5x10^8$ cells were counted and resuspended in 5 ml polystyrene round bottom tube (12x75 mm) (Corning, Product# 352058) with 1 ml recommended media 2 percent FBS (Gibco, REF # 10270-106), 1 mM EDTA (J.T.Baker Product # 15548494) in PBS (Gibco, REF# 14190-094). Then cells were labelled with 100 µl selection cocktail (EasySep Human CD34 Positive Selection Cocktail Cat #17856C) and incubated for 10 min at room temperature (RT). Before adding to 75 µl rapid spheres (EasySep, Dextran Rapid Spheres Cat #50100) were mixed with vortex motion then cells were incubated with them for 3 min at RT. Recommended medium was added to top up to 2.5 ml, mixed them by gently pipetting for two to three times. Tube was placed into the magnet (EasySep Magnet Cat # 18000) and incubated for 3 min. and then supernatant was poured. This washing step was repeated two more times. At the end of these steps, tube was taken from the magnet and the inner surface of tube was washed with recommended media and cells were counted with hemacytometer (Merch, Product # Z359629- 1EA). Isolated cells were stained with CD34-PE (Biolegend, Cat #343606) and CD45- APC-A700 (Biolegend, Cat # 368514) markers and the purity of HSCs were observed with flow cytometry (Beckman CytoFLEX S).

2.2. DIFFERENTIATION OF HEMATOPOIETIC STEM CELLS TO CLASSICAL DENDRITIC CELLS AND THEIR FLOW CYTOMETRY ANALYSIS

Approximately 62500 cells/well were cultured with a differentiation medium which contains 20 ng/ml GM-CSF (R&D Systems, Cat #7954-GM/CF), 20 ng/ml SCF (R&D Systems, Cat #7466-SC/CF), 20 ng/ml IL-4 (PeproTech, Cat # AF-200-04- 20UG) and 100 ng/ml Flt-3L (GenScript Cat # Z02926-10) in X-VIVO medium (LONZA, Cat# BE02-060Q) in 12 well plate (ThermoFisher Cat#150628) at 37°C, 5 percent CO_2 for two weeks.

Differentiation medium was renewed at the beginning of the second week. Apart from this setup, besides all the cytokines, 1 nM VitD3 (Haver Farma İlaç A.Ş.) were also added into VitD3 groups.

2.2.1. Flow Cytometry Analysis of HSC to DCs

After two weeks incubation, to determine the effect of VitD3 on cDCs differentiation, cells were harvested from plate and stained with HLA-DR II (APC- Cy7 Biolegend, Cat #307618), CD11c (PerCP/Cyanine 5.5, Biolegend, Cat #337210), CD86 (PE/Dazzle - ECD), PD-L1 (FITC, Biolegend, Cat #393606), CLEC9-A (DNGR-CD370)(APC, Biolegend, Cat #353806) in 100 µl FACS buffer for 15 min. after the staining, cells were washed with FACS buffer two times and analysed with flow cytometry.

2.3. PLASMACYTOID DC ANALYSIS

To determine the effect of VitD3 on pDC, differentiated control and VitD3 treated cells were stained with CD34 (PE, Biolegend,Cat#343606), CD45 (Alexa Fluor 700 , Biolegend,Cat#368514), CD11c (PE/CY7, Biolegend,Cat#331516), CD123 (PE/Dazzle, Biolegend, Cat#306041), CD303 (PerCp/Cy5.5, Biolegend, Cat #354209) and HLA-DR II-(APC- Cy7 Biolegend, Cat #307618) markers in 100µl FACS buffer for 15 min after the stained cells were washed with FACS buffer two times and analysed with flow cytometry.

2.4. STIMULATION OF DCS WITH LPS AND R848

Cells were harvested and stimulated with 100ng/ml lipopolysaccharide (LPS)(TLR4 agonist) from *Escherichia coli* (Merck, Cat# 297-473-0) and 2,5 µg/ml R848 (TLR 7/8 agonist, InvivoGen Cat#tlrl-r848) for overnight at 37°C, 5 percent CO_2. After the incubation cells were harvested and stained with HLA-DR class II (APC- Cy7 Biolegend, Cat #307618), CD11c (PE/CY7, Biolegend, Cat#331516) in 100 µl FACS buffer for 15 min. After the staining, cells were washed with FACS buffer two times and observed with flow cytometry.

2.5. CD3 ISOLATION

Approximately $100x10^6$ PBMCs were counted and suspended in 5 ml (12x75 mm) polystyrene round bottom tube with 1 ml recommended medium (2 percent FBS, 1 mM EDTA in PBS), 100µl/ml selection cocktail(EasySep, Human CD3 positive Selection Coctail II, Cat# 1785C) was added and incubated three min at RT then 60µl/ml rapid spheres (EasySep, Dextran Rapid Spheres, Cat#50100) were added and incubated at RT for three min. After that recommended medium was added to top up the sample to 2,5 ml and gently mixed with pipetting. The tube was placed into the magnet and incubated three min then in one continuous motion, magnet and tube were turned together and the supernatant were removed. This step was repeated two more times. After the washing step, polystyrene tube was taken from the magnet and the inner surface of the tube was washed with recommended media to collect the pure $CD3^+$ cells. The purity of isolated cells was controlled with flow cytometry.

2.6. CD8 ISOLATION

Approximately $50x10^6$ PBMCs were counted and suspended in 5 ml (12x75 mm) polystyrene round bottom tube with 1 ml recommended medium (2 percent FBS, 1 mM EDTA in PBS), 50 µl/ml selection cocktail (EasySep, Human naïve $CD8^+$ T cell isolation cocktail, Cat# 19258C) was added and incubated three min at RT. Then 50 µl/ml rapid spheres (EasySep, Dextran Rapid Spheres, Cat #50103) were added and incubated at RT for three min. Then recommended medium was added to top up the sample to 2,5 ml and gently mixed with pipetting. The tube was placed into the magnet without any incubation magnet and tube were turned together, supernatant was collected in another polystyrene tube and the recommended media added again to top up to 2,5 ml and placed into the magnet, then supernatant was collected into another polystyrene tube. The purity of isolated cells were observed with flow cytometry.

2.7. DC- T CELL MIX COCULTURE

2.7.1. Cell Proliferation Assay for T Lymphocytes

Approximately $1x10^6$ $CD3^+$ T cells were labelled with 2,5 μM Carboxyfluorescein Succinimidyl Ester (CFSE) (Santa Cruz, Cat#sc-202096) in PBS for 15min at 37ºC. Cells were incubated 5 min with its own medium (which have to contain 10 percent FBS to inhibition of the staining process) on ice then washed with PBS two times. Then 50.000 untreated and PMA (25 ng/ml), ionomycin (750 ng/ml) [61] treated $CD3^+$ T lymphocytes (50000 cell/well) were cocultured with both of LPS and non-stimulated cDCs (5000 cell/well). Flow data were taken at zeroth and fifth day.

2.7.2. Intracellular Staining of Cocultured cDCs/CD3 and CD8 T Lymphocytes

Sorted $5x10^3$ cDCs were co-cultured with PMA (25 ng/ml)(Merck, Ca #P1585- 5 MG), ionomycin (CAYMAN, Ca#56092- 81- 0)(750ng/ml) treated $5x10^4$ CD3- CD8 and untreated CD3- CD8 T lymphocytes in 96 well plate U bottom shape (Corning, Cat#3799). After three days of co- culture, cell were treated with 1 µl Brefeldin A. (Biolegend, Cat# 420601) at 37°C for 5 h. After the incubation, cells were stained with CD3 (APC), CD8 (PE) markers and intracellular compartments were stained with INF-γ (PC7) (Biologend, Cat# 502527) and TNF- α (ECD) (Biologend, Cat# 502945) and analysed with flow cytometry.

2.8. LYSATE PRODUCTION

Approximately $1x10^7$ A549 cell pellet (passage 21) were suspended in 1 ml PBS which were frozen and thawed five times from -80 ºC to 56 ºC. Then concentration of lysate was determined with Bradford assay (Fermentas, Cat# R1271).

2.9. CELL VIABILITY ASSAY OF HUMAN LUNG CARCINOMA CELLS (A549) AFTER TREATMENT OF $CD8^+$ T LYMPHOCYTES

VitD3 treated and control cDCs (5000 cell/well) were loaded with 300 μg/ml A549 lysate till 3h. then cells were stimulated with 2,5 μg/ml R848 till overnight. After that DCs cocultured with overnight PMA (25 ng/ml)- Ionomycin (750 ng/ml) treated $CD8^+$ T lymphocytes for five days. Then cells and A549 cell (1:1) were cocultured with an overnight incubation. Then apoptotic and necrotic populations were observed with Annexin/PI (Santa Cruz Cat# sc-4252 AK) staining according to manufacturer instruction. Cells were stained with annexin like flow cytometry staining procedure for 15 min and washing steps were performed, before each observation cells were stained with PI dye.

2.10. STATISTICAL ANALYSIS

Statistical analysis were performed with GraphPad Prism 8, some of experiments were analysed with at least 3 replicates. To compare VitD3 and control groups' unpaired student T test were performed.

3. RESULTS

3.1. VITAMIN D SUPPRESS THE HSC DERIVED DC DIFFERENTIATION AND INDUCE IMMUNE SUPPRESSIVE MARKERS ON THE DC SURFACE

To show how VitD3 affects HSC derived DC differentiation, HSCs were isolated from PBMC with a commercial $CD34^+$ isolation kit and then isolated cells were stained with CD34 (PE), CD45 (APC-A700) antibodies. The purification ratio of HSCs was determined 83.4 percent (Figure 3.1.). Then HSCs were differentiated into cDCs in vitro condition for 14 days in the presence of 20 ng/ml GM-CSF, 20 ng/ml SCF, 20 ng/ml IL-4 and 100 ng/ml Flt-3L. VitD3 only added one experiment groups and without VitD3 groups are accepted as a control groups. Following DCs' differentiation cells were stained with main DC markers (CD11c (PE), HLA- DR (APC-CY7)) to determine DC percentage in the culture.

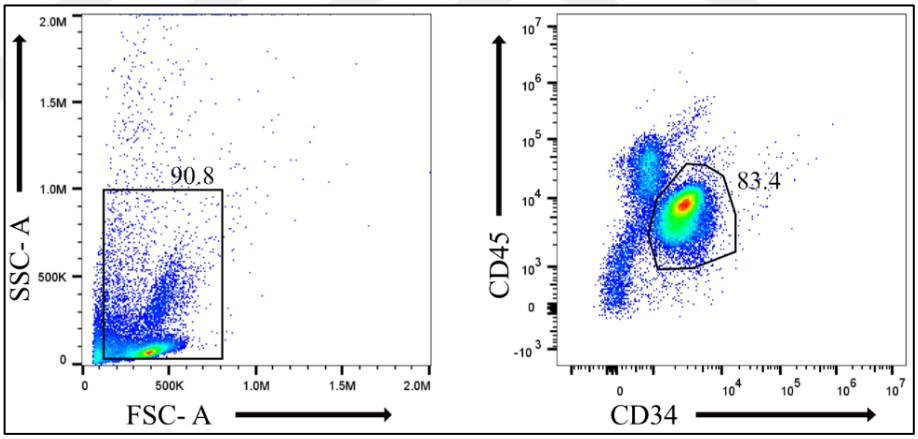

Figure 3.1. $CD34^+$ $CD45^+$ HSC gate without debris (CD34/PE, CD45/ APC-A700)

After discarding cell debris, HLA-DR^{high} $CD11c^+$ cells were accepted as DCs. It was found that VitD3 treated group had less DC percentage than control group. Percentage of DCs in VitD3 treated and control group was observed as 1.16 and 17.7, respectively. (Figure 3.2. and 3.3.). HLA- DR^{high} $CD11c^+$ cell percentage was slightly decreased from VitD3 treated group. Following figure out, VitD3 negatively affects DC differentiation, HLA- DR and CD11c expression levels were compared. Histogram plot and MFI value were used for the analyses (Figure 3.4. and 3.5.) It was found that VitD3 treated group had less HLA- DR

(Figure 3.4.) but more CD11c expression (Figure 3.5.).

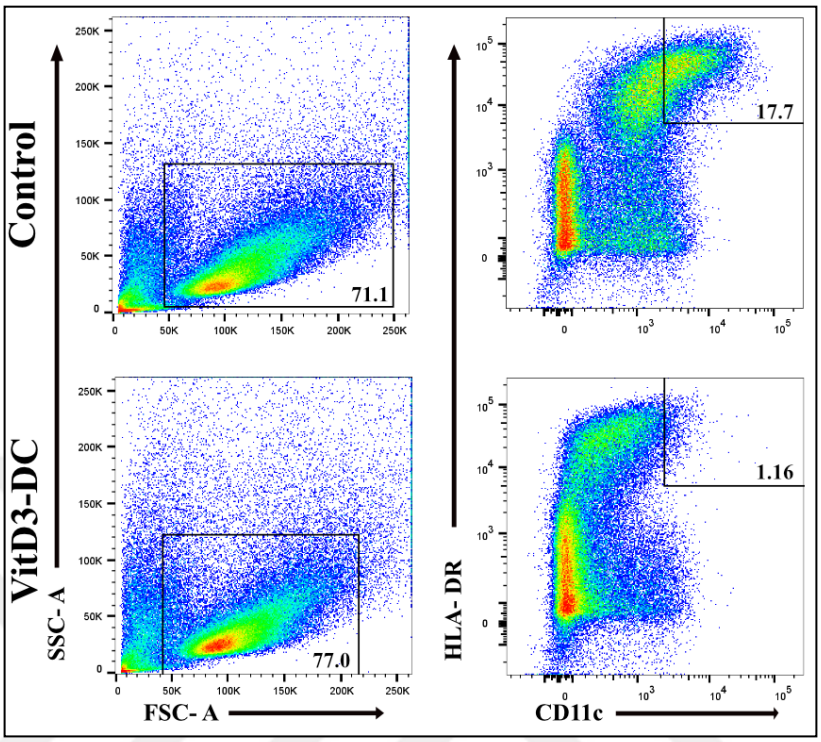

Figure 3.2. The gating strategy of DCs, HLA-DR^{high} CD11c^{+} cells from alive cells without debris. CD11c marker expression of control group was shifted from left to right

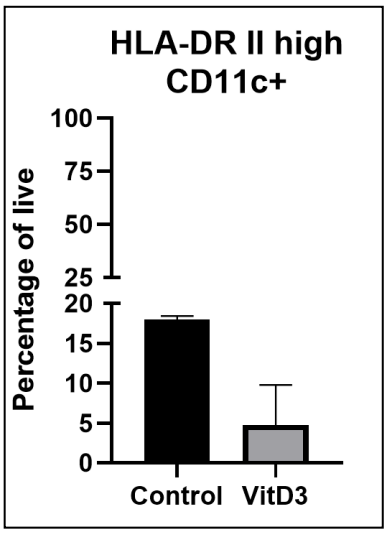

Figure 3.3. HLA-DR ^{high}CD11c^{+} cell percentage comparison graph of VitD3 treated and control groups

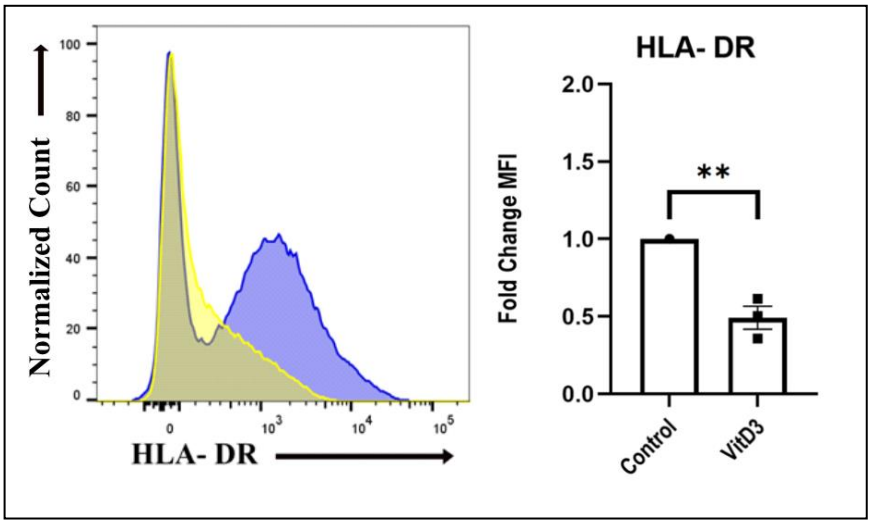

Figure 3.4. Histogram overlay and fold change MFI value of HLA- DR ($p<0.05$, n=3) expressions of control (purple) and VitD3 (yellow) DCs

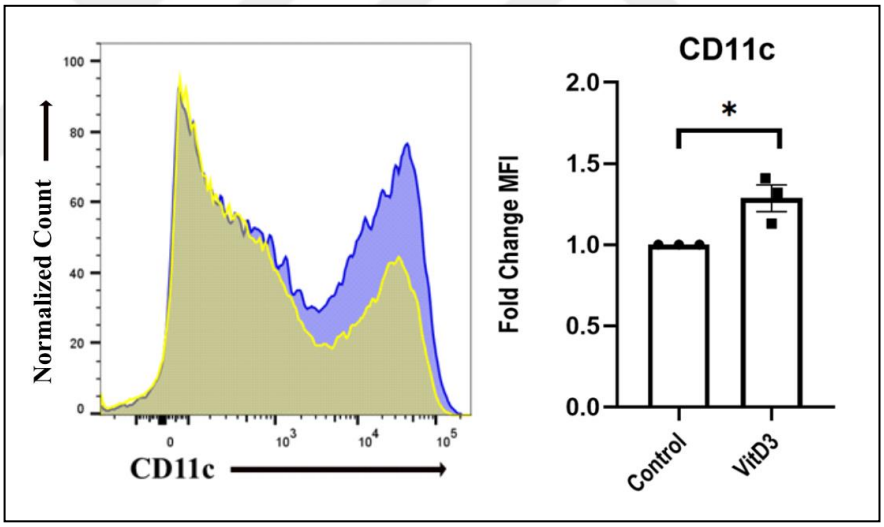

Figure 3.5. Histogram overlay and fold change MFI value of CD11c ($p<0.05$, n=3) expressions of control (purple) and VitD3 (yellow) DCs

Expression levels of DC surface markers are one of the main indicators for their maturation stage and function. To compare how VitD3 affected surface markers on DCs, CD86, PD-L1 and CLEC9a expressions were observed with flow cytometry. According to surface markers from MFI value and histogram plots, DCs were derived from HSC with VitD3 had more CD86 (Figure 3.6.) but not PD-L1 and Clec9a (Figure 3.7. and 3.8.) expressions as their control groups.

From the histogram overlay data, VitD3 treated group was increased their CD86 expression (Figure 3.6.) that were shifted from left to right.

From the fold change MFI graph (Figure 3.6.) of CD86, VitD3 treated cDC group is significantly higher than the control group.

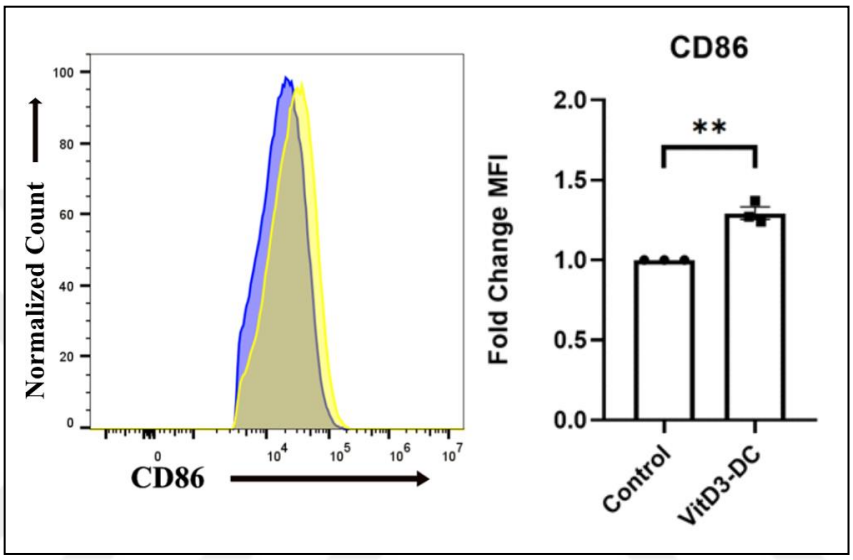

Figure 3.6. Histogram overlay (left) and fold change MFI (right) value of CD86 expressions of control (purple) and VitD3 (yellow) DCs ($p<0.05$, n=3)

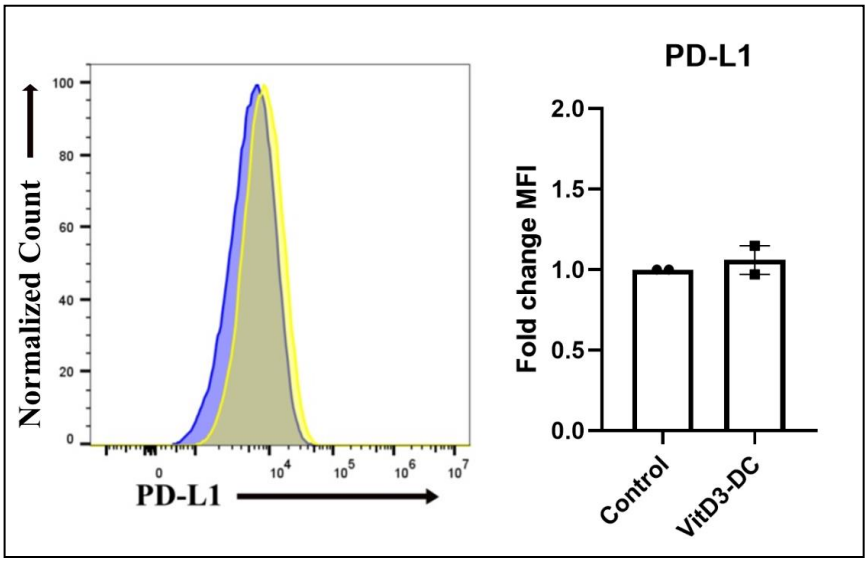

Figure 3.7. Histograms overlay (left) and fold change MFI (right) value of PD-L1 expressions of control (purple) and VitD3 (yellow) DCs ($p<0.05$, n=2)

From the histogram overlay and fold change MFI values of PD-L1 and CLEC9a, the expression levels was similar in both of control and VitD3 treated groups (Figure 3.7. and 3.8.).

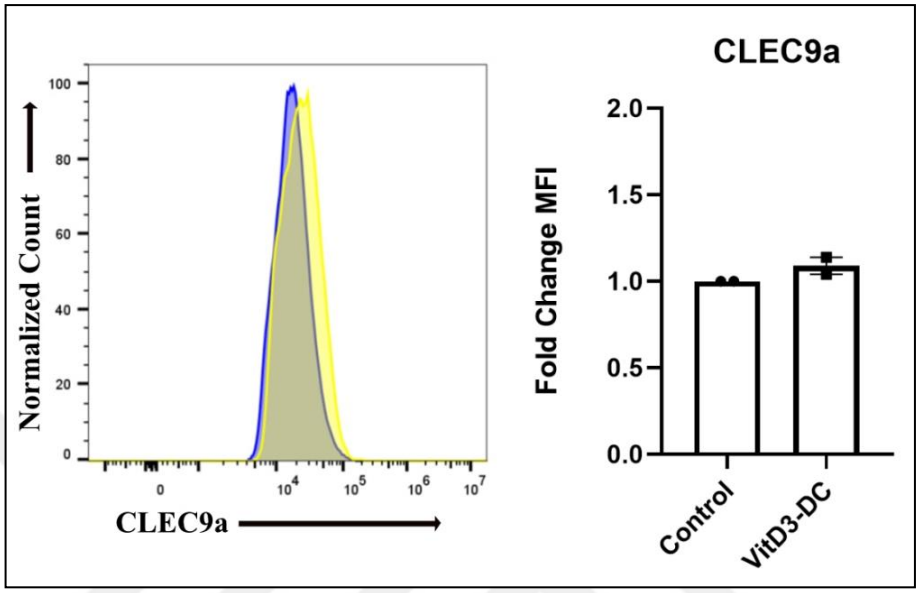

Figure 3.8. Histogram overlay of CLEC9a (left) expression of control (purple) and VitD3 treated (yellow) DCs and its MFI values of control and VitD3 treated group ($p<0.05$, n=2)

3.2. VITD3 NEGATIVELY AFFECT PLASMACYTOID DC (PDC) DIFFERENTIATION

pDC is another DC subset that is main source of type I IFN. The effect of VitD3 on its differentiation was determined at day 14 with flow cytometry. Cells were stained with CD45 (APC- A700), CD1c (PE/Cy7), CD123 (PE/Dazzle), CD303 (PerCp/Cy5.5) and HLA-DR (APC- A750). $CD45^+$ $CD123^+$ $CD303^+CD1c^-$ cells were accepted as plasmacytoid DCs.

pDC percentage and MFI values (from geometric mean) of HLA- DR marker were compared between VitD3 treatment and control group (Figure 3.9, Figure 3.10.). VitD3 treated group had less (20 percent) pDCs percentage than its control group (34.4 percent). But HLA-DR expression was similar between two groups (Figure 3.12.).

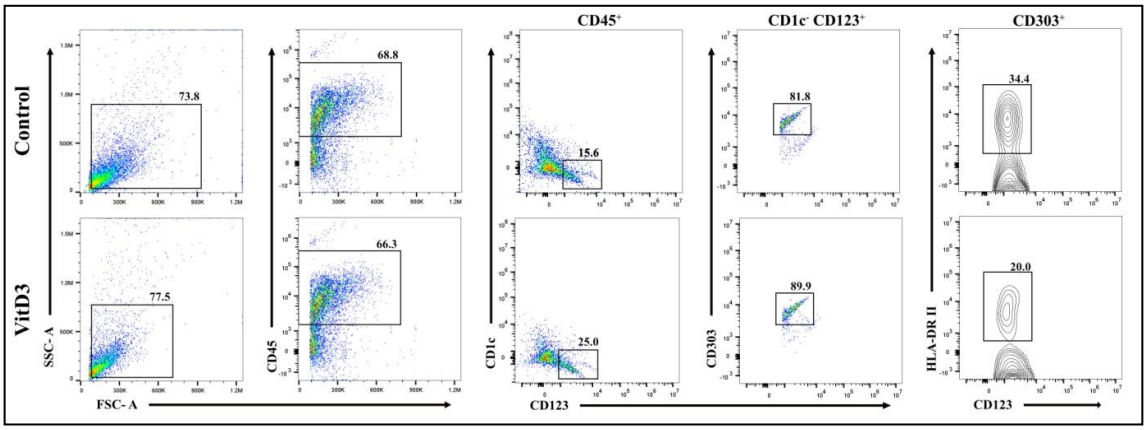

Figure 3.9. The gating strategy of CD1c^{-} CD123^{+} CD303^{+} plasmacytoid DCs based on HLA- DR II receptor. Upper alignment demonstrates control group, below alignment demonstrates VitD3 treated groups

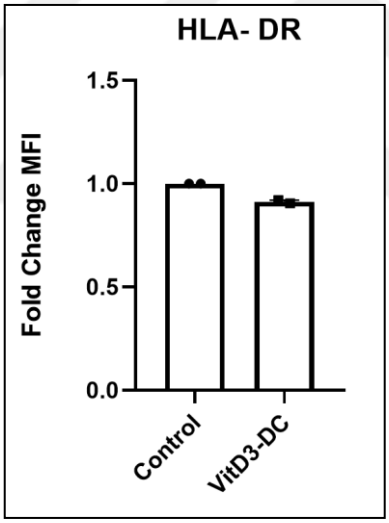

Figure 3.10. Flow cytometry analysis of pDC fold change MFI values of control and VitD3 treated groups based on HLA- DR marker ($p<0.05$, n=2)

3.3. STIMULATION OF DC WITH LPS AND R848

It was showed that VitD3 was affected DC differentiation based on HLA-DR and CD86 expressions on HSC derived DCs in this study. In this part of thesis, functional differences between HSC-DCs and their control groups were observed. To determine how DCs are able to be stimulated by TLR ligands, LPS and R848 were used. Each of these molecules are TLR ligands and they can bind TLR4 and TLR7/8, respectively. These two TLR

stimulant have been used for DC vaccine studies [195–197].

DCs were treated with LPS (100 ng/ml) [198] and R848 (2 μg/ml) [199] in the culture condition for overnight. When HLA-DR^{high} CD11c^{+} gates of control and VitD3 groups were compared with each other (Figure 3.11.), HLA-DR expressions were increased for each control and stimulant groups but VitD3 treated groups' expressions are not as high as their control groups (Figure 3.12.).

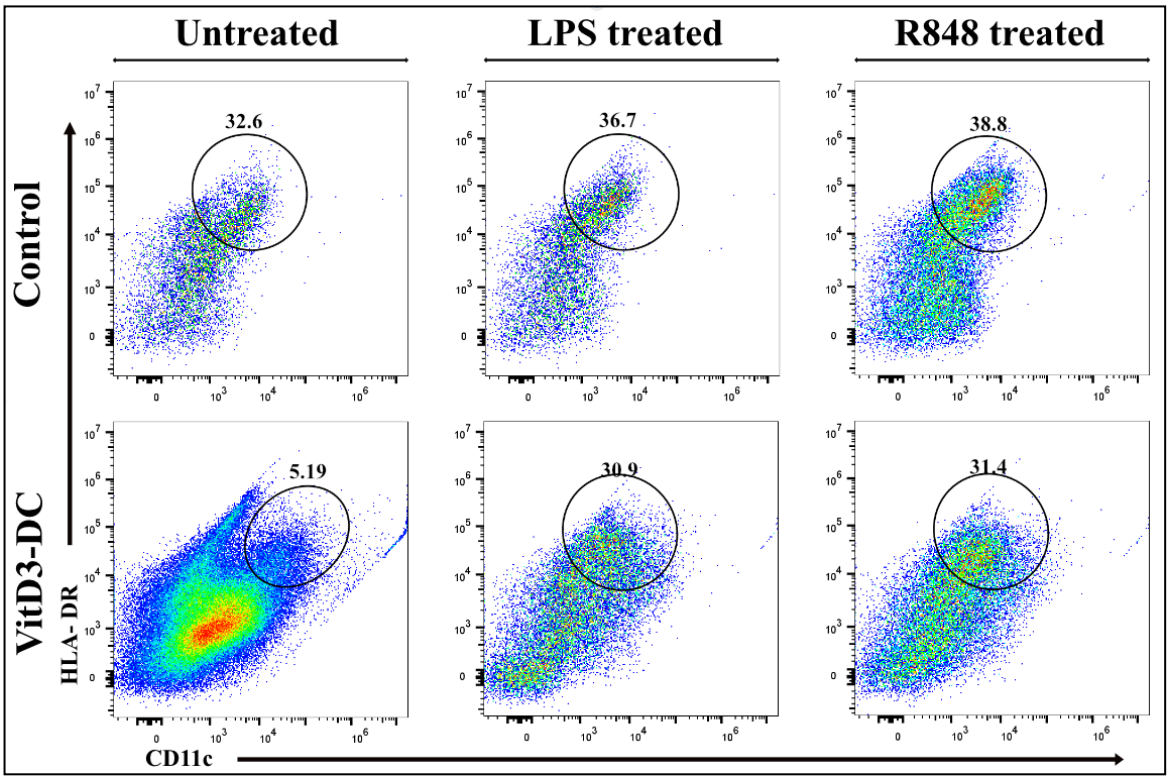

Figure 3.11. The gating strategy of untreated, LPS and R848 treated HLA- DR^{high}, CD11c^{+} cells from alive cells without debris

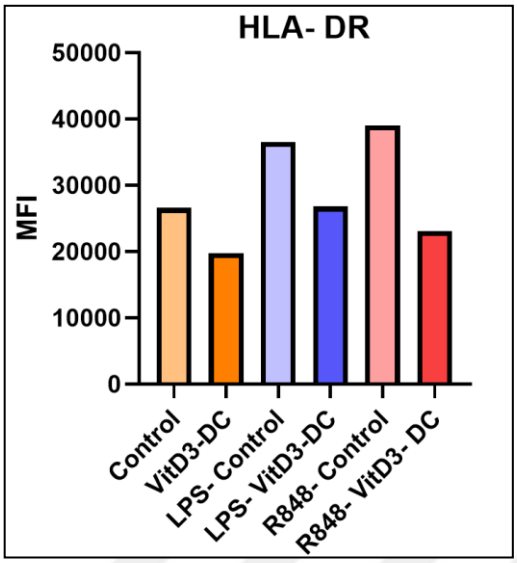

Figure 3.12. MFI value graphs of HLA- DR II marker of LPS and R848 treated control and VitD3 groups

3.4. VITD3-DC INDUCE LESS T CELL STIMULATION THAN CONTROL GROUP

DCs are the main T cell stimulator among the other APC [200,201]. Determining how VitD3-DC and control group induce T cell stimulation is important to find how VitD3 affects HSC derived DC differentiation and their function. To address the question, total naïve T cells ($CD3^+$) and $CD8^+$ naïve T cells were isolated from PBMCs and stained with CD3 (APC) and CD8 (PE) markers to conform purity. 51 percent $CD3^+$ T cells (Figure 3.13) and 72.8 percent $CD8^+$ T cells were isolated (Figure 3.14.).

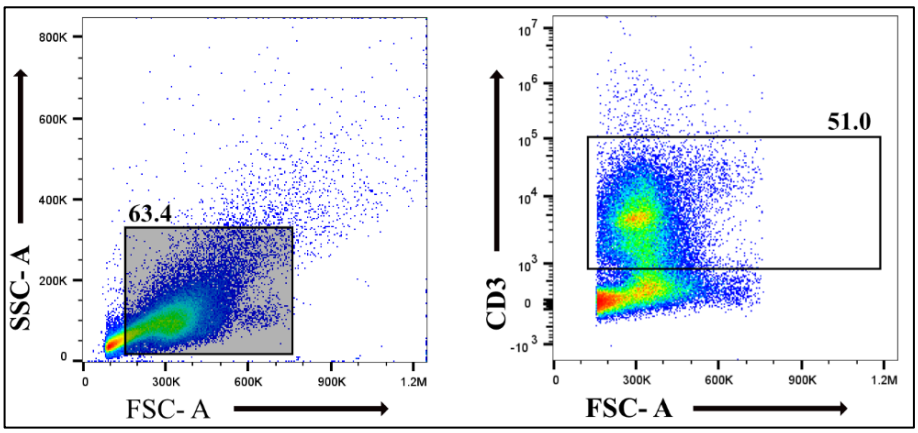

Figure 3.13. $CD3^+$ cell population without debris

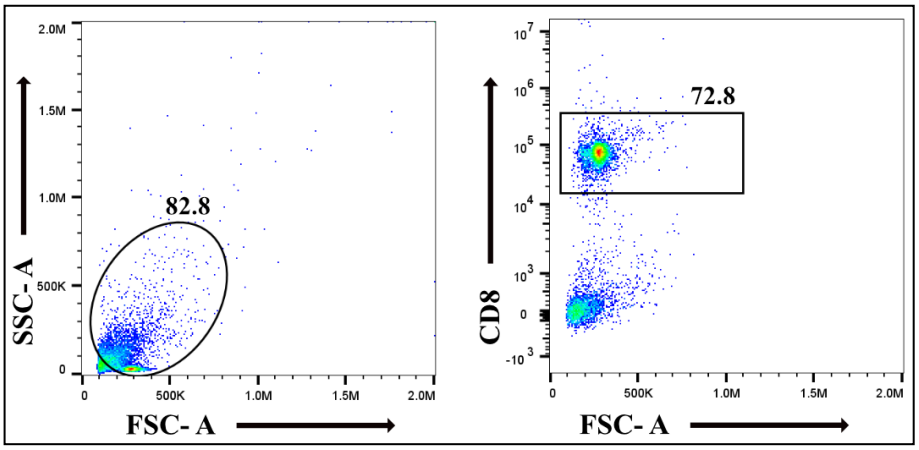

Figure 3.14. $CD8^+$ cell population without debris

Following to $CD3^+$ T cell isolation, two different experiments were planned to find how VitD3-DC induce T cell proliferation. In the first experimental plan, allogenic CFSE labelled naïve T cells combined with VitD3-DCs and their control group. In the second experiment set up, allogenic CFSE labelled T cells were stimulated with PMA-Ionomycin and then combined with VitD3-DCs and their control groups for three days. After the incubation CFSE low cells were accepted as proliferated cells and their percentage were compared between VitD3 and control groups DC-T cell coculture. It was determined that VitD3-DC (3.72 percentage) induce less naïve T cell stimulation than control group (7.46 percentage) beside that VitD3-DCs blocked the T cell proliferation after PMA-Ionomycin stimulation. Control groups had 43.6 percentage CFSE low cells but VitD3-T cells had 34 percentage CFSE low T cells (Figure 3.15.). From the statistical analysis of PMA-Ionomycin stimulated and non-stimulated groups, both of the groups VitD3 treatment blocked the proliferation capacity of $CD3^+$ T lymphocytes (Figure 3.16.).

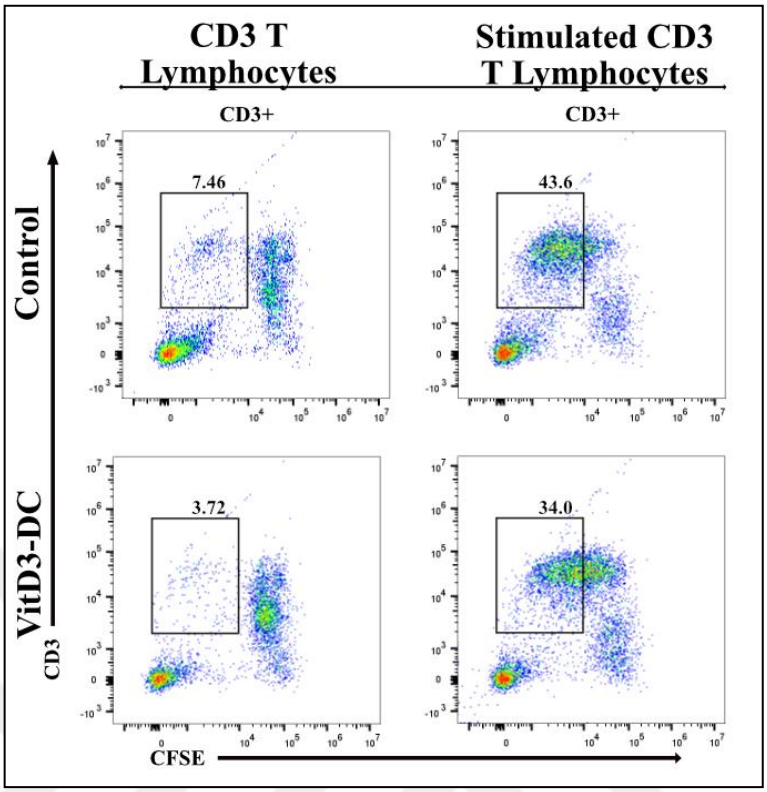

Figure 3.15. $CD3^+$ $CFSE^{low}$ gate demonstration of unstimulated (left) and PMA-Ionomycin stimulated (right) $CD3^+$ T lymphocytes which were cocultured with control and VitD3 DCs

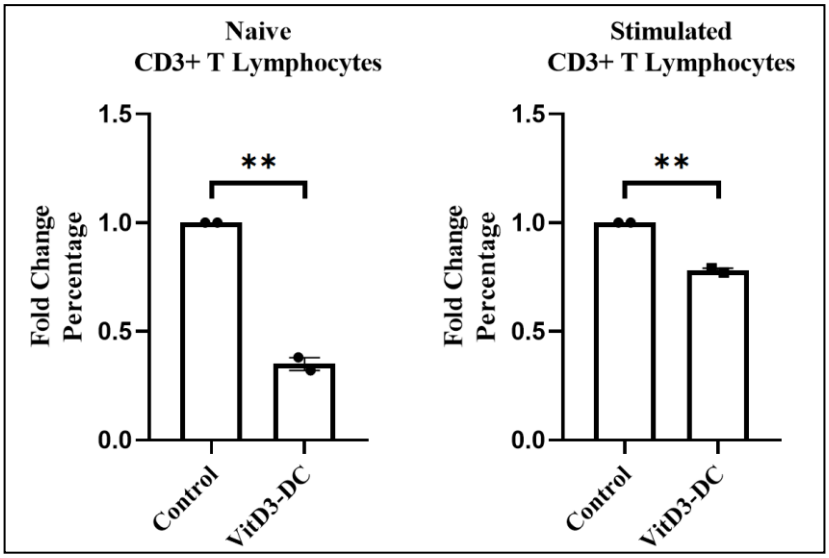

Figure 3.16. Flow cytometry analysis of $CD3^+$ CFSE low gate of naïve (left) and PMA-Ionomycin stimulated (right) fold change percentage comparisons of control and VitD3 groups($p<0.05$, n=2)

3.4.1. Intracellular Staining

As is known, activated T cells produce TNF-α and IFN- γ which are inflammatory cytokines. Thus determination production of TNF-α and IFN- γ by naïve or stimulated total $CD3^+$ T cells and $CD8^+$ T cells after DC: T cell coculture as another way to find VitD3-DCs function.

VitD3-DCs and control group DCs were cocultured with naive and PMA-Ionomycin stimulated $CD3^+$ and $CD8^+$ T lymphocytes and stained with CD3 (APC), CD4 (PC5.5), CD8 (PE), INF-γ (PC7) and TNF- α (ECD) markers. VitD3-DCs induced less TNF-α and IFN-γ production than control DCs in total $CD3^+$ T lymphocytes (Figure 3.17.). In addition to that VitD3-DCs more suppressed IFN-γ production in the PMA- Ionomycin activated $CD3^+$ total T cells than control group. But suppression rate of TNF- α production in the PMA- Ionomycin activated $CD3^+$ total T cells was similar with VitD3-DCs and control group (Figure 3.18.).

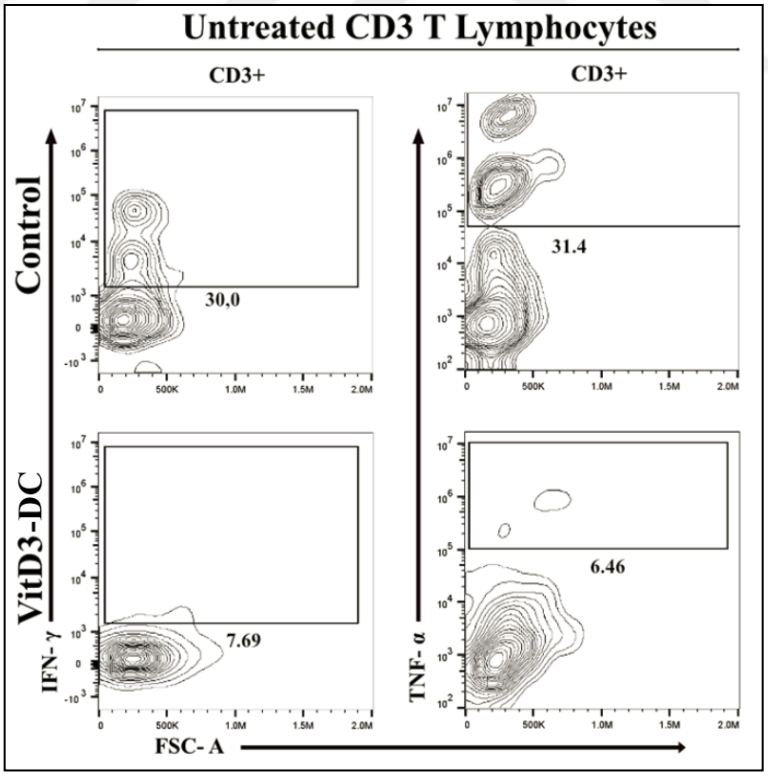

Figure 3.17. Gating strategy of untreated $CD3^+$ T lymphocytes based on INF-γ and TNF- α markers

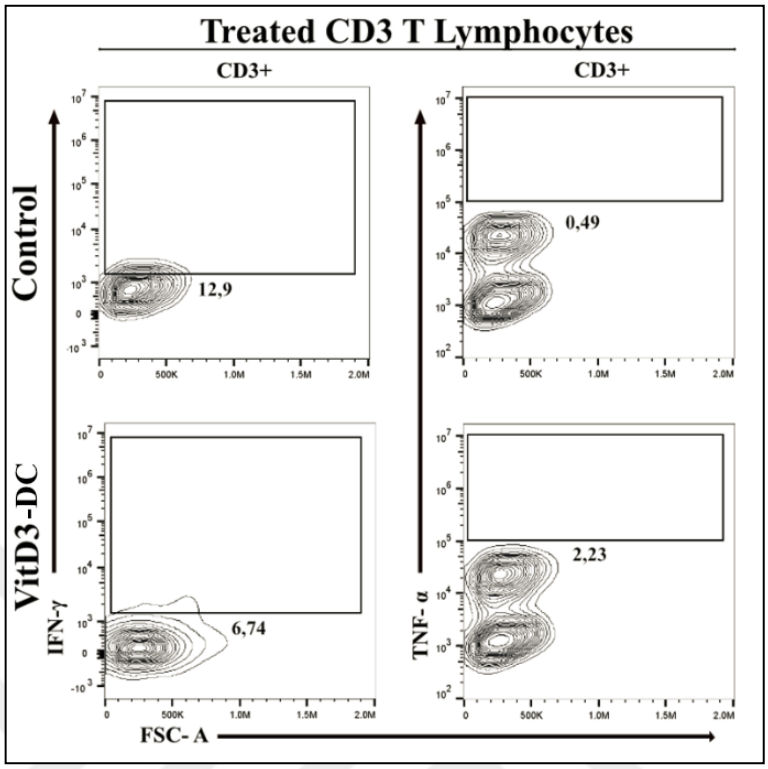

Figure 3.18. Gating strategy of PMA- Ionomycin treated $CD3^+$ T lymphocytes based on INF-γ and TNF- α marker

Same experimental strategies were used for naïve and PMA- ionomycin activated $CD8^+$ T cells. Similar result was found that VitD3-DCs had less capacity for IFN- γ and TNF- α induction in naïve $CD8^+$ T lymphocytes and suppression of these cytokines in activated $CD8^+$ T cells (Figure 3.19. and Figure 3.20.).

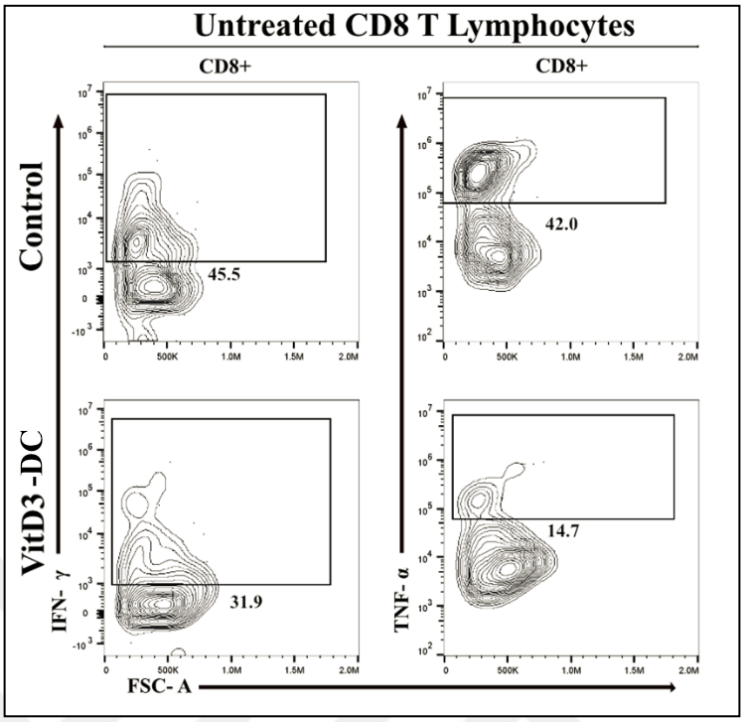

Figure 3.19. Gating strategy of untreated $CD8^+$ T lymphocytes based on INF-γ and TNF- α markers

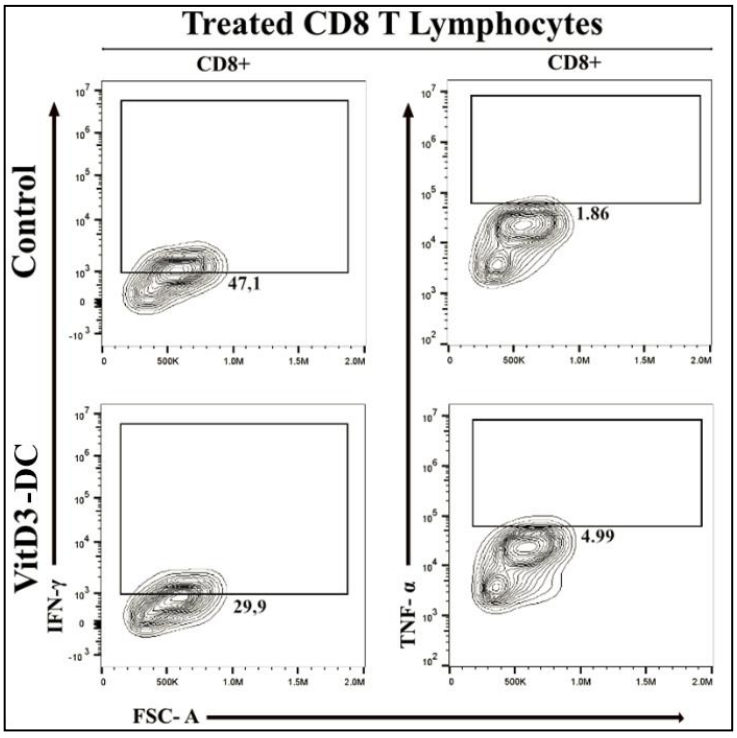

Figure 3.20. Gating strategy of PMA- Ionomycin treated $CD8^+$ T lymphocytes based on INF-γ and TNF- α markers

3.4.2. PI-Annexin

$CD8^+$ T cells are also known as cytotoxic T cells, which can kill cancer cells after the stimulation by APCs. This function of $CD8^+$ T cells were used to find out function of VitD3-DCs. Control DCs and VitD3-DCs were loaded with A549 cancer cell lysate, stimulated with R848 (TLR 7/8 ligand) and incubated with naïve $CD8^+$ T cell for 5 days then $CD8^+$ T cells combined with A549 cells and apoptotic cells were determined with PI-Annexin V staining.

The late apoptotic area of graph (Q2) (PI^+ $Annexin^+$) VitD3 treated population percentage was (3.13) is lower than the control group (8.73). From the early apoptotic area (Q1) (only $Annexin^+$) VitD3 treated $CD8^+$ T cell population percentage (22.9) lower than the control group (42.5) (Figure 3.21.).

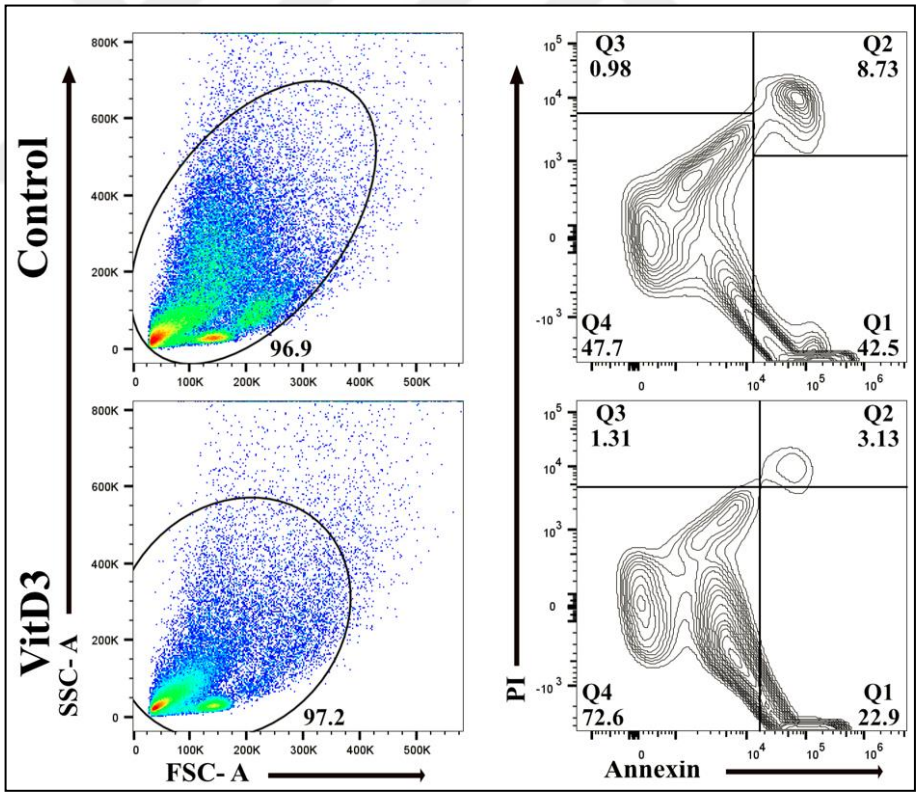

Figure 3.21. Gating strategy of Annexin/PI staining of A549 lysate and A549 encountered R848 stimulated DC- $CD8^+$ T cell coculture

4. DISCUSSION

DCs are main T cell activator and antigen presenting cells, while the semi-mature or immature tol-DCs are important for the tolerance of the peripheral reactive T cell suppression. Various mechanisms are used for T cell suppression by tol-DCs. For example; decrease in their T cell stimulatory molecules that provides the suppression of immune reactivity [202,203], T cell anergy [204] or de nova induction of Tregs due to the decrease in the co-stimulatory molecules and cytokines [176]. These mechanisms suppress T cell proliferation [205] as well as the secreted cytokines that become inhibited [206,207]. Thus, tol-DCs have been used for the treatment of autoimmune diseases [161–163]. Monocytes are used to generate tol-DCs in the presence of IL-4 (Interleukin-4), GM-CSF (granulocyte/macrophage colony stimulating factor), and different tolerogenic agents [180,185,208–212].

Nonetheless, cDCs constitute a better DCs subtype for T cell stimulation, therefore, adaptation of cDCs for tol-DC studies may help to create more effective treatment based on tol-DC vaccine. In our study, we aimed to find a novel way to generate tol-DCs. We used HSCs to generate tol-DCs in the presence of Flt-3L (FMS- like tyrosine kinase 3 ligand), SCF (Stem cell factor), IL-4, and GM-CSF. Vitamin D also was used as a tolerogenic agent. VitD3 was chosen because it has been used also in monocyte-derived tol-DC studies. Furthermore, it is known that both Vitamin D receptor (VDR) and 1α25 (OH) $_2D_3$ (active form of VitD3) have immunosuppressive effects on immune-mediated diseases such as systemic lupus erythematosus [213], multiple sclerosis [214], encephalomyelitis, diabetes mellitus, and allograft rejection [215–217].

In this thesis, it was found that VitD3 negatively affects HSC-derived DC differentiation and suppress their T cell activation capacity by decreasing HLA-DR expression, TNF-α, and IFN- γ production. Our findings are correlated with those from the tol-moDCs studies in the literature. It is shown that from other studies, VitD3 [181,210,218,219] inhibits differentiation of moDCs based on the reduced HLA-DR expression [181,205,220–222].

HLA-DR is the first and the essential signal for the antigen presentation of DCs to T lymphocytes which means that T cell stimulation capacity of DC mainly depends on this interaction. After VitD3 treatment, expressed HLA-DR was decreased from DCs like the

literature, can be observed from figure 3.4. CD86 is one of the co- stimulatory molecules found in the mature DCs and it is important for the second signal of T cell activation process. Depending on the experiments, CD86 expression of tol-moDCs is lower than control groups. Only in the experiment by Tatjana Nikolic and Bart O. Roep [223], the expression of CD86 is significantly increased in response to VitD3 in moDCs, in agreement with our results (Figure 3.6.). It is known that CD86 binds CD28 co-stimulatory receptor on naïve T cells, but also CD86 binds to CTLA-4, which is co-inhibitory receptor on T cells. It thought that VitD3 may induce CD86 expression on HSC-derived VitD3-DCs and CD86 binds CTLA-4 on T cells to induce T cell suppression.The most tol-DCs and tumour cells increase the expression of PD-L1 inhibitory molecule [224–226]. PD-L1 which are expressed from DCs binds to PD-1 receptor on T cells that induce T cells' exhaustion and promotes immune resistance [227,228]. From the Figure 3.7. VitD3 did not changed the expression level of PD-L1 on DCs.

To examine VitD3-DCs effects on T cell proliferation and activation, cells were cocultured with $CD3^+$ T lymphocytes and stained with carboxyfluorescein succinimidyl ester (CFSE) that is a fluorescent cell staining dye. CFSE can pass through cell membrane and can cut off acetate groups of fluorescein via the esterase activity, thus cells can fluoresce, but dye lose its translocation property through the cell membrane. For each cell division, dye passes to the daughter cell and, thereby, its amount will be split during each division, so the intensity of dye will decrease continuously. To detect the divided T lymphocyte population, CFSE low area was selected and the dye intensities were evaluated (Figure 3.16). The percentage of VitD3-DC group was significantly lower than the control group which means that, VitD3-DCs had less of $CD3^+$ T cell activation capacity. This data is correlated with moDC studies [184,205,229]

The profile of the secreted cytokines by the T lymphocytes was also determined in this study. $CD3^+$ and $CD8^+$ T lymphocytes were stained with IFN- γ and TNF-α. Both of T lymphocyte types were significantly decreased in the production of IFN- γ [205] and TNF-α after VitD3 treatment (Figure 3.17., for $CD3^+$ T lymphocytes, Figure 3.19. for $CD8^+$ T lymphocytes). It was also investigated whether VitD3-DCs can maintain this effect on stimulated T lymphocyte or not. Thus, VitD3-DCs and control DCs were cocultured with PMA- Ionomycin stimulated T lymphocytes (Figure 3.18. for $CD3^+$ T lymphocytes, Figure 3.20. for $CD8^+$ T lymphocytes). IFN- γ secretion was significantly suppressed again for

both of the VitD3-DCs in the coculture. The expression of TNF-α, CD3, and CD8 stimulated groups did not changed compared to that of the unstimulated groups. IFN-γ has a significant role based on $CD8^+$ T cell expansion, contraction, and in the memory phases of $CD8^+$ T cell responses. Thus, VitD3-induced DCs displayed a decrease in IFN-γ cytokine for both of naive and stimulated T lymphocytes. This means that, VitD3 stimulation provides a decrease of T lymphocyte proliferation and that it can also change the secreted cytokine profile.

On the other hand, TNF-α acts as a immunomodulatory cytokine that can be either a pro-inflammatory or anti- inflammatory agent, so it may induce apoptosis of highly activated effector cells or may have a divergent effect on regulatory T cells [230–232]. $CD8^+$ T cell stimulation capacity of VitD3-DCs as well as their antigen presenting capacity was determined together with anti-tumor effect of $CD8^+$ T cell. R848 stimulated VitD3-DC and control groups of DCs were loaded with A549 human lung carcinoma lysate overnight and then cocultured with naïve $CD8^+$ T lymphocytes. After 5 days, DC-$CD8^+$ T cell population were cocultured with A549 cells. Based on the Annexin V and PI data (Figure3.21.) both of VitD3-treated cells induced significantly lower early and late apoptotic A549 cell populations compared to the control groups. This suggests that, VitD3-DCs may have decreased the tumor destruction capacity of $CD8^+$ T lymphocytes due to their lowered antigen presentation capacity in response to VitD3 treatment, therefore, $CD8^+$ T lymphocytes did not destroy A549 cells like they did in the control group.

These results can be attributed to some causes. For example, produced tol-DC cytokines may change the fate of $CD4^+$ T lymphocytes from Th1/Th17 to Th2 [189,233]. The shifting process from Th1 to Th2 may provide a beneficial effect to avoid tissue damage of Th1 and secreted cytokines such as IL-2, IFN-γ, TNF-α, IL-5 may also be inhibited [234,235]. On the other hand, tol-DCs can decrease the proliferation of autoreactive T lymphocyte because of early and late apoptosis or autoreactive T cells might leaned to be Tregs [188,236,237] which may begin to secrete IL-10 [205].

At the same time, if VitD3 treatment increases the activity from the FoxP3 promoter region, leading to the increase in CTLA-4 expression [188,190,233,238] (a key factor for the regulatory mechanisms of Treg cells), the observed acquirement of a more tolerogenic property can be understood [188]. IL-10 blocks the synthesis of IL-12 by the DCs that promotes a decrease in the co-stimulatory molecule secretion, favoring a more tolerogenic

potential. IL-10 may also inhibit pro-inflammatory mediators such as TNF- α, IL-6, IL-8, IL-1β, GM-CSF, and G-CSF.

In this study, T cell proliferation, the secretion of TNF- α, and IFN-γ cytokines were decreased, suggesting that a probability for Treg conversion should also be addressed in future studies.

On the other hand, some of DCs may account for a killing mechanism, which are called killer DCs (KDC). Also, bone marrow derived DCs can kill T lymphocytes or tumor cells using Fas ligand (FasL) [239–241] or TNF-related apoptosis- inducing ligand (TRAIL) [242]. Some DCs may express IDO and that may induce IDO-dependent apoptosis of T lymphocytes [243]. Hence, these genes may be investigated in future studies.

In a nutshell, active form of VitD3 treatment promotes an inhibitory effect on DC maturation and VitD3-DCs have tolerogenic phenotype compared to their control groups. It is crucial for the regulation of immune response based on immune homeostasis, exhaustion, depletion, anergy of T lymphocyte or Th differentiation, and Treg production. Thus, HSC-derived VitD3-DCs may be an alternative for tol-cDCs generation, in the sense that they may be used as an immune suppressor cell-based therapy modality to treat autoimmune diseases and to avoid rejection or to prolong graft survival.

5. CONCLUSION

In this study, active form of VitD3 treatment promotes an inhibitory effect on DC maturation and these cells have a tolerogenic phenotype compared to their control groups. After VitD3 treatment the presentation capacity of DCs to T lymphocytes were decreased and that caused reduced T cell proliferation and cytokine secretion. As a consequence of this, VitD3- DCs are very significant on the regulation of the immune response, these may be an alternative tool for tol-cDC generation and may be used for autoimmune disorders and to prevent rejection or prolong graft survival.

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