THE ROLE OF CD8 T-CELLS IN THE DIFFERENTIATION OF TNF/INOS-PRODUCING DENDRITIC CELLS AND TH1 RESPONSES

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Abstract

In this study, we showed that activated human CD8 T-cells could induce DCs to produce IL-12p70 *in vitro* and this interaction also resulted in the production of a cytokine milieu that promoted the differentiation of monocytes into TNF/iNOS-producing (Tip) DCs. These Tip-DCs expressed high levels of MHC class II and upregulated co-stimulatory molecules CD40, CD80, CD86 and the classical DC maturation marker CD83. Tip-DCs exhibited T-cell priming ability and were capable of further driving Th1 responses, through their expression of TNF-α and iNOS, by priming naive CD4 T-cells for IFN-γ production. Finally, we showed that the ability of CD8 T-cells to differentiate monocytes into Tip-DCs also occurred in an *in vivo* mouse model of allergic contact hypersensitivity (CHS). This differentiation and activation of Tip-DCs during CHS responses were observed to be compromised in $\beta_2 m^{-/-}$, IFN-$\gamma^{-/-}$ and CCR2$^{-/-}$ mice and mice that were depleted of CD8, but not CD4, T-cells. In particular, the presence of Tip-DCs was significantly increased in mice that have been treated with a Th1-inducing topical sensitizer, DNCB, but not in mice that have been treated with a Th2-inducing sensitizer, TMA. Collectively, our results identify a role for CD8 T-cells in orchestrating Th1-mediating signals, not only through the rapid initiation of DC IL-12p70, but also through the differentiation of monocytes into Tip-DCs.

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Abbreviations

7AAD	7-amino-actinomycin D
AF488	Alexa Fluor 488
AF647	Alexa Fluor 647
AP	Alkaline Phosphatase
APC	Antigen presenting cell
APC	Allophycocyanin
BSA	Bovine serum albumin
β_2m	beta-2-microglobulin
CCR	C-C motif Receptor
CD	Cluster of differentiation
CHS	contact hypersensitivity
CTL	Cytotoxic T lymphocyte
DC	dendritic cell
DMEM	Dulbecco's modified eagle's medium
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme Linked Immunosorbent assay
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
FITC	Fluorescein-5-isothiocyanate
Fig	Figure
GM-CSF	Granulocyte Macrophage-Colony Stimulating Factor
HBSS	Hanks Buffer Salt Solution
IFN	Interferon

IL	Interleukin
iNOS	inducible nitric oxide synthase
LPS	lipopolysaccharide
M-CSF	Macrophage Colony Stimulating Factor
MHC	Major Histocompatibility Complex
MFI	Mean Fluorescence Intensity
NK	Natural Killer
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PB	Pacific Blue
PBS	Phosphate buffered saline
PE	Phycoerythrin
PerCP	Peridinin-chlorophyll protein
PFA	Paraformaldehdye
PMA	phorbol 12-myristate-13-acetate
pNPP	p-nitrophenyl phosphate
Myd88	Myeloid differentiation primary response gene 88
PBS	Phosphate Buffered Saline
RPMI	Roswell park memorial institute
RT-PCR	Real Time Polymerase Chain Reaction
TCR	T-cell receptor
TGF-β	Transforming growth factor beta
Th	T-helper
TLR	Toll-like receptor
TNF	tumor necrosis factor
WT	Wild type

Publications

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Li R, Cheng C, **Chong SZ**, Goh YQ, Locht C, Kemeny DM, Angeli V, Wong WSF, Alonso S (2012) Attenuated B. pertussis BPZE1 protects against allergic 1 asthma and contact dermatitis in murine models. *Allergy* (manuscript in revision)

CHAPTER 1: Introduction

1.1 Immunity

"The sick and the dying were tended by the pitying care of those who had recovered, because they knew the course of the disease and were themselves free from apprehensions. For no one was ever attacked a second time, or not with a fatal result"

--- Thucydides, 430 B.C. A description of the plague which hit Athens.

Immunity was originally described as a condition that permits resistance and protection from a disease. However, it is now clear that many of the crucial mechanisms governing a body's immune response to infections are also involved in the individual's response to non-infectious foreign substances. Importantly, the immune system consists of different layers of defenses with increasing specificity which can be subdivided into the innate and adaptive immune response.

1.1.1 Innate Immunity

Innate immunity is the natural resistance a person is born with which does not discriminate between most foreign agents. It provides resistance through several physical, chemical, and cellular approaches. The first consists of physical barriers such as skin and mucous membranes while the subsequent general defenses include cytokines,

complement, fever, and phagocytic activity. Phagocytes such as dendritic cells (DCs) and macrophages express pattern recognition receptors (PRR) such as Toll-like receptors (TLRs) which bind and respond to common molecular patterns expressed on invading microbes. Granulocytes (i.e. neutrophils, basophils and eosinophils) can release a variety of toxic substances that kill or inhibit growth of bacteria and fungi while basophils, eosinophils and mast cells secrete histamine which is important for parasite eradication. Lastly, Natural Killer (NK) cells destroy compromised host cells, such as tumor cells or virus-infected cells by recognizing low levels of MHC class I (major histocompatibility complex) expressed on cells. Through these approaches, the innate immune system can prevent the colonization, entry, and spread of microbes. However, it does not confer long-lasting immunity to the host as they operate through receptors encoded in the genome; therefore our body has a second line of defense known as the adaptive immunity.

1.1.2 Adaptive immunity

Thought to have arisen in the first jawed vertebrates, the adaptive or "specific" immune system is initiated by cells of the innate immune system, such as DCs. The adaptive immune response caters the ability to recognize and remember specific pathogens so as to mount stronger defense each time the pathogen is encountered. The system is highly adaptable because of somatic hypermutation, a process of accelerated somatic mutations, and V(D)J recombination, which is an irreversible genetic recombination of antigen receptor gene segments. This mechanism allows a small number of genes to generate a

vast number of different antigen receptors, which are then uniquely expressed on each individual lymphocyte. B cells and T cells are the major types of lymphocytes. T cells provide important cell mediated immunity by participating in the cytolysis of infected cells through the recognition of antigen presented on MHC class I. They do so through the T cell receptor (TcR) which is composed of two different polypeptide chains, α and β, linked together by disulphide bonds. They can also recognize antigen on MHC class II molecules and provide help to other immune cells by secreting cytokines. On the other hand, B cells provide humoral immunity by producing antibodies or immunoglobulins (Ig) which are used by the immune system to identify and neutralize foreign particles. Like T cells, B cells express a unique B cell receptor (BCR), which recognizes and binds to only one particular antigen. While T cells recognize their cognate antigen in a processed form such as a peptide in the context of a MHC molecule, B cells recognize antigens in their native form. Once a B cell encounters its antigen, it receives help from T cells which results in isotype class switching from IgM to either IgG, IgA, IgE isotypes. In this way, the adaptive immune system allows the formation of memory such that these cells can be called upon to respond quickly upon a re-infection; while the host experiences few, if any, symptoms.

1.2 CD8 T-cells

The role of CD8 T-cells was first discovered when it was demonstrated in 1960 that lymphocytes, as opposed to antibodies made from B-cells in sera, destroyed allogeneic targets *in vitro* (GOVAERTS, 1960). Subsequently, Golstein, Wigzell, and colleagues

(Golstein et al., 1971) demonstrated that these lymphocytes were both cytotoxic and specific. This killing mechanism was then further explained by Rolf Zinkernagel and Peter Doherty (Zinkernagel and Doherty, 1974a; Zinkernagel and Doherty, 1974b), who observed that cytotoxic T-cells exert their killing functions by using their TCRs to recognize antigenic peptides presented on MHC class I. Finally, depletion of Ly-2 (CD8α) and Ly-3 (CD8β) bearing lymphocytes by treatment with anti-sera and compliment abolished cell mediated cytotoxic function, thereby establishing the cytotoxic role of CD8 T-cells (Cantor and Boyse, 1975; Kisielow et al., 1975; Shiku et al., 1975)

1.2.1   Activation of CD8 T-cells

By virtue of a defined set of homing receptors such as CD62L and the chemokine receptor CCR7, naive CD8 T-cells circulate between the blood and secondary lymphoid organs. Upon encounter with antigen presented on DCs, CD8 T-cells proliferate and differentiate into effector T-cells. These activated effector CD8 T cells express killing molecules such as Fas Ligand (FasL) and secrete the pore forming molecule perforin and granule enzymes granzyme A and B (Berke, 1995). They then induce cytolysis of infected cells either by the granule exocytosis pathway through co-ordinated delivery of perforin and granzymes into target cells, or by upregulation of FasL which initiates programmed cell death by aggregation with Fas (CD95) on target cells. Notably, while all nucleated cells express MHC class I, CD8 T-cells only kill infected or tumor cells that present the appropriate antigenic peptides on MHC class I. Effector CD8 T-cells also elaborate cytokines, including IFN-γ and TNF-α, as well as chemokines, such as

RANTES, MIP-1α and MIP-1ß, that function to recruit and activate mononuclear cells and granulocytes (Harty and Bevan, 1999).

During an infection, the CD8 T-cell response typically peaks about 7 days after encounter with antigen and is followed by a contraction phase, when 90–95% of the effector cells die in the ensuing days and weeks and the remaining 5–10% become long-lived memory cells (Harty and Badovinac, 2008; Joshi and Kaech, 2008; Prlic et al., 2007). These memory cells are able to mobilize their effector mechanisms very rapidly upon re-encounter with the same antigen which serves as a basis for protective vaccination against infectious diseases. Human CD8 T-cells can be distinguished into naive, effector and memory T-cells based on the expression of CCR7 and CD45RA; naive CD8 T-cells are CCR7$^+$ and CD45RA$^+$; effector memory CD8 T-cells are CCR7$^-$and CD45RA$^-$ while central memory CD8 T-cells are CCR7$^+$ and CD45RA$^-$ (Sallusto et al., 1999). In addition, a population of "memory revertants" has been shown to express CD45RA but are CCR7$^-$. In mice, the expression of CD44 is also an indicator of the memory status of CD8 T-cells (Gerberick et al., 1997).

1.2.2   CD8 T-cell subsets

Just like the Th1/Th2 classification of CD4 T cells, activated CD8 T cells can also be broadly classified into Tc1 or Tc2 cells upon activation by stimuli based on their cytokine expression profiles. Tc1 cells secrete IFN-γ and TNF-α while Tc2 cells predominantly secrete IL-4 and IL-5 (Croft et al., 1994; Noble et al., 1995; Sad and Mosmann, 1995).

Despite the similarities between CD4 and CD8 T-cells, CD4 Th1 cells require the presence of IL-12 for IFN-γ production while CD8 Tc1 cells are capable of producing IFN-γ independently of IL-12 (Carter and Murphy, 1999; Croft et al., 1994). Since CD8 T-cells are predominantly IFN-γ producers and rarely make Th2 cytokines, they are strongly biased towards the Tc1 phenotype (Fong and Mosmann, 1990). However, under the appropriate conditions such as in the presence of IL-2 and IL-4, CD8 T-cells can produce significant amounts of IL-4 thereby resembling the Tc2 phenotype (Seder et al., 1992; Vukmanovic-Stejic et al., 2000). Tc2 cells have also been observed clinically, especially in lesions of lepromatous leprosy patients (Salgame et al., 1991), in asthma (Ying et al., 1997), in chronic obstructive pulmonary disorder (Barczyk et al., 2006), in graft-versus-host disease (Fowler et al., 2006) and in several forms of cancer (Dobrzanski et al., 2006; Ito et al., 2005a; Sasaki et al., 2007). Recently, CD8 T cells expressing IL-17 have also been documented and have been termed Tc17 cells (Burrell et al., 2008; Yen et al., 2009). As with cytolytic effector mechanisms, expression of cytokine molecules by CD8 T cells is tightly regulated through TCR-dependant signals (Harty et al., 2000).

1.3 Dendritic cells

Dendritic cells (DCs) were discovered in 1973 by Ralph Steinman and Zanvil Cohn when they observed an unusual looking population of cells with a distinct stellate morphology. They had long cytoplasmic processes containing large spherical mitochondria which, in the living state *in vitro*, are continually elongating, retracting, and reorienting themselves, leading to a wide variety of cell shapes (Steinman and Cohn, 1973). Thereafter, the

authors found that these DCs had an unprecedented ability to activate naive T cells (Steinman and Cohn, 1974). These cells are now known as the primary instigators of the adaptive immunity as they are vital for detecting, alerting and priming the adaptive immune system to invading pathogens (Banchereau et al., 2000; Banchereau and Steinman, 1998)

1.3.1   Activation of DCs

According to the classical paradigm, DCs are located in peripheral tissues in an immature state where they act as immune sentinels, exemplified by their ability to sample their local environment for pathogens through macropinocytosis and endocytosis (Guermonprez et al., 2002; Sallusto et al., 1995). However, immature DCs are poor APCs, because they retain most of their MHC molecules intracellularly and are unable to form peptide–MHC class II complexes (Cella et al., 1997; Pierre et al., 1997). Nevertheless, upon encounter with pathogens, they undergo activation and "maturation" and migrate via lymphatic vessels to the draining lymph nodes where they interact with T-cells (Randolph et al., 2005). This maturation process also transforms immature DCs from efficient antigen capturers into professional antigen presenters through carefully orchestrated alterations in membrane traffic. Besides the downregulation of endocytosis controlled by Rho family GTPases, such as Cdc42 (Garrett et al., 2000), cathepsin S activity is activated upon maturation resulting in the release of MHC class II from Ii chain and the subsequent delivery of peptide-$\alpha\beta$ dimers to the plasma membrane (Turley et al., 2000). Internalized antigens are directed to specialized intracellular compartments

where they are degraded and subsequently loaded onto MHC class II molecules. MHC class II-peptide complexes are then directed to the surface for presentation to CD4 T cells. Mature DCs can also process and cross present antigenic peptides by transporting them via the TAP transporter to the endoplasmic reticulum (ER) where they bind to nascent MHC class I and are directed to the surface for priming of CD8 T cells (Cresswell et al., 1999; Suh et al., 1994). These mature DCs also increase the expression of co-stimulatory molecules and secrete cytokines that have an influential impact on the outcome of the adaptive immune response (Banchereau and Steinman, 1998).

1.3.2 Heterogeneity of DCs in mice

DCs are heterogeneous and exist in various locations, such as in the peripheral tissues, lymphoid organs and the blood. In mice, conventional DCs (cDCs) can be classified as migratory where they are mainly located in peripheral tissues or residential where they reside in lymphoid organs (Shortman and Liu, 2002). In lymphoid organs such as the spleen, there are three distinct populations of lymphoid resident DCs, namely $CD4^+CD8\alpha^-$, $CD4^-CD8\alpha^+$ and $CD4^-CD8\alpha^-$ (Vremec et al., 2000). Skin draining lymph nodes also contain three extra DC subsets representative of skin DC migrants derived from the periphery. These are Langerhans DCs ($CD8\alpha^{lo}CD11b^+$ $CD205^{hi}Langerin^+$) and 2 subsets of dermal DCs distinguished by their expression of CD11b and CD103 ($CD4^-CD8\alpha^-CD11b^+CD103^-$ and $CD4^-CD8\alpha^-CD11b^-CD103^+$) (Bursch et al., 2007; Ginhoux et al., 2007; Poulin et al., 2007). There are also other non-conventional DC subsets including the plasmacytoid DCs (pDCs) ($CD11c^{lo}B220^+Gr-1^+Ly-6C^+$) (Asselin-Paturel et

al., 2001; Grouard et al., 1997; Liu, 2005; Nakano et al., 2001)and interferon-producing

killer DCs (IKDCs)(CD49b$^+$NK1.1$^+$NKG2D$^+$B220$^+$CD11c$^+$) (Chan et al., 2006; Taieb et

al., 2006) which respond rapidly to viruses by the rapid production of type I interferons.

Fig 1.1 illustrates the different DC subsets that can be commonly found in the mouse.

Lymphoid resident DCs

CD4$^-$ CD8α$^-$
CD4$^-$CD8α$^-$
CD11b$^+$

CD4$^+$
CD4$^+$CD8α$^+$
CD11b$^+$33D1$^+$
IFN-γ production
Preferentially primes
CD4 T-cells

CD8α$^+$
CD4$^-$CD8α$^+$
CD11b$^-$DEC205$^+$
TLR7$^-$
Cross presentation
IL-12 production

Migratory skin DCs

Langerhan Cells
CD8^{int}CD11b$^+$
DEC205^{hi}Langerin$^+$
Epidermis
Transfer of antigens to
lymphoid resident DCs

CD11b$^+$
CD4$^-$CD8α$^-$
CD11b$^+$DEC205^{mid}
Dermis
Primes CD4 T- cells

CD103$^+$
CD4$^-$CD8α$^-$
CD11b$^-$CD103$^+$
Langerin$^+$
Dermis
Cross presentation
Primes CD8 T- cells

Inflammatory monocyte-derived DCs

Exist during
inflammatory conditions
Generated with IL-4 and
GM-CSF *in vitro*
Produces high levels of
inflammatory cytokines

Non-classical DCs

pDC
CD11c^{lo}B220$^+$
GR1(Ly6C)$^+$mPDCA-1$^+$
TLR7$^+$TLR9$^+$
Type I IFN-α
Activation induces MHC
class II^{hi} and T cell priming
ability

IKDC
CD49b$^+$NK1.1$^+$NKG2D$^+$
B220$^+$CD11c$^+$
NK cell activity
Activation inudces MHC
class II^{hi} and T cell priming
ability

Fig 1.1 Known Mouse DC subsets and their characteristics

1.3.3 Heterogeneity of DCs in humans

In humans, at least three DC subsets have been described in the blood: CD141^{+} myeloid DCs, CD1c^{+} myeloid DCs and pDCs (Collin et al., 2011; Grage-Griebenow et al., 2001; MacDonald et al., 2002). The non-classical CD16^{+} human monocyte may also be considered a blood myeloid DC (MacDonald et al., 2002). However, CD8α, the major marker used to segregate mouse cDC subsets, is not expressed by human cDCs (Dzionek et al., 2000). CD141^{+} myeloid DCs found in human blood are thought to have equivalent antigen cross-presenting function to mouse DCs that can be identified by CD103 expression in most tissues and by CD8 expression in lymphoid organs. In both species, C-type lectin 9A (CLEC9A), XC-chemokine receptor 1 (XCR1) and Toll-like receptor 3 (TLR3) are conserved on these cells (Bachem et al., 2010; Crozat et al., 2010; Jongbloed et al., 2010; Poulin et al., 2010). While tissue equivalents of the blood CD141^{+} DC have not yet been described in humans, most organs contain a sizeable proportion of migratory CD1c^{+} myeloid DCs that are distinct from macrophages (Ochoa et al., 2008; Zaba et al., 2007) and monocyte-derived DCs that express CD14 and CD209 (also known as DC-SIGN) (Angel et al., 2007). A recent study has also shown that human DC subsets in the spleen were functionally and phenotypically similar to those in human blood (Mittag et al., 2011). However due to the small numbers of such DCs in the blood, human studies of DCs are often conducted using monocyte-derived DCs generated by culturing monocytes with IL-4 and GM-CSF (Sallusto and Lanzavecchia, 1994). Fig 1.2 illustrates a comparison of human DCs with known mouse equivalents

Myeloid DCs

Human
CD141+
CLEC9A+
CD11b-
XCR1+
TLR3+
FLT3+
CD11c^{low}

Mouse
CD8+ (lymphoid)
CD103+(tissue)
CLEC9A+
CD11b-
XCR1+
TLR3+
FLT3+
CD11c^{+}

•MHC class I restricted antigens
•Cross presentation
•CD8 T cell responses

Human
CD11c^{hi}
CD11b+
CD1c+
CD1a-
(blood)
CD1a+
(tissue and
lymph node)

Mouse
CD11c+
CD11b+
M-CSFR+
CX$_3$CR1+

•MHC class II restricted antigens
•CD4 T cell responses

Monocyte-derived DCs

Human
CD14+
CD11b+
CX3CR1+
CD209+

Mouse
CD11b+
CX3CR1+
(intestine)

Tip-DCs
(inflammation)
CD11b+
CD206+
CD209+

•TNF-α and iNOS
responses
•Inflammatory
cytokines

Plasmacytoid DCs

Human
CD123+
CD303+
CD304+
CD11c-
TLR7
TLR9

Mouse
BST2+
B220+
Siglec-H+
TLR7
TLR9

•Type-I interferons
•Durable memory
responses

Fig 1.2 A comparison of known DC subsets in mice and humans

1.4   T helper responses

A principal component of the adaptive immune response is the CD4 T helper cell which differentiates according to distinct stimuli. To date, at least four different T helper subsets have been described. These main subsets are known as Th1, Th2, Th17 and Treg cells (Harrington et al., 2005; Langrish et al., 2005; Mosmann and Coffman, 1989; Sakaguchi, 2004).

1.4.1   Th1 and Th2 cells

Before the discovery of Th17 and Treg cells, effector CD4 T-cells were classically divided into two distinct lineages: Th1 cells, characterized by their production of IFN-γ, are essential for eradicating intracellular pathogens while Th2 cells, distinguished by their production of IL-4, IL-5, IL-10 and IL-13, are potent activators of B-cell IgE production and eosinophil recruitment for eradicating parasites (Mosmann and Coffman, 1989). Immune pathogenesis that results from dysregulated Th1 responses to self or commensal floral antigens can promote tissue destruction and chronic inflammation while dysregulated Th2 responses results in allergy and asthma.

1.4.2 Th17 and Treg cells

Recent studies have suggested a greater diversification of the CD4 T-cell effector repertoire than that encompassed by the Th1/Th2 paradigm. New studies that link the

cytokines IL-23 and IL-17 to immune pathogenesis previously attributed to the Th1 lineage have delineated a new effector CD4 T-cell arm — referred to as Th17 (Cua et al., 2003). Th17 cells produce IL-17, IL-21 and IL-22 and are important for immunity against extracellular bacteria and fungi species. However, excessive numbers of these cells are known to cause tissue injury in autoimmune diseases (Harrington et al., 2005; Stockinger et al., 2007). In addition, another subset known as the T regulatory (Treg) cells characterized by CD25 and Foxp3 expression were found to produce IL-10 and TGF-ß (Fehérvari and Sakaguchi, 2004). These cells are crucial for dampening the immune response during infections to prevent excessive inflammation but have also been involved in the pathogenesis of leishmaniasis, AIDS, and certain cancers (Cools et al., 2007).

1.4.3   Control of T helper responses

The response to a pathogen at the early stages of an infection or disease is typically dominated by one T cell subset, and T cell subsets may be antagonistic to each other. This highlights the importance of mounting a proper protective T helper response during an infection. Since DCs are the principle cell type that prime T cells, controlling DC activity is the key to mounting a proper T cell response. The differentiation of CD4 Th1 cells by DCs is largely mediated by IL-12p70 (Trinchieri, 2003). Additionally, IL-18 (Nakanishi et al., 2001; Salagianni et al., 2007) and type I interferons (Brinkmann et al., 1993; Parronchi et al., 1992) also contribute to Th1 polarization. Th2 polarization is mediated by IL-4 (Le Gros and Erard, 1994; Swain et al., 1990), OX40L (Ito et al., 2005b), IL-25 (Fort et al., 2001) and IL-33 (Schmitz et al., 2005). The notch ligand

families, Delta and Jagged, also regulate Th1 and Th2 polarization respectively (Amsen et al., 2004). On the other hand, DC production of TGF-ß, IL-6, IL-21 and IL-23 were shown to favour differentiation of Th17 cells (Dong, 2008; Stockinger and Veldhoen, 2007) while the presence of TGF-ß, IL-4 and IL-10 can induce Treg cells (Chatenoud, 2006).

1.4.4   Transcription factors

T-bet is the central transcription factor involved the development of Th1 cells (Szabo et al., 2000). T-bet potently activates the transcription of the IFN-γ gene, and its expression is correlated with IFN-γ production by Th1 and NK cells. IL-12 and IFN-γ triggers STAT1 and STAT4 which eventually leads to the activation of T-bet (Afkarian et al., 2002; Grogan et al., 2001; Lighvani et al., 2001). GATA-3 is the critical regulatory transcription factor involved in Th2 differentiation controlling IL-4, IL-5, IL-10 and IL-13 transcription (Ouyang et al., 1998; Zhang et al., 1997; Zheng and Flavell, 1997). STAT6 is also a central mediator of IL-4 signaling and Th2 development that acts upstream of GATA-3 (Hou et al., 1994). In contrast, STAT3, ROR-α and ROR-γ mediates the development of Th17 cells (Dong, 2008). Fig 1.3 illustrates the development of the four main T helper subsets.

IFN-γ
STAT-4
T-bet
Th1 cell

IL-4
STAT-6
GATA-3
Th2 cell

IL-10
TGF-ß
Foxp3
Treg cell

IL-17
STAT-3
RORγt
Th17 cell

Peptide-
MHC
molecule

IL-12

IL-4

TGF-ß

TGF-ß and
IL-6 (IL-1
and IL-23)

DC

**Naive
T cell**
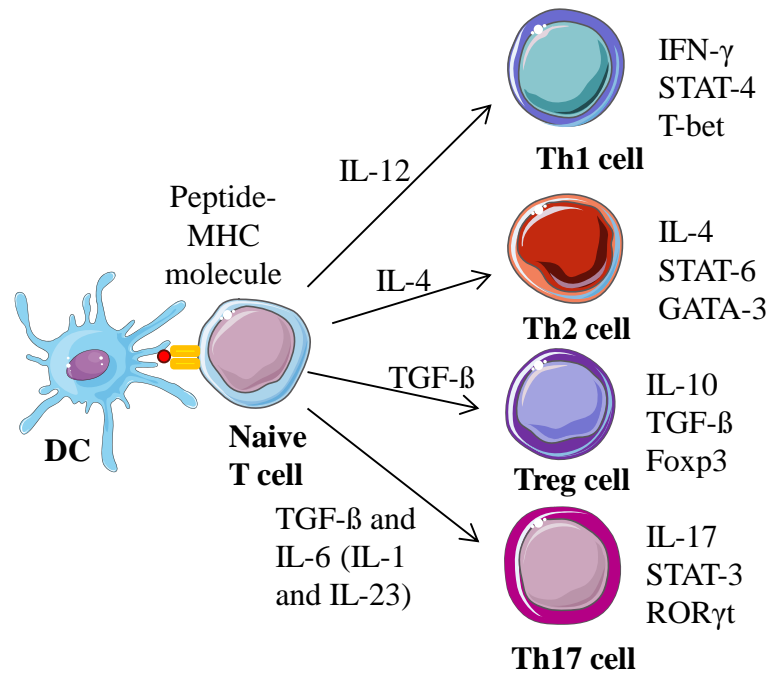
Fig 1.3 Development of T helper subsets after interacting with DCs

1.5 Interleukin-12

IL-12 is a pivotal pro-inflammatory cytokine comprising of the p35 and p40 subunits whose genes are located on separate chromosomes, namely chromosome 3 and 5 in humans, and chromosome 6 and 11 in mice respectively (Trinchieri, 1998). These two genes are regulated independently (Trinchieri, 1998) and have to be expressed in a highly coordinated fashion in the same cell to produce the biologically active heterodimer, IL-12p70 (Wolf et al., 1991). IL-12 is mainly produced by APCs (Trinchieri, 2003) and differential control of p35 and p40 subunit transcription results in modest IL-12p40, but not IL-12p70, production with a single stimulus. For example, only stimulation of DCs

with LPS together with IFN-γ or CD40 Ligand, but not either alone, results in the production of IL-12p70 (Snijders et al., 1998).

1.5.1 IL-12p40

While the Th1 inducing capacity is only restricted to the IL-12p70 heterodimer (Gubler et al., 1991; Kobayashi et al., 1989; Trinchieri, 2003), the p40 subunit is often produced in excess over the p70 heterodimer and has also been shown to have important immunological functions. For example, IL-12p40 homodimers are known to bind to the IL-12 receptor, IL-12Rβ1, which antagonizes the activity of IL-12p70 (Gately et al., 1996; Gillessen et al., 1995; Mattner et al., 1993). It also activates T-cells, acts as a chemokine that attracts macrophages (Ha et al., 1999) and induces DC migration (Khader et al., 2006). It can also induce the expression of TNF-α and iNOS in myeloid cells (Jana et al., 2003; Pahan et al., 2001).

1.5.2 Importance of IL-12p70

Notably, IL-12p70 is the primary factor produced by DCs that drives the development of Th1 responses (Heufler et al., 1996; Macatonia et al., 1995; Trinchieri, 2003). IL-12p70 binds to the IL-12 receptor which is composed of two chains, the IL-12Rβ1 and IL-12Rβ2. The IL-12 receptor is expressed by T cells and NK cells (Presky et al., 1996), DCs and B cell lines (Airoldi et al., 2000; Grohmann et al., 1998). CD4 Th1 cells, but not Th2 cells, express IL-12Rβ2 and respond to IL-12 (Rogge et al., 1997; Szabo et al.,

1997). Upon activating the IL-12 signalling cascade, IL-12p70 causes NK cells to produce IFN-γ, which enhances the bactericidal activity of macrophages during the early phases of infection (Gazzinelli et al., 1994). It also plays an important role by activating bystander resting T cells and maintaining the antigen specific Th1 response (Kubin et al., 1994). IL-12p70 also enhances the cytotoxic activity of CTLs through upregulation of adhesion molecules and transcription of genes that encode perforin and granzymes (Kobayashi et al., 1989; Trinchieri, 1998).

The importance of IL-12 in the development of functional Th1 responses has been established in several *in vivo* studies with IL-12 neutralizing antibodies, IL-12 knockout mice or STAT-4-deficient animals (Magram et al., 1996; Stern et al., 1996; Trinchieri and Scott, 1994). These examples highlight its function as an immunoregulatory cytokine that bridges the innate resistance and adaptive immunity (Trinchieri, 2003). While IL-12p35$^{-/-}$ mice are deficient in Th1 responses, IL-12p40 deficient mice are also susceptible to infections with several intracellular pathogens. However IL-12p35$^{-/-}$ mice, but not IL-12p40$^{-/-}$ mice, developed experimental autoimmune encephalomyelitis (Becher et al., 2002; Gran et al., 2002). These observations are due to the association of the p40 subunit with the p19 subunit which forms IL-23 (Oppmann et al., 2000). IL-23 promotes the differentiation of Th17 cells (Park et al., 2005) and is involved in the development of inflammatory and autoimmune associated diseases (Cua et al., 2003; Langrish et al., 2005; Murphy et al., 2003; Wiekowski et al., 2001). However IFN-γ, which can be induced by IL-12p70, has been shown to inhibit Th17-cell development and this finding

may help to explain how IL-23-induced Th17-cell-mediated pathology might be negatively regulated by IFN-γ or IL-12p70 (Bettelli et al., 2007; Weaver et al., 2007).

1.6   Modulation of DCs by immune cells

Since DCs form an important bridge between the innate and adaptive immune systems, the microenvironment where DCs receive their first signals is important for their subsequent T-cell inducing properties. For example, the lung microenvironment, mediated by lung resident pDCs, conditions DCs to induce tolerance to harmless inhaled antigens, thereby suppressing the potential of lung derived myeloid DCs to generate effector T cells (de Heer et al., 2004). During an allergic reaction, however, stimulation of lung epithelial cells via pathogen recognition receptors (PRRs) results in the production of thymic stromal lymphopoietin (TSLP) that directly activates DCs to differentiate CD4 T-cell into Th2 cells in an OX40L dependent manner (Hammad and Lambrecht, 2008; Ito et al., 2005b; Wang et al., 2006b).

1.6.1   Modulation of DCs by B-cells

It has been shown that B cells can generate Th2 promoting DCs by producing IL-10 (Mizoguchi et al., 2002; Moulin et al., 2000; Skok et al., 1999). DCs from B cell deficient mice were shown to produce increased levels of IL-12 and exhibit enhanced Th1 responses (Moulin et al., 2000). Conversely, cell mediated immunity can be enhanced by

the binding of antigen IgG complexes on FcγRI and FcγRIII on DCs that promotes cross-presentation of these antigens (Regnault et al., 1999).

1.6.2   Modulation of DCs by mast cells and fibroblasts

Mast cells are found abundantly in mucosal tissues and are located in close proximity to immature DCs where they may have a Th2 directing effect on DCs. Mast cells secrete histamine in response to IgE-immune complexes that binds to Fcε receptors. Histamine has a profound effect on DCs by suppressing IL-12 and promoting the production of IL-10 (Caron et al., 2001; Mazzoni et al., 2001; van der Pouw Kraan et al., 1998). Similarly, prostaglandin D2, another major product of activated mast cells, strongly suppresses DC's production of IL-12 (Faveeuw et al., 2003). More recently, the presence of fibroblasts in the tissue environment can promote DC maturation (Saalbach et al., 2007) and migration (Saalbach et al., 2010), and have also been shown to induce DCs to produce IL-23 in an inflammatory condition *in vitro*, thereby promoting Th17 responses (Schirmer et al., 2010).

1.6.3   Modulation of DCs by NK cells

NK cells have been shown to promote the activation of DCs for anti-tumor effects (Fernandez et al., 1999) and viral immunity (Andrews et al., 2003). Activated NK cells produce TNF-α and IFN-γ that promotes DC maturation and Th1 polarization (Gerosa et al., 2002; Mailliard et al., 2003; Mocikat et al., 2003). Alternatively, NK cells can

negatively regulate DCs by killing them (Piccioli et al., 2002; Wilson et al., 1999). Neutrophils have also been shown to activate DCs to trigger Th1 responses and this interaction is driven by the binding of the DC-specific, C-type lectin DC-SIGN to the β2-integrin Mac-1 (van Gisbergen et al., 2005).

1.6.4 Modulation of DCs by CD4 T-cells

Mice deficient in CD4 T-cells are unable to mount effective cytotoxic responses as they are important for "licensing" DCs to activate the killing ability of CD8 T-cells (Bennett et al., 1998; Ridge et al., 1998; Schoenberger et al., 1998). This licensing process is dependent on CD40L as mice lacking CD40 or CD40L did not mount helper-dependent cytotoxic responses. In particular, pre-stimulation of DCs with anti-CD40 *in vitro,* or the injection of anti-CD40 antibodies into helper T-cell deficient mice, restored the ability of DCs to produce inflammatory cytokines such as IL-12 and thereby stimulating CD8 T cell killer responses (Caux et al., 1994; Cella et al., 1996).

1.6.5 Modulation of DCs by CD8 T cells

Besides their role in cytotoxic killing, CD8 T-cells have been shown to be important for the promotion of Th1 responses. In particular, CD8 T cells were demonstrated to induce protective Th1 responses against microbial infections *in vivo*. It was observed that low dose *Lesihmania major* infection resulted in Th2 responses and that CD8 T cells were required for the eventual down modulation of low dose induced Th2 responses and

resistance to *L. major* (Uzonna et al., 2004). Likewise, depletion of CD8 T cells abrogated the protective Th1 response induced by DNA vaccination (Gurunathan et al., 2000), suggesting that CD8 T cells were vital for the polarization of Th1 cells. This was also supported by studies demonstrating the involvement of CD8 T cells in the generation of protective CD4 Th1 responses during retroviral infection (Peterson et al., 2002).

Importantly, CD8 T cells have the ability to deviate immune responses away from allergic Th2 phenotypes. Previous work from our group have demonstrated that depletion of CD8 T-cells *in vivo* in OVA/alum sensitized and challenged animals caused a drastic increase in IgE responses. On the other hand, adoptive transfer of OVA specific CD8 T-cells resulted in a significant reduction in IgE (MacAry et al., 1998). Subsequently, it was shown that CD8 T-cell mediated IgE suppression was abolished in IL-12 and IL-18 deficient hosts, but could be restored by the transfer of wildtype DCs (Salagianni et al., 2007; Thomas et al., 2002). These results indicate that CD8 T cells can exert their pro-Th1 response by stimulating DCs to produce IL-12 and IL-18. Above all, CD8 T cells are able to induce DCs to mature (Ruedl et al., 1999) and enhance their Th1 capabilities by utilizing IFN-γ, GM-CSF, TNF-α and CD40L, to induce IL-12p70 from DCs (Mailliard et al., 2002; Thomas et al., 2002; Wong et al., 2008; Wong et al., 2009). Importantly, CD8 T-cells are shown to interact with DCs in lymph nodes (Mempel et al., 2004) and peripheral tissue sites (Aldridge et al., 2009; McGill et al., 2008) where they exert their pro-Th1 influence.

Similarly to NK cells, CD8 T cells can also negatively regulate DC activity by inducing their apoptosis *in vivo* (Guarda et al., 2007). However, DCs are also protected from killing by CD8 T-cells by expressing protease inhibitors, such as protease inhibitor-9 and serpin serine protease inhbitor-6 (Hirst et al., 2003; Medema et al., 2001). These proteases are up-regulated by factors that activate DCs including LPS and TNF-α. Interestingly, recent studies have shown that memory CD8 T cells protected DCs from killing through cytotoxic granules by releasing TNF-α early during interaction, thereby inducing the expression of granzyme B inhibitor PI-9 in these DCs (Nakamura et al., 2007; Watchmaker et al., 2008).

1.7 Bystander mediated effects

While most immune mechanisms involve specific cell-cell interactions, studies have shown that the activation and suppression of other immune cells in the nearby vicinity can also occur without physical interaction. Of note, such "bystander mediated effects" often occur during inflammation and is mediated by cytokines being produced by activated cells which further modulate the immune response.

1.7.1 Bystander mediated effects on uninfected cells

Virus-specific T-cells have been shown to migrate to areas of infection in peripheral sites where they encounter viral peptides presented by viral infected cells. CD8 T-cells recognizing such cells would then release cytotoxic granules resulting in the killing of

these infected cells. Under these circumstances, the dying cells, CD8 T-cells and other APCs such as macrophages and DCs within the inflammatory focus would release cytokines such as TNF-α, TNF-ß, lymphotoxin (LT), and reactive oxygen species such as nitric oxide (NO), which would lead to bystander killing of the uninfected neighboring cells. This results in additional immunopathology at sites of infection (Duke, 1989; Smyth and Sedgwick, 1998). Such bystander mediated effects also appear to occur for CD4 T cells that recognize peptides on MHC class II (Yasukawa et al., 1993). In this case, CD4 T-cells can release cytokines that would not only kill uninfected cells but may also stimulate nearby macrophages which would kill uninfected cells in a bystander manner (Mitrovic et al., 1994).

1.7.2   Bystander activation of T-cells

Bystander activation of T cells, *i.e.* the stimulation of unrelated, heterologous T-cells by cytokines during an antigen-specific T-cell response can also occur for CD8 T-cells and to a lesser extent, CD4 T-cells. T cell proliferation *in vivo* is presumed to reflect a TCR-mediated polyclonal response. However, the massive proliferation of T cells seen in viral infections is suggestive of a cytokine-driven bystander reaction instead of the TCR as such proliferation was not associated with up-regulation of CD69 or CD25 (Tough et al., 1996). In particular, memory CD44^{hi} CD8 T-cells are activated through such bystander mediated mechanisms when they are stimulated by IFN-α/β (Tough et al., 1996), IFN-γ, IL-12, IL-15, and IL-18 produced by APCs interacting with antigen specific T-cells (Tough et al., 1999; Zhang et al., 2001). These CD8 T-cells produce IFN-γ rapidly and

while they may be protective in some bacteria models (Lertmemongkolchai et al., 2001; Yajima et al., 2002), they are often dispensable and may even contribute to the induction of autoimmune diseases due to their cross reactivity (Fujinami et al., 2006; Gilbertson et al., 2004; Mueller and Jenkins, 1997).

1.7.3 Bystander activation or suppression of immune responses

Bystander mediated activation or suppression of DCs can also influence the outcome of an immune response. Oral infection with *Salmonella enterica* serovar typhi causes typhoid and in particular, *Salmonella* flagellin, which is recognized by host cells through TLR 5 has been identified as a mediator of inflammation (Honko and Mizel, 2005; Salazar-Gonzalez and McSorley, 2005). However splenic DCs express little or no TLR5 and are not responsive to flagellin (Means et al., 2003; Uematsu and Akira, 2006). It was subsequently discovered that the activation of such splenic DCs occurred in a bystander mediated fashion through activation of bone marrow-derived cells and required MyD88 expression (Salazar-Gonzalez et al., 2007). Such innate immune responses to flagellin is highly deleterious to the growth of *Salmonella in vivo*, and offers an explanation for bacterial modulation of flagellin expression *in vivo* (Cummings et al., 2006; Eriksson et al., 2003). Similar bystander activation of DCs has also been described with LPS (Yadav et al., 2006). In contrast, pathogens such as *Mycobacterium tuberculosis* can evade immune responses by inhibiting the differentiation of bystander non-infected monocytes into DCs through the release of interleukin-10 (Remoli et al., 2011). Therefore, the

presence of different cytokines in the microenvironment can directly affect the outcome of bystander mediated immune responses.

1.8 Monocytes

Monocytes are heterogeneous bone marrow-derived cells that circulate in the blood and rapidly extravasate into inflamed tissues upon sensing environmental signals.

1.8.1 Monocyte heterogeneity

Two major subsets of blood monocytes have been described in mice and humans (Geissmann et al., 2003; Sunderkötter et al., 2004; Ziegler-Heitbrock, 2007). In mice, these two subsets are described as classical ($Ly6C^{++}CD43^+$) and non-classical ($Ly6C^+CD43^{++}$)(Ziegler-Heitbrock et al., 2010). Analogously, human monocytes can also be separated into these two major subsets whereby classical monocytes are $CD14^{++}CD16^-$ while non-classical monocytes are $CD14^+CD16^{++}$ (Ingersoll et al., 2010; Ziegler-Heitbrock, 2007; Ziegler-Heitbrock et al., 2010). Differential gene expression profiles between monocyte subsets are well conserved between mice and humans, suggesting that mouse models of trafficking may reveal clues to human monocyte behavior in disease (Ingersoll et al., 2010). However, despite their similarities, the subset ratio in the blood is vastly different between these two species. While the ratio of classical to non-classical subsets in mice is about 1:1, classical monocytes account for about 90% of total blood

monocytes and the non-classical monocytes account for the remaining 10% in humans (Ziegler-Heitbrock et al., 2010).

1.8.2 Developmental relationship of monocyte subsets

A developmental relationship between the two subsets of monocytes has been proposed, supported by the discovery of a small subset of monocytes, known as the "intermediate" subset which are $CD14^{++}CD16^{+}$ (Ancuta et al., 2003), and is proposed to be a transitional population bridging the classical and non-classical monocyte subsets (Ziegler-Heitbrock et al., 2010). In humans, during the course of an infection or in the presence of macrophage colony-stimulating factor (M-CSF) treatment, there is an increase first of the intermediate cells followed by an increase of the non-classical subset (Weiner et al., 1994). Cell-depletion studies in mice have also shown that the non-classical monocytes in mouse blood are more mature and are derived from the classical monocytes (Sunderkötter et al., 2004). Furthermore, the intermediate subset was shown to express a handful of surface markers at levels in between the classical and non-classical subsets (Ancuta et al., 2009; Ancuta et al., 2003; Cros et al., 2010). Fig 1.4 illustrates the development of monocyte subsets.
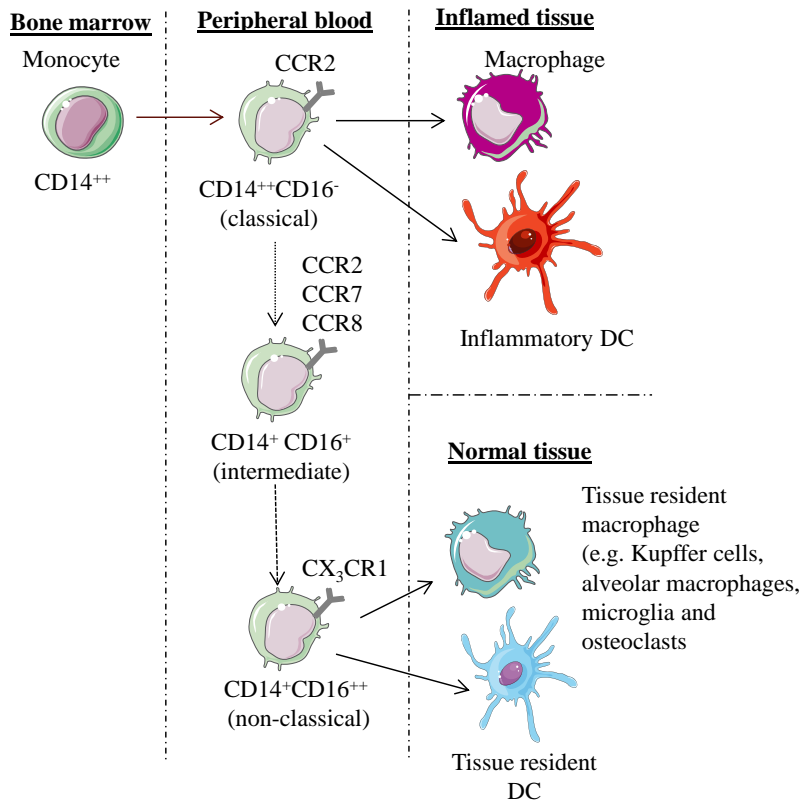
Fig 1.4 Development of monocyte subsets

1.8.3 Functional heterogeneity of monocyte subsets

Importantly, monocyte subsets also exhibit functional heterogeneity. Non-classical monocytes demonstrate a patrolling behaviour along blood vessel walls (Auffray et al., 2007) and accumulate in non-inflamed peripheral tissues (Geissmann et al., 2003) where they contribute to resident macrophage populations (Jakubzick et al., 2008). In contrast, increasing evidence suggest that the recruitment of classical monocytes dominates early in inflammatory responses. For example, classical monocytes preferentially infiltrate into inflamed tissues, such as in sterile peritonitis models, than their non-classical counterparts in mice and rats. In addition, they are specifically increased in the

circulation during systemic and chronic infection (Geissmann et al., 2003; Sunderkötter et al., 2004; Tacke et al., 2007; Yrlid et al., 2006). This is further supported by evidence showing the migration of only classical monocytes, and not non-classical monocytes, into the skin of mice receiving an intra-cutaneous injection of latex microspheres (Qu et al., 2004) and in a model of skeletal muscle injury (Arnold et al., 2007). However, after engulfing dying cells, classical monocytes have also differentiate into cells that resemble non-classical monocytes, which mediate tissue repair mechanisms (Arnold et al., 2007).

1.8.4   Trafficking of monocyte subsets

In particular, the two subsets are under the control of distinct trafficking mechanisms, with the classical subset being recruited via CCR2 in response to MCP-1 and non-classical monocytes utilizing a CX_3CR1-dependent pathway (Nahrendorf et al., 2007). The importance of CCR2 in mediating the trafficking of classical monocytes is apparent in disease studies such as *Listeria monocytogenes* (Serbina et al., 2003), *Mycobacterium tuberculosis* (Peters et al., 2004) and West Nile virus infections (Getts et al., 2008) whereby the inability of these cells to migrate into inflamed tissues in $CCR2^{-/-}$ mice is detrimental to the host; although it may also be protective in certain circumstances as seen in dengue virus infections (Guabiraba et al., 2010).

1.9 Monocyte derived cells

Monocytes are known for their plasticity and ability to differentiate into osteoclasts, macrophages or DCs upon migrating into tissues (Gordon and Taylor, 2005).

1.9.1 Monocyte derived cells in the steady state

However, monocytes are not the precursors of all DC and macrophage subsets, especially those that reside in the steady state. Several studies have shown that steady state DCs such as splenic conventional DCs (cDCs) originate from a pre-cDC subset that is distinct from monocytes (Naik et al., 2006). In addition, Langerhans cells (Merad et al., 2002) and plasmacytoid DCs (Corcoran et al., 2003) in the steady state do not originate from monocytes but develop from a distinct pathway. Moreover, while it is generally believed that monocytes are the main precursors of tissue residential macrophages, studies of many tissue-resident macrophage populations have shown that local proliferation has a considerable role in the renewal and maintenance with the recruitment of circulating precursors having little, if any, role under steady-state conditions (Gordon and Taylor, 2005; van Furth et al., 1985).

1.9.2 Monocyte derived cells during inflammation

Nevertheless, while the general consensus suggests that monocytes are not the main precursors of steady state DCs and residential macrophages, they are recruited in large

numbers into peripheral sites during inflammation and tissue injury. Their differentiation into DCs and macrophages are thus only as a consequence of inflammation (Geissmann et al., 2003; Naik et al., 2006; Sunderkötter et al., 2004). Classical monocytes have been shown to differentiate into macrophages that resemble classically activated M1 macrophages which are associated with high microbicidal activity, pro-inflammatory cytokine production and cellular immunity (Mosser, 2003; Van Furth et al., 1973). In contrast, non-classical monocytes are recruited at late phases of inflammation and differentiate into alternatively activated M2 macrophages (Geissmann et al., 2010; Martinez et al., 2009; Nahrendorf et al., 2007). These M2 macrophages express vascular-endothelial growth factor (VEGF), and promote tissue repair, angiogenesis and deposition of collagen, as well as humoral immunity. Such macrophages can also be cultured *in vitro* by differentiating human monocytes with M-CSF and stimulating them with IFN-γ or LPS to achieve M1 macrophages; or by stimulating them with IL-4, IL-10, IL-13 or TGF-ß to attain M2 macrophages (Gordon and Taylor, 2005; Mosser, 2003; Taylor et al., 2005).

1.9.3   Monocyte-derived DCs

The clearest example of a distinct DC type that is produced during inflammation are those that derived from CCR2-expressing classical monocytes (Gordon and Taylor, 2005; Sunderkötter et al., 2004; Sánchez-Torres et al., 2001). These classical monocytes produced no detectable DC progeny in lymphoid organs or tissues when transferred intravenously into sterile, non-irradiated mice, indicating that they make no contribution

to steady-state DCs (Geissmann et al., 2003; Naik et al., 2006; Sunderkötter et al., 2004). However, when transferred into mice that have been subjected to a vigorous inflammatory response, these monocytes produced DCs in the peritoneum and entered the spleen where they differentiated into a distinctive DC type (Geissmann et al., 2003; Naik et al., 2006). These monocyte-derived inflammatory DCs can be distinguished from steady-state splenic cDCs by their intermediate expression of CD11c, high expression of CD11b, MAC3 (a glycoprotein also found on activated macrophages) and the absence of CD4 or CD8 expression (Naik et al., 2006; Shortman and Naik, 2007). In particular, an *in vitro* study comparing steady-state DCs with inflammatory DCs by culturing bone marrow precursors with fms-like tyrosine kinase 3 ligand (Flt3L) or GM-CSF and IL-4 (GM/IL-4 DCs) respectively showed phenotypic and functional differences between these two subsets (Xu et al., 2007). In contrast to steady state FL-DCs, inflammatory GM/IL-4 DCs were larger and more granular and although comparable in T cell activation, inflammatory GM/IL-4 DCs produced more inflammatory mediators such as TNF-α, IL-10, CCL-2 and nitric oxide (NO) upon TLR ligation. Notably, unlike GM/IL-4 DCs, FL-DCs cannot be differentiated from classical monocytes, suggesting that the differentiation of DCs under inflammatory conditions is indeed from the classical subset. In addition, the discovery of a subset of inflammatory DCs known as TNF/iNOS-producing dendritic cells (Tip-DCs) that appear in mice infected with *L. monocytogenes* highlights the important role of such cells during inflammation and infection (Serbina et al., 2003).

1.10   TNF/iNOS-producing dendritic cells (Tip-DCs)

The presence of Tip-DCs was first discovered in a model of *Listeria monocytogenes* where classical monocytes were shown to migrate into *Listeria*-infected spleen using CCR2 where they differentiate into Tip-DCs (Serbina et al., 2003). These Tip-DCs were the key producers of TNF-α and iNOS; expressed intermediate levels of CD11b and CD11c, high expression of Mac-3 and MHC class II and were morphologically similar to CD11c^{hi} DCs by displaying pleomorphic nuclei and cytoplasmic extensions. However, unlike splenic macrophages that were infected with *L. monocytogenes*, the majority of Tip-DCs were not directly infected, suggesting that they differed functionally from residential macrophages and were activated indirectly.

1.10.1 Tip-DCs during infection

Cells resembling Tip-DCs have also been identified in several other bacterial infection models such as *Brucella melitensis* (Copin et al., 2007), Uropathogenic *Escherichia coli* (Engel et al., 2006), and *Salmonella* (Rydström and Wick, 2007). Development of Tip-DCs has also been observed in viral infections such as influenza (Aldridge et al., 2009), where they are important for supporting the proliferation of influenza-specific CD8 T-cells in the lung. Studies in parasitic infection models, including *Trypanosoma brucei* (Bosschaerts et al., 2010), *Leishmania major* (De Trez et al., 2009), and *Toxoplasma gondii* infection (Dunay et al., 2008) have also shown the importance of Tip-DCs in

eliminating these parasites as CCR2$^{-/-}$ and CCL2$^{-/-}$ mice were shown succumb to infection more rapidly.

1.10.2 Tip-DCs in mucosal immunity

Tip-DCs have also been shown to regulate IgA production in mucosal immunity (Tezuka et al., 2007) as IgA class-switch recombination (CSR) was showed to be impaired in iNOS$^{-/-}$ mice. The authors demonstrated that iNOS was preferentially expressed in DCs present in the mucosa-associated lymphoid tissues (MALT) in response to the recognition of commensal bacteria by TLR and further analysis revealed that these MALT DCs were Tip-DCs resembling those identified in mice infected with *Listeria monocytogenes*. Notably, iNOS regulates the T-cell-dependent IgA CSR through expression of TGF-β receptor, as well as the T-cell-independent IgA CSR through production of a proliferation-inducing ligand (APRIL, also called Tnfsf13) and a B-cell-activating factor of the tumour necrosis factor (TNF) family (BAFF, also called Tnfsf13b). Thus, the presence of a naturally occurring TNF-α and iNOS producing DC subset may explain the predominance of IgA production that is critical for gut homeostasis in the MALT.

1.10.3 Tip-DCs in skin diseases

Tip-DCs have also been demonstrated to contribute to disease pathogenesis. Studies in psoriasis have shown the presence of Tip-DCs in skin lesion samples of psoriasis patients (Lowes et al., 2005). These cells expressed HLA-DR, CD40, and CD86, lack Langerin

and CD14, and to a significant extent expressed the DC maturation markers DC-LAMP and CD83. In particular, treatment of psoriasis with efalizumab (anti-CD11a) strongly reduced infiltration of these Tip-DCs, suggesting that Tip-DCs are an important target for suppressive therapies. Pathogenicity of these Tip-DCs in psoriasis was further confirmed by the rapid downmodulation of Tip-DC products during effective treatment with TNF-blocking drugs (Zaba et al., 2007). In mouse models of psoriasis (Stratis et al., 2006; Wang et al., 2006a), TNF-α and iNOS expressing cells characterized as activated macrophages were also found to be present at the dermal/epidermal border where they may contribute to hyperkeratosis and parakeratosis, terming these cells as *epithelium-lining macrophages* (van den Oord and de Wolf-Peeters, 1994; Weber-Matthiesen and Sterry, 1990). However these cells were also shown to express CD83, which suggests that they may resemble Tip-DCs seen in human psoriasis studies (Stratis et al., 2006).

1.11 Factors modulating monocyte differentiation

Since the differentiation of monocytes into DCs or macrophages relies on the microenvironment, the presence of different cell types and cytokines is a critical feature in determining the outcome of differentiation. While the reference method for obtaining monocyte-derived DCs and macrophages in humans consist of the addition of IL-4/GM-CSF and M-CSF respectively, increasing studies have demonstrated the use of other cytokine cocktails to obtain these cells.

1.11.1 Factors that favour macrophage differentiation

Studies have shown that the presence of IL-6 and IL-32 favors the differentiation of monocytes into macrophages. IL-6, produced by fibroblasts when in contact with monocytes, was demonstrated to up-regulate the expression of functional M-CSF receptors on monocytes. This allows monocytes to consume their autocrine M-CSF, thereby switching monocyte differentiation into macrophages rather than DCs (Chomarat et al., 2000). In contrast, the ability of IL-32 to induce the differentiation of monocytes into macrophages is mediated through non-apoptotic, caspase-3-dependent mechanisms (Netea et al., 2008). Notably, the presence of ox-LDL (Fuhrman et al., 2008), influenza virus induced apoptotic cells (Uchide et al., 2002), cholera toxin (Veglia et al., 2011) and CXC-chemokine platelet factor 4 (Scheuerer et al., 2000) were shown to favour the differentiation of monocytes into macrophages. These cells showed characteristics of macrophages as they downregulated the expression of HLA-DR and exhibited robust phagocytic activities.

1.11.2 Factors that favour DC differentiation

The differentiation of monocytes into DCs has also been characterized by many studies. In particular, factors such as IL-2 (Sanarico et al., 2006), IL-15 (Mohamadzadeh et al., 2001), IFN-α (Mohty et al., 2003), TNF-α (Chomarat et al., 2003; Iwamoto et al., 2007), CD40 Ligand (Zhang et al., 2007), CD137 Ligand (Kwajah M M and Schwarz, 2010) and Delta-1 (Ohishi et al., 2001) have all been shown to favour the differentiation of

monocytes with DC characteristics. Notably, DCs obtained by differentiating monocytes in the presence of IFN-α (IFN-DCs) expressed a large number of Toll-like receptors (TLRs), including TLR7, which is classically found on plasmacytoid DCs (Mohty et al., 2003). Like plasmacytoid DCs, IFN-DCs could also secrete IFN-α following viral stimulation or TLR7-specific stimulation. Interestingly, addition of IL-15 during the differentiation process results in DCs exhibiting features of LCs. However, unlike gold standard LCs obtained by differentiating monocytes with TGF-ß1, IL-4 and GM-CSF (Geissmann et al., 1998), LCs obtained by differentiation with IL-15 do not exhibit bona fide Birbeck granules.

1.12   Contact Hypersensitivity

Contact hypersensitivity (CHS), also known as allergic contact dermatitis, is one of the most frequent skin diseases characterized by erythema, edema, and vesiculation. CHS is a T cell-mediated inflammatory reaction that occurs after cutaneous exposure to haptens (i.e. chemicals and reactive metal ions) which become immunogenic after binding to discrete amino acid residues of proteins or peptides (Lepoittevin, 1999).

1.12.1   Mechanism of CHS

The CHS response is antigen specific and develops in two distinct phases. In the sensitization phase, haptens penetrating the skin are captured by resident DCs, such as LCs and dermal CD103+ DCs, which migrate to regional lymph nodes and induce the

activation of specific T cells (Bursch et al., 2007). The next phase, known as the elicitation phase of CHS, is induced by re-exposure to the same hapten at a remote skin site. This leads to the rapid recruitment and activation of T cells within a few hours after challenge and to the constitution of a local inflammatory response peaking at 24–48 h which progressively decreases via active down-regulating mechanisms. A schematic representation of the CHS response is illustrated in Fig. 1.5.
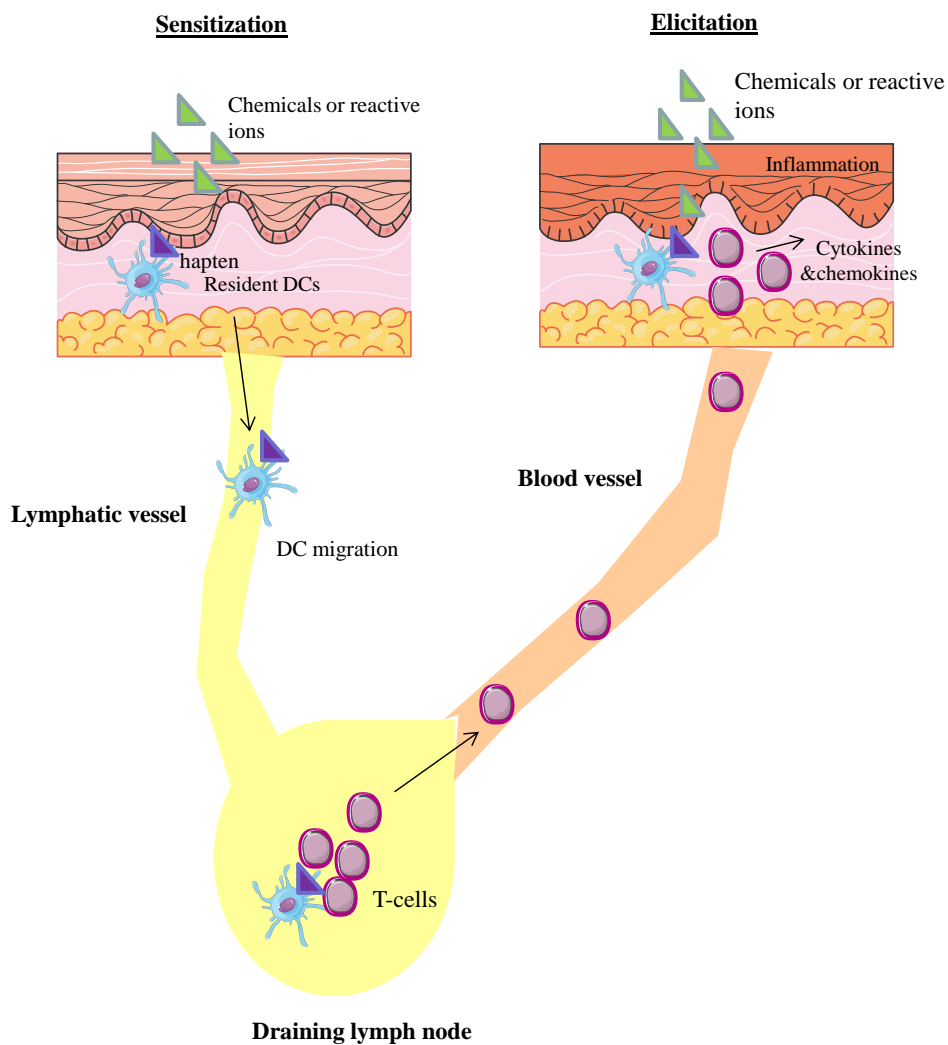
Fig. 1.5 A schematic illustration of the mechanism behind a CHS response

Clinical symptoms associated with CHS only appear in the elicitation phase. Re-exposure to the same hapten causes cross-linking of IgM molecules which activates the complement pathway and generates C5a (Tsuji et al., 2002). This act on platelets, mast cells, and macrophages (Tsuji et al., 2000) which results in TNF-α production and microvasculature activation (McHale et al., 1999). This facilitates the infiltration of a first wave of leukocytes, mainly CD8 T-cells, within 2 hours (Akiba et al., 2002). These hapten-primed CD8 T-cells enter the dermis at the site of hapten exposure in response to chemokines such as IP-10 or CXCL10 and become re-stimulated by resident APCs (Dufour et al., 2002) or hapten-presenting vascular endothelial cells (Kish et al., 2011). CD8 T-cells then release pro-inflammatory mediators triggering endothelial cells to produce CXCL1 and CXCL2 which directs neutrophils into the site (Kish et al., 2011). At the same time, chemokines such as CXCL2 and MCP-1 would lead to monocyte infiltration and a massive second wave of leukocyte infiltration (Wang et al., 2006a). Importantly, treatment of allergic contact dermatitis with glucocorticoids showed reduction in clinical symptoms and its therapeutic action is directed at macrophages and neutrophils (Tuckermann et al., 2007).

1.12.2 CD8 T-cells in CHS

Several studies by independent investigators using different haptens (i.e. such as DNFB, DMBA, TNP and oxazolone) have shown that CHS is principally mediated by CD8 T-cells (Akiba et al., 2002; Anderson et al., 1995; Bouloc et al., 1998; Bour et al., 1995; Xu et al., 1996). The use of monoclonal antibodies to deplete CD4 or CD8 T-cells selectively

have also established that CHS reactions are mediated by CD8 T-cells and down-regulated by CD4 T-cells (Gocinski and Tigelaar, 1990). Further evidence to support this can be drawn from studies in mice that lacked MHC class I which were unable to support CHS responses to dinitrofluorobenzene (DNFB), while mice that were deficient in MHC class II developed an exaggerated CHS reaction (Bour et al., 1995). Moreover, MHC class I+/II- dendritic cells (DCs) could induce hapten-specific immune responses (Kolesaric et al., 1997), whereas MHC class I-/II+ DCs down-regulated CHS response (Krasteva et al., 1998). CD8 T-cells are recruited early into the skin where they induce inflammation and keratinocyte apoptosis (Akiba et al., 2002; Kehren et al., 1999; Xu et al., 1996). Their cytotoxicity is critical as mice lacking the perforin and Fas/Fas ligand pathways were unable to mount a CHS response (Kehren et al., 1999). Priming of CD8 T-cells during CHS does not require CD4 T-cells or CD40/40L interaction as mice deficient in CD40L or had CD4 T-cells depleted with monoclonal antibodies did not have decreased CHS responses (Gorbachev et al., 2001a; Krasteva et al., 1998; Martin et al., 2000). These CD8 T-cells produce IFN-γ or IL-17 and form distinct populations with important effector functions (He et al., 2006; He et al., 2009; Kish et al., 2009).

1.12.3 CD4 T-cells in CHS

On the other hand, CD4 T-cells are known to be important for regulating the immune suppression of CHS. CD4 T-cells were found to express Th2 cytokines such as IL-4, IL-5 and IL-10 and notably, CD4$^+$CD25$^+$ regulatory T-cells were found to be an important down-regulator as mice that had CD4 T-cells depleted *in vivo* had enhanced

inflammatory reactions during CHS (Desvignes et al., 1996; Dubois et al., 2003; Gocinski and Tigelaar, 1990; Xu et al., 1996). It is noteworthy that CD4 T-cells are recruited into challenged skin hours after recruitment of CD8 T-cells (Akiba et al., 2002), suggesting that their entry into the skin is responsible for the inactivation of CD8 T-cell's cytotoxic activity, thereby limiting the development pathogenicity. However, despite studies showing the role of CD4 T-cells in down-regulating the CHS response, a small subset of CD4 T-cells that are polarized towards the Th1 phenotype may still express IFN-γ, albeit at a much lower level than CD8 T-cells (He et al., 2009). In addition, CD4 T-cells could also act as effector cells in CHS to some haptens when the CD8 T-cell population is deficient (Martin et al., 2000).

1.13   Aims of this study

While it has been shown previously that the interaction of CD8 T cells and DCs results in a Th1 response through IL-12p70 production in mice and humans (Mailliard et al., 2002; Thomas et al., 2002; Watchmaker et al., 2008; Wong et al., 2008), it is unclear if CD8T-cells can induce the production of IL-12p70 from DCs in the absence of CD4 T-cells or CD40L-expressing cells in humans. It has also been shown that CD8 T cells can secrete chemokines such as RANTES, MIP1α and MIP-1ß for HIV suppression (Cocchi et al., 1995) while DCs can secrete MCP-1 in addition to RANTES, MIP1α and MIP-1ß (Lebre et al., 2005). This suggests that the CD8 T-cell-DC interaction can result in the recruitment of other immune cells such as monocytes which can be influenced to differentiate by environmental cytokine factors. Indeed, studies have shown that CD8 T-

cells are able to infiltrate quickly into inflamed sites (Akiba et al., 2002) which may facilitate their early interaction with DCs. In human psoriasis studies, administration of alefacept, an antibody which reduces infiltration of activated memory CD8 T-cells, showed reduced monocyte-derived DC content, inflammation and expression of iNOS and IFN-γ in the skin (Chamian et al., 2005). These data suggest that CD8 T-cells, upon interacting with DCs, may have an impact on the differentiation and function of monocytes which may help support Th1 responses. However, the precise mechanism underlying these observations remains unclear.

Specific aims:

1) To determine if human CD8 T-cells can induce DCs to produce IL-12p70 in the absence of CD4 T-cells and determine the conditions that are necessary *in vitro*.

2) To determine if CD8 T-cells and DCs can create a cytokine milieu that favors the differentiation of monocytes

3) To determine the outcome of this monocyte differentiation and the functional properties of these monocyte derived cells

4) To determine if these monocyte derived cells can support Th1 responses

5) To demonstrate *in vivo* using a contact hypersensitivity model that CD8 T-cells are principally involved in the differentiation of these monocyte-derived cells.

CHAPTER 2: Materials and methods

2.1 Media and buffers

PBS buffer

PBS buffer was commercially obtained from 1st base as a 10 x stock. The 10 x stock consisted of 137mM NaCl, 2.7mM KCl and 10mM phosphate buffer. This stock was diluted with 9 parts water to give 1 x PBS. The pH was 7.2 to 7.4

Buffers for human blood processing

To prevent peripheral blood from coagulating, freshly drawn blood was collected with PBS + 4% sodium citrate (10:1 blood: buffer ratio). Subsequent washing steps were performed with PBS + 0.4% sodium citrate. All buffers were adjusted to a pH of 7.2 to 7.4 under sterile conditions.

Culture Medium

All human cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Invitrogen, Singapore) supplemented with 10% autologous serum or heat-inactivated human AB serum (Biowhittaker, Cambrex, Singapore).

All mice cells were cultured in complete medium. This was prepared from RPMI-1640 with L-Glutamine (Gibco) with the addition of the following:

a) 10 % v/v FCS, heat inactivated for 30min at 55oC.

b) 1 % v/v Non-essential amino acid (100 x from stock)

c) 1 mM Sodium pyruvate (100 x from 100 mM stock)

d) 5 µM β-Mercaptoethanol (2 860 000 x from 14.3 M stock)

e) 100 IU/ml Penicillin and 0.1mg/ml Streptomycin (100 x from 10 000 IU/ml Penicillin

10mg/ml Streptomycin stock)

Buffers for isolation of mouse skin cells

1-Dispase (Gibco) was diluted with RPMI-1600 (Gibco) to a working concentration of

2.4mg/ml (4 U/ml) for the separation of epidermis and dermis. Collagenase Type IV

(Gibco) was diluted in HBSS (w/o Ca^{2+} and Mg^{2+}) to a working concentration of 1mg/ml

(4 U/mg) for further digestion of dermis cells.

MACS/FACS buffer:

MACS/FACS buffer consisted of 1 x PBS with 2% FCS and 5mM EDTA adjusted to pH

7.2 to 7.4 at 4°C and was prepared under sterile conditions.

Permeabilization/washing buffer for intracellular staining:

For intracellular staining, the buffer used consisted of PBS containing 0.5% BSA, 0.1%

saponin and 0.1% sodium azide (perm/wash buffer), adjusted to a pH of 7.2 to 7.4. This

was used for the permeabilization and washing steps when cells were prepared for

intracellular analysis using FACS. Alternatively, a commercial permeabilization/washing

buffer purchased from BD biosciences (BD Perm/Wash™ buffer) was used according to manufacturer's instructions.

Annexin-V binding buffer:

10mM Hepes/NaOH with 140mM NaCl, 2.5mM $CaCl_2$, pH 7.4. 5ml of 1 M Hepes/NaOH stock was added to 495ml of sterile deionised water. 4.09g of NaCl and 0.184g of $CaCl_2$ were added to this buffer. pH was adjusted to 7.4 and stored at 4°C

Buffers for ELISA:

Wash buffer consisted of PBS with 0.5% v/v Tween-20, pH adjusted to 7.2 to 7.4. The block buffer consisted of PBS with 1% v/w BSA, pH adjusted to 7.2 to 7.4. Diethanolamine buffer was used to dissolve the pNPP substrate for ELISA.

Buffers for immunostaining:

Wash buffer consisted of PBS with 10mM of glycine, pH adjusted to 7.2 to 7.4. Antibodies were diluted in permabilization buffer. For staining of tissue cryosections, blocking buffer consisted of 0.2% BSA in PBS. Buffer used to dilute antibodies was PBS with 1% normal mouse serum and 10mM glycine. PBS was used to wash samples in between each step.

2.2. Human Studies

2.2.1 Cell isolation

Isolation of Human Peripheral Blood Mononuclear Cells (PBMCs):

All blood was collected after informed consent in accordance with the Declaration of Helsinki and under an approved protocol by the National University of Singapore Institutional Review Board (NUS IRB code: 05-024). Briefly, PBMCs were isolated from blood samples by Ficoll-Paque^{TM} (GE Healthcare, Singapore) density gradient sendimentation.

1. 50ml of peripheral blood was collected from healthy donors by venipuncture into 5ml of 4% sodium citrate in a 50ml falcon tube.

2. Blood was diluted with equal volume of PBS + 0.4% sodium citrate.

3. 35ml of diluted blood was layered carefully onto 15ml of Ficoll-Paque^{TM}.

4. Cells were centrifuged at 650 x g for 20 min, with brakes off at room temp.

5. Cell interface was harvested and volume diluted at least 3 fold with PBS + 0.4% sodium citrate and centrifuged at 450 x g for 15 min.

6. Washes were repeated 2 more times at 300 x g, 10 min with PBS + 0.4% sodium citrate until supernatant was clear.

7. Cells were counted by trypan blue exclusion method for selective isolation through MACS column

Blood CD8 T-cell isolation

To isolate CD8 T-cells, contaminating NK cells were first depleted with anti-CD56 conjugated magnetic beads as NK cells express low levels of the CD8 molecule. CD8 T-cells were then positively selected from PBMCs by anti-CD8 conjugated magnetic beads (Miltenyi biotec, Singapore). This routinely gives a purity of 97 ± 1.5% of $CD3^+CD8^+$ T-cells.

1. PBMCs were centrifuged at 350 x g for 5 min at 4^oC.

2. MACS buffer was added to the cell pellet and washing was repeated by centrifugation at 350 x g for 5 min at $4°C$.

3. Supernatants were discarded and anti-CD56 conjugated microbeads were added to the cell pellet at a volume of 10μl per 10 x 10^6 cells. Clumping was prevented by pipetting several times.

4. The mixture was incubated at 4^oC for 20 min.

5. After incubation, the cells were washed by adding 3ml of MACS buffer. Cells were centrifuged at 350 x g for 5 min at 4^oC.

6. A LS MACS column was inserted into the magnetic stand. This was pre-wetted with 3ml of MACS buffer.

7. After centrifugation, supernatants were discarded and cells were resuspended with 1ml of MACS buffer. Cell clumps which will cause column blockage were prevented by vigorous pipetting several times.

8. Cells were loaded into the column and allowed to flow into the column. An additional 3ml of MACS buffer was added to wash the column once the reservoir was dry. Washing was repeated thrice.

9. The effluent was collected and the column was washed 3 times. Each flowthrough was collected in the same tube as the effluent.

10. Cells were washed by centrifugation at 350 x g for 5 min at 4^oC and subsequently resuspended with appropriate media before cell counting was performed.

11. Anti-CD8 conjugated microbeads were added to the cell pellet at a volume of $10\mu l$ per 10×10^6 cells. Clumping was prevented by pipetting several times.

12. The mixture was incubated at 4^oC for 20 min.

13. After incubation, the cells were washed by adding 3ml of MACS buffer. Cells were centrifuged at 350 x g for 5 min at 4^oC.

14. After discarding the supernatant, cells were resuspended in 1ml of MACS buffer and loaded into the pre-wet LS column on the magnetic stand.

15. Cells bounded in the column were harvested by removing the column from the magnetic stand. 3ml of MACS buffer or culture media (depending on experimental purpose) was added into the reservoir and cells were forced out of the column using a plunger. This fraction represented the final enriched population of $CD3^+CD8^+$ cells.

Blood naive CD4 T-cell isolation

Naive CD4 T-cells were negatively selected from PBMCs using the Naive CD4 T cell Isolation Kit II (Miltenyi Biotec, Singapore) according to the manufacturer's instructions. This routinely gave a purity of 98 ± 0.7% CD3$^+$CD4$^+$CD45RA$^+$ T-cells.

1. Cell number was first determined and cell pellet was resuspended in 40µl of MACS buffer per 10 x 10^6 total cells

2. 10µl of Biotin-Antibody Cocktail II per 10 x 10^6 total cells was added.

3. The cell suspension was mixed well and was incubated for 10 min at 4°C.

4. Cells were washed by adding 3ml of MACS buffer per 10 x 10^6 cells and centrifuged at 350 x g for 5 min. Supernatant was aspirated completely.

5. Cell pellet was resuspended in 80µl of MACS buffer per 10 x 10^6 total cells.

6. 20µl of Anti-Biotin MicroBeads per 10 x 10^6 total cells was added.

7. The cell suspension was mixed well and incubated for 15 min at 4°C.

8. Cells were washed by adding 3ml of MACS buffer per 10 x 10^6 cells and centrifuged at 350 x g for 5 min. Supernatant was aspirated completely.

9. Cells were resuspended in 1ml of MACS buffer. Cell clumps which will cause column blockage were prevented by vigorous pipetting several times.

10. Cell suspension was loaded onto a LS column that was pre-wet with 3ml of MACS buffer on a magnetic stand.

11. The column was washed with 3ml of MACS buffer and the effluent was collected together with the flowthrough for cell counting. This contained the enriched naive CD4 T-cell fraction.

Blood monocyte isolation

Monocytes were negatively selected from PBMCs using the Monocyte Isolation Kit II (Miltenyi Biotec, Singapore) according to the manufacturer's instructions. This routinely gave a purity of $97 \pm 0.5\%$ CD14$^+$cells.

1. Cell number was first determined and cell pellet was resuspended in 30µl of MACS buffer per 10 x 10^6 total cells.

2. 10µl of FcR Blocking Reagent per 10 x 10^6 total cells was then added.

3. Next, 10µl of Biotin-Antibody Cocktail per 10 x 10^6 total cells was further added to the mixture.

4. The cell suspension was mixed well by pipetting several times to prevent cell clumping. Cells were incubated for 10 min at 4°C.

5. After incubation, 30µl of MACS buffer per 10 x 10^6 total cells was added, followed by 20µl of Anti-Biotin MicroBeads per 10 x 10^6 total cells into the cell mixture.

6. The cell suspension was mixed well by pipetting several times to prevent cell clumping and incubated for an additional 15 min at 4°C.

7. Cells were washed by adding 3 ml of MACS buffer and centrifuged at 350 x g for 5 min. Supernatant was discarded completely.

8. The cell pellet was then resuspended in 1 ml of MACS buffer and cell clumps which will cause column blockage were prevented by vigorous pipetting several times.

9. Cell suspension was loaded into a LS column that had been pre-wet with 3 ml of MACS buffer on a magnetic stand

10. Cells were allowed to pass through and the column was washed 2 times with 3 ml of MACS buffer before the entire effluent was collected. This fraction represented the enriched population of monocyte cells.

2.2.2 Preparation of cells for flow cytometry

Surface staining of cells for flow cytometry analysis:

1. Cells were transferred into FACS tubes. Typically 1×10^5 to 1×10^6 cells would be used per tube.

2. Cells were centrifuged at 350 x g, 4^oC for 5 min.

3. Supernatants were discarded by inversion of the tubes.

4. Cells were washed by adding 1.5 ml of FACS buffer into each tube, and centrifuged at 350 x g, 4^oC for 5 min.

5. Supernatants were discarded by inversion of the tubes.

6. 0.5 µg per million cells of FcBlock (1µl of 0.5 mg/ml stock per million cells) was added and incubated for 5 min at 4^oC. This prevents non-specific binding of antibodies to Fc receptors.

7. Antibodies directly conjugated to fluorophores or appropriate isotype controls were added directly to cell pellet (typically 0.1µg of antibody per million cells), and mixed by raking gently on tube racks.

8. Cells were incubated in the dark at 4^oC for 30 min.

9. After incubation, cells were washed by adding 3 ml of FACS buffer and centrifuged at 350 x g, 4°C for 5 min. Supernatants were discarded and washing was repeated.

10. After the final wash, cells were raked and cells resuspended in 0.5 ml of FACS buffer for immediate analysis, or fixed with 1% PFA in PBS if analysis was performed after several hours. For some experiments, 1μl of 1 mg/ml of 7-AAD were added per 1 ml of cells for 5 min at 4°C. 7-AAD$^+$ cells are dead cells that were gated out of analysis.

11. Cells were filtered through a 61 μm gauze into a new FACS tube prior to analysis.

Intracellular staining of cells for flow cytometry analysis:

1. Cells for intracellular staining were first restimulated with 10 ng/ml PMA and 400 ng/ml ionomycin for T-cells or 100 ng/ml of LPS for monocytes. Protein secretion was blocked with Golgi stop (BD PharMingen) containing monensin. Golgi stop was used at 1 μl per 1 ml of culture.

2. After 6 h, cells were harvested, transferred into FACS tubes and washed by adding FACS buffer and centrifugation at 350 x g, 5 min at 4°C. Washing was repeated once more.

3. Cells were stained for surface markers using the "Surface staining of cells for FACS analysis" protocol.

4. Cells were fixed using 500 μl of 1 % PFA in PBS per tube. Tubes were vortexed immediately after addition to prevent cell clumping. Tubes were incubated in the dark for 15 min.

5. Fixed cells were permeabilized by the addition of 2 ml of permeabilization/wash buffer. Cells were then centrifuged at 350 x g for 5 min. Washing was repeated.

6. The appropriate antibodies specific for intracellular targets or isotype controls were added to the tubes. These were incubated for 30 min at r.t in the dark.

7. After incubation, cells were washed twice with 3 ml of perm/wash buffer.

8. Cells were subsequently resuspended in FACS buffer for immediate analysis or 1% PFA in PBS for future analysis.

Preparation of cells for sorting:

1. Cells for sorting where prepared and placed into sterile FACS tubes.

2. 0.5 μg per million cells of FcBlock (1μl of 0.5 mg/ml stock per million cells) were added and incubated for 5 min at 4^oC. This prevents non-specific binding of antibodies to Fc receptors.

3. Antibodies directly conjugated to fluorophores were added directly to cell pellet and mixed by raking gently on tube racks.

4. Cells were incubated in the dark at 4^oC for 30 min.

5. After incubation, cells were washed by adding 3 ml MACS buffer and centrifugation at 350 x g, 4^oC for 5 min. Supernatants were discarded and washing was repeated.

6. Cells were raked and resuspended to 10×10^6/ml, filtered through a 61 μm guaze and sorted using a MOFLO cell sorter (Dako).

2.2.3   Generation of monocyte-derived DCs and macrophages

Monocytes were cultured with appropriate cytokines to induce their differentiation into DCs or macrophages. Monocyte-derived DCs were used in co-cultures with CD8 T-cells to study their interaction and induction of IL-12. These DCs, together with monocyte-derived macrophages, were also used as controls to compare the morphology and phenotype of Tip-DCs in later studies.

1. Isolated blood monocytes were seeded in 6-well plates (Nunc) at 2×10^6 cells per ml, resulting in 6×10^6 monocytes in 3 ml of culture medium per well. For generation of cells with smaller monocyte numbers, monocytes were seeded in 24-well plates (Nunc) at 5×10^5/well in 1 ml of culture medium.

2. To generate immature DCs, 1000 U/ml of *rh*GM-CSF and 1000 U/ml of *rh*IL-4 (Peprotech, Gene-Ethics, Singapore) was added to the culture medium. To generate macrophages, 1000 U/ml of *rh*M-CSF (Peprotech) was added to the culture medium. All cells were incubated for 6 days at 37°C/5% CO_2.

3. To induce their maturation, LPS (100ng/ml) was added for a further 24 h where indicated.

2.2.4   Generation of influenza specific CD8 T-cells

CD8 T cells specific for the influenza peptide (Flu M1; 58-66; GILGFVFTL) in the context of HLA-A2.01, were obtained by tetramer isolation and kept in culture through

re-stimulation with peptide-loaded DCs and with growth cytokines every week. The percentage of tetramer-binding cells was >80%.

1. To synthesize the tetramers, 50ng of biotinylated monomeric recombinant HLA-A2.01 (containing the influenza A matrix 1 protein encoded peptide, GILGFVFTL) were incubated with extravidin-PE (Sigma-Aldrich, Singapore). 20μl were added every 10 min for a total of 150 min to form 200ng/ml of each tetramer.

2. CD8 T-cells were isolated from fresh blood of healthy donors that have been tested to be HLA-A2.01 positive.

3. The CD8 T cell fraction was subsequently stained with the PE-labeled tetramer and sorted using a MoFlo cell-sorter (Beckman Coulter, Singapore).

4. To prepare peptide-loaded DCs for re-stimulation, autologous immature monocyte-derived DCs (from the same donor as the CD8 T-cells) was pulsed with 5μM of influenza peptide in 1ml of DMEM without serum at 37°C for 1 h. DCs were subsequently γ-irradiated at 3000 rad (gamma irradiation chamber 4000a) and washed three times with DMEM to remove excess peptide.

5. Sorted CD8 T-cells that stained positive for the influenza peptide were cultured for 14 days in the presence of IL-2 (50 U/ml) and IL-7 (5 ng/ml) and re-stimulated with peptide-loaded autologous DCs every week.

6. The cells were then harvested and used for co-culture studies

2.2.5 CD8 T-cell and DC co-cultures

Allogeneic co-cultures

1. Freshly isolated CD8 T-cells were activated with 10ng/ml of PMA and 400ng/ml of ionomycin, seeded at 1×10^6 cells/well in 24-well plates (Nunc) and incubated at 37°C/5% CO_2 for 48 hours. Alternatively, CD8 T-cells were used unactivated in some studies.

2. Activated CD8 T-cells were washed extensively with pre-warmed DMEM three times to remove excess PMA and ionomycin. Cells were resuspended with culture medium to a final concentration of 1.33×10^6 cells/ml.

3. Allogeneic immature monocyte-derived DCs were harvested and washed with pre-warmed DMEM three times to remove excess IL-4 and GM-CSF. Cells were resuspended with culture medium to a final concentration of 0.67×10^6 cells/ml.

4. 100 µl of activated or freshly isolated CD8 T-cells (1.33×10^5 cells) were then added to 100 µl of allogeneic DCs (0.67×10^5 cells) such that CD8 T-cells and DCs were cultured in a ratio of 2:1 to a total of 2×10^5 cells in 200µl of culture medium per well in triplicates in U-bottom 96-well plates (Costar). Cells were then incubated at 37°C/ 5% CO_2 for 18 h.

5. Where indicated, LPS (100ng/ml) was added into the co-cultures simultaneously.

Autologous co-cultures

1. Immature HLA-A2.01 restricted DCs were pulsed with 5µM influenza peptide (Flu M1; 58-66; GILGFVFTL) for 1 h in 1ml of DMEM without any serum. Alternatively, the irrelevant peptide (EV 71 protein-encoded peptide, KLTDPPAQV) was used as a control.

2. DCs were washed with 5ml of DMEM three times to remove excess peptide.

3. Influenza specific CD8 T-cells were subsequently added with peptide-pulsed DCs in a ratio of 2:1 to a total of 2×10^5 cells in 200ul of culture medium per well in triplicates in U-bottom 96-well plates (Costar) and incubated at 37°C/ 5% CO_2 for 18 h.

4. Where indicated, LPS (100ng/ml) was added to co-cultures simultaneously.

2.2.6 Detection of cytokines in supernatants

ELISA:

IL-12p40 and IL-12p70 levels were detected using sandwich ELISA with DuoSet ELISA development kit from R&D systems (Singapore) according to the manufacturer's protocol. The substrate, 1 mg/ml of pNPP was dissolved in 0.05 M diethanolamine buffer.

1. The appropriate capture antibody was diluted in PBS to the concentration recommended by the manufacturer (typically 180 x dilutions)

2. The diluted capture antibodies were dispensed into 96 well Maxisorb plates at 50 μl per well and incubated O/N at r.t.

3. Capture antibody coated wells were then washed thrice with wash buffer

4. Coated wells were blocked with 300μl block buffer for 1 h at r.t.

5. Wells were washed thrice with wash buffer

6. Pre-diluted standards and supernatants diluted with block buffer to 50μl were added to the appropriate wells and incubated for 2 h at r.t.

7. Wells were washed thrice with wash buffer

8. The appropriate detection antibody pair was diluted in block buffer to the recommended concentration (typically 180 x).

9. Diluted detection antibodies were dispensed into wells at 50 μl per well and incubated for 2 h at r.t.

10. Wells were washed thrice with wash buffer

11. Avidin conjugated AP was diluted 1000 x with block buffer and dispensed into wells at 50 μl per well and incubated for 1 h at r.t

12. Wells were washed 4 x with wash buffer

13. 50μl of 1 mg/ml of PNPP dissolved in 0.05 M diethanolamine per well were dispensed.

14. Color intensity at 405 nm was determined using a microplate reader (Biorad, model 680). Levels of cytokines present were determined by reference to standard curves.

Multiplex bead array:

Multiplex bead arrays were performed for cytokines IL-1α, IL-1β, IL-4, IL-6, IL-10, GM-CSF, IFN-γ, TNF-α and chemokines IP-10, RANTES, MCP-1, MIP-1α, MIP-1β using Bio-rad multiplex assay kit according to the manufacturer's instruction. However, we used 2 x less of the stock reagents (for example, 50 x dilution of beads becomes 100 x dilution of beads). This gave the same results as the dilution factor recommended by the manufacturer.

1. The master standard stock was reconstituted with 500 ul of complete RPMI-1640 with 10 % FCS, and vortexed for 1-3 seconds. This was incubated on ice for 30 min prior to use.

2. 4 fold serial dilutions of the standard were made with complete media.

3. Antibody conjugated beads were vortexed for 30 s before being diluted with Bioplex assay buffer according to the recommended dilution factor. Diluted beads were kept in the dark on ice.

4. 96 well sterile filter plates (Millipore, Multiscreen HTS, BV, 1.2 μm hydrophil low protein binding, durapore membrane) were pre-wet with 100 μl Bioplex assay buffer.

5. Buffer was removed using a vacuum manifold (Millipore, MultiscreenTM HTS), with pressures not exceeding 2.5 Hg or 85 mBar

6. Diluted beads were vortexed for 20 s and 50 μl of beads were added to each well.

7. Buffer was removed using a vacuum manifold

8. 100 µl per well of Bioplex wash buffer was added to each well. Buffer was removed using vacuum manifold.

9. 50 ul of pre-diluted standards and samples diluted in complete media were added into the appropriate wells. The filter plates were sealed with the sealing tape provided and placed onto a plate shaker. Plates were shaken at 1100 rpm for the first 30 s and then 300 rpm for 1 h. Plates were covered with aluminum foil to prevent photobleaching.

10. After incubation, supernatants and standards were removed using vacuum manifold. 100 µl of Bioplex wash buffer was added to each well and removed using the vacuum manifold. Washing was repeated 2 more times.

11. Detection antibodies were diluted in detection antibody diluent according to the recommended dilution factor given by the manufacturer.

12. 25 µl of diluted detection antibody was added to each well and sealed using sealing tape. Plates were shaken at 1100 rpm for the first 30 s and then 300 rpm for 1 h. Plates were covered with aluminum foil to prevent photobleaching.

13. After incubation, detection antibody solution was removed using vacuum manifold. 100 µl of Bioplex wash buffer was added and removed using vacuum manifold. Washing was repeated twice more.

14. 50 µl of streptavidin-PE was diluted with assay buffer at the manufacturer's recommended dilution and added to each well. Plates were shaken at 1100 rpm for the first 30 s and then 300 rpm for 20 min. Plates were then covered with aluminum foil to prevent photobleaching.

15. After incubation, streptavidin-PE solution was removed using vacuum manifold, and wells were washed thrice with 100 μl Bioplex wash buffer.

16. Wells were resuspended with 125 μl of Bioplex assay buffer, sealed and shaken at 1100 rpm for 30 s. Analysis were performed with the Luminex 100 (Qiagen). At least 60 events were collected for each cytokine bead.

2.2.7   Viability Assays

Annexin-V and 7-AAD viability assay

This assay was used to measure the viability of DCs in co-cultures with CD8 T-cells. As such, it also functions as a measure of CTL killing in these co-cultures.

1. Cells were transferred to FACS tubes and washed with PBS.

2. Cells were resuspended with 100 μl of Annexin-V binding buffer at a concentration of 1×10^6 cells/ml.

3. 5 μl of Annexin-V FITC and 1 μl of 7-AAD were added to each tube. Cells were incubated for 15 min at r.t in the dark.

4. After incubation, 400 μl of Annexin-V binding buffer were added to each tube. Cells were analyzed by flow cytometry immediately.

LIVE/DEAD fixable violet dead cell stain kit viability assay

This assay was used to measure the viability of monocytes after activation with LPS and IFNγ. The LIVE/DEAD fixable violet dead cell stain kit (Invitrogen, Singapore) is based

on the reaction of a fluorescent reactive dye with cellular amines from necrotic cells which results in intense fluorescent staining. The difference in intensity between the live and dead cell populations is typically greater than 50-fold and is completely preserved following fixation of the sample.

1. One vial of the fluorescent reactive dye and one vial of the anhydrous DMSO from the kit were allowed to sit at room temperature before the caps were removed.

2. 50 µl of DMSO was added to the vial of reactive dye. The solution was mixed well and visually confirmed that all of the dye had been dissolved. The solution of reactive dye was used as soon as possible, ideally within a few hours of reconstitution.

3. Cells that were to be used for analysis were first counted to determine the cell number and centrifuged at 350 x g for 5 min before supernatant was discarded.

4. Cells were washed once with 1 ml of PBS, centrifuged at 350 x g for 5 min and supernatant was aspirated completely.

5. 400 µl of PBS was added to the cell suspension.

6. Next, 0.4 µl of the reconstituted fluorescent reactive dye (from step 2) was added to the cell suspension and pipetted several times to ensure that the solution was mixed well. Ideally, the reconstituted fluorescent reactive dye should be used at a 1000 x dilution.

7. Cells were incubated at 4°C for 20 min, protected from light.

8. If other surface markers were to be stained, cells were washed twice with 1 ml of FACS buffer before proceeding to the "surface staining of cells for FACS analysis" protocol. Otherwise, cells were washed once with 1 ml of PBS and fixed with 1% PFA.

9. The fixed cell suspension was analyzed by FACS using the channel which corresponds to 405 nm excitation and ~450 nm emission (450/50 nm)

2.2.8 CTL killing assay

The ^{51}Cr release assay was used as a measure to determine the ability of CTLs to lyse target cells. These CTLs were specific for the Flu M1 peptide in the context of HLA-A2.01 and were generated according to the protocol "Generation of Influenza-specific CD8 T-cells".

1. The assay was performed under sterile conditions and with culture medium (DMEM + 10% human AB serum)

2. Target cells (e.g. T2 cells) were prepared in a 15 ml Falcon tube. 4×10^3 T2 cells per well were used.

3. Cells were spun down at 300 x g for 5 min, r.t. Supernatants were discarded and 50 μCi of ^{51}Cr were added. Cells were placed into a 37°C/5% CO_2 incubator for 1 h.

4. Labeled cells were washed with 5 ml of pre-warmed media. Cells were split into two 15 ml Falcon tubes (e.g. 2 x 450 μl). Antigenic peptide Flu M1 (up to 5 μM)

was added to one of the tubes. Both tubes were incubated for 30 min in a 5% CO_2 incubator at 37°C.

5. Effector cells were plated out at the desired ratios onto a 96 U bottom plate. E.g. 10:1, 5:1, 1:1. For wells without effectors, 100 µl pre-warmed media were added. For total lysis controls, 80 µl of pre-warmed media with 20 µl of 10% Triton-X were added.

6. Peptide-pulsed target cells were washed with 5 ml of pre-warmed media at 300 x g, r.t. for 5 mins. Supernatants were discarded.

7. Target cells were counted and resuspended to 4 x 10^4 cells/ml.

8. 100 µl of target cells (4x 10^3 per well) were added to each well in a 96 U-bottom plate.

9. Plates were incubated for 4 to 6 h at 37°C/5% CO_2 incubator.

10. Supernatants were harvested from the assay plate using the cell harvester and transferred into appropriate wells of 96 well Luma plate. The Luma plate was dried in oven at 50°C for 2 h or O/N at r.t. Radioactive counts were made with the Beckman Top Count.

11. Cytotoxic activity is defined as %: (sample lysis – spontaneous lysis)/(total lysis- spontaneous lysis) x 100

2.2.9   Quantitative RT-PCR

To detect the presence of CD40 ligand mRNA in T-cells, quantitative RT-PCR was performed on T-cells that have undergone activation in co-cultures.

1. Cells were harvested and total RNA isolated using the QiaShredder and RNeasy kits (Qiagen), followed by cDNA conversion using reverse transcription with the Quantitect Reverse Transcription kit (Qiagen), according to manufacturer's instructions.

2. 100ng of cDNA per sample was used for Real Time PCR, which was done in 96-well format using the ABS Power SYBR kit (Applied Biosystems, Singapore) in the ABS 7500 Real time PCR machine.

3. Cycling conditions were 95ºC for 10mins in the first cycle followed by 40 cycles of 95ºC for 30s; 60 ºC for 1min; 68 ºC for 1 min.

4. Results were analyzed using the ddCt method for relative quantification of mRNA, with the appropriate endogenous and calibrator controls. The primer sequences used are as follows: CD40L Forward 5'-AGTGGGCTGAAAAAGGATACTACACC-3' and CD40L Reverse 5'-TCTCGAATCTACCGGGGGACTTTAG-3'; GAPDH Forward 5'-TGCCCCCATGTTCGTCA -3' and Reverse 5'- CTTGGCCAGGGGTGCTAA -3'; and 18S RNA Forward 5'- CCGGCGGCTTTGGTGACTCT -3' and Reverse 5'- TTCCTTGGATGTGGTAGCCGTTTCT -3'.

2.2.10 Preparation of cytokine milieus for monocyte differentiation studies

To study the effect of cytokines produced during the CD8 T-cell-DC interaction on the differentiation of monocytes, supernatants of CD8 T-cell-DC co-cultures were harvested

and used as cytokine milieus which would be added to freshly isolated monocytes for 48 h. The preparation of CD8 T-cell-DC co-cultures in this protocol was slightly modified from the previous protocol on co-cultures to reduce the presence of LPS in the supernatants. Supernatants and culture medium (in a ratio of 3:1) were then added to freshly isolated monocytes seeded at a density of 1.3×10^6 cells/ml and incubated for 48 h at 37°C/ 5% CO_2.

1. Immature monocyte-derived DCs that were previously grown in 6-well plates were used.

2. About three quarters of the culture medium in half of the total number of wells (e.g. 2 out of 4 wells of DCs) was carefully aspirated by placing the tip of the pipette at the corner edge of each well. Care was taken not to aspirate too quickly to prevent cells from being aspirated.

3. A fresh amount of culture media (typically about 2 ml) containing 100ng/ml of LPS was added into each well. Leave the other half of the number of wells untouched.

4. Cells were incubated at 37°C/ 5% CO_2 for about 2-3 h.

5. The DCs that have had their culture medium changed were checked for maturation under the microscope for long spindle dendrites and adherence

6. These DCs were carefully harvested using a cell scraper into a 15 ml falcon tube and labeled as "activated DCs" while wells that did not have their culture medium changed were also harvested at the same time into a different falcon tube and labeled as "non-activated DCs".

7. Both tubes of cells were centrifuged at 350 x g at r.t. for 5 min.

8. Cells were washed 3 x by adding 5 ml of pre-warmed DMEM and centrifuged at 350 x g at r.t. for 5 min each time.

9. Activated and non-activated DCs were resuspended in culture medium to a final concentration of 0.67×10^6 cells/ml.

10. Allogeneic CD8 T-cells that have been pre-activated previously (see Chapter 2.2.5) were harvested and washed 3 x with DMEM.

11. CD8 T-cells were resuspended in culture medium to a final concentration of 1.33×10^6 cells/ml

12. 100 µl of activated CD8 T-cells (1.33×10^5 cells) were then added to 100 µl of allogeneic DCs (0.67×10^5 cells) such that CD8 T-cells and DCs were cultured in a ratio of 2:1 to a total of 2×10^5 cells in 200ul of culture medium per well in triplicates in U-bottom 96-well plates (Costar). Cells were then incubated at 37°C/ 5% CO_2.

13. After 18 h, 96-well plates were centrifuged at 350 x g at r.t. for 5 min. Supernatants were harvested and can be stored at -80°C in 1.5 ml eppendorf tubes for future use.

14. To use the supernatants for monocyte studies, supernatants were used either right after harvesting from co-cultures or thawed out from -80°C at r.t.

15. Supernatants and fresh culture medium was added in a ratio of 3:1 to freshly isolated monocytes and incubated for 48 h at 37°C/ 5% CO_2 for differentiation studies. Freshly isolated monocytes were cultured at a density of 1.3×10^6 cells/ml in 24-well plates.

2.2.11 Nitric Oxide Assay

For the detection of intracellular nitric oxide (NO) produced by differentiated monocytes, a pH insensitive fluorescent dye DAF-FM diacetate (Calbiochem, Merck, Singapore) was used. This compound is essentially nonfluorescent until it reacts with NO to form a fluorescent benzotriazole. DAF-FM diacetate is cell-permeant and passively diffuses across cellular membranes. Once inside cells, it is deacetylated by intracellular esterases to become DAF-FM. DAF-FM fluorescence emission, which is related to the intracellular NO content, was then analyzed by flow cytometry.

1. Monocytes that have been previously differentiated for 48 h with cytokine milieus (see Chapter 2.2.10) were used.

2. The media from each well was aspirated gently by placing the tip of a pipette at the corner edge of each well.

3. Cells were washed once by adding 1.5 ml of PBS to each well and gently aspirating by placing the tip of the pipette at the corner edge of each well. Although differentiated monocytes were generally adherent to the plate, care must still be taken not to aspirate too quickly.

4. 2 ml of phenol-red free DMEM (Gibco, Invitrogen) containing 10% human AB serum and 100ng/ml of LPS was added to each well.

5. Cells were incubated for 6 h at 37°C/ 5% CO_2.

6. 5µM of the the pH insensitive fluorescent dye DAF-FM diacetate (Calbiochem, Merck, Singapore) was added directly to the cells and placed back into the incubator for another 30min at 37°C/5% CO_2.

7. The media in each well was gently aspirated and cells were washed once by adding 2 ml of PBS into each well. The PBS was then aspirated completely.

8. 2 ml of phenol-red free DMEM (Gibco, Invitrogen) containing 10% human AB serum was added to the cells and incubated for another 15-30 min at 37°C/5% CO_2 to allow complete de-esterification of the intracellular diacetates.

9. Cells were then gently harvested from the plates by using a cell scrapper, transferred to FACS tubes and centrifuged at 350 x g for 5 min.

10. Supernatants were discarded and cells resuspended in about 250 µl of FACS buffer for FACS analysis. The fluorescence excitation and emission maxima are 495 and 515 nm, respectively, which was detected in the FITC channel.

2.2.12 Phagocytosis

Phagocytosis of differentiated monocytes was determined by incubation with *Escherichia coli* Alexa Fluor 488 conjugate bioparticles at 100 µg/ml (Molecular Probes, Invitrogen, Singapore) in culture medium. Stained cells were washed thrice with cold PBS, resuspended in PBS plus trypan blue 0.4% as a quencher, and analyzed by flow cytometry (Cyan-ADP, Beckman Coulter, Singapore). Phagocytosis was expressed as a percentage of mononuclear cells that were fluorescent.

1. 2 plates of monocytes that have been previously differentiated for 48 h with cytokine milieus (see Chapter 2.2.10) were used.

2. The media from each well was aspirated gently by placing the tip of a pipette at the corner edge of each well.

11. 100 µg/ml of *Escherichia coli* Alexa Fluor 488 conjugate bioparticles was added into each well in fresh culture medium. 1 plate of cells was incubated for 1 h at 37°C/5% CO_2 while the other plate of cells was incubated at 4°C or on ice as a negative control.

3. After incubation, both plates of cells were placed on ice and harvested by gently scraping using a cell scraper. Cells were transferred into FACS tubes and centrifuged at 350 x g for 5 min at 4°C. Supernatants were then discarded.

4. Cells were subsequently washed with cold FACS buffer 3 times.

5. Next, cells were resuspended in 250 µl of PBS + 0.4% trypan blue to quench the fluorescence of BioParticles® conjugates that were bound to the surface but not internalized. Cells were then analyzed by flow cytometry.

2.2.13 Endocytosis

For measurement of endocytosis, differentiated monocytes were incubated with FITC-labeled dextran (40,000 molecular weight; Molecular Probes) in culture medium. After washing 4 times, cells were analyzed by flow cytometry (Cyan-ADP).

1. 2 plates of monocytes that have been previously differentiated for 48 h with cytokine milieus (see Chapter 2.2.10) were used.

2. The media from each well was aspirated gently by placing the tip of a pipette at the corner edge of each well.

3. 1 mg/ml of FITC-labeled dextran was added into each well in fresh culture medium. 1 plate of cells was incubated for 30 min at 37°C/5% CO_2 while the other plate of cells was incubated at 4°C or on ice as a negative control.

4. After incubation, both plates of cells were placed on ice and harvested by gently scraping using a cell scraper. Cells were transferred into FACS tubes and centrifuged at 350 x g for 5 min at 4°C. Supernatants were then discarded.

6. Cells were washed 4 times with cold FACS buffer, subsequently resuspended in 250 µl of FACS buffer and analyzed by flow cytometry.

2.2.14 Proliferation Assay

Naïve CD4 T cells (1x 10^5/well) were co-cultured with decreasing numbers of differentiated monocytes in U-bottom 96-well plates and the T cell proliferative response was measured 6 days after co-culture by an 18 h pulse with [^{3}H]thymidine (1 μCi/well; Amersham Pharmacia Incorporation).

1. Naive CD4 T-cells were resuspended in culture medium at a concentration of 1 x 10^6 cells/ml. 100 µl of the CD4 T-cell suspension (1x 10^5 cells) was pipetted into each well of a U-bottom 96 well plate.

2. Differentiated monocytes were resuspended in culture medium at a concentration of 1 x 10^5 cells/ml and added at appropriate ratios. 100ng/ml of LPS was added where indicated.

3. Cells were incubated for 6 days at 37°C/5% CO_2.

4. 1mCi/ml [methyl-^{3}H]-thymidine (Amersham Biosciences) was diluted in culture medium to obtain a working concentration of 50µCi/ml.

5. Each well was pulsed with 20µl of [methyl-^{3}H]-thymidine (1µCi/well) 18 h before harvesting. They were collected onto a harvesting plate (Unifilter-96, GF/C from Perkin Elmer) using Filtermate Harvester (Perkin Elmer).

6. The plate was then placed in a 50°C oven for 2h to dry. The bottom of the plate was then sealed with Backing Tape (Perkin Elmer) and 20µl of Microscint 20 (Perkin Elmer) was added to each well containing the harvested cells.

7. The plate was covered with TopSealTM (Perkin Elmer) and transferred to TopCount NXT (Perkin Elmer) for counting. Results were expressed as counts per minute (cpm).

2.2.15   Microscopy

Haematoxylin and eosin staining

Monocytes were differentiated on glass coverslips and fixed with acetone/methanol. Haematoxylin and eosin staining was performed to study the morphology of these cells.

1. Round-shaped 10mm glass coverslips were first dipped in 70% ethanol and allowed to dry on a C-fold towel. They were then placed at the bottom of plastic 24-well culture plates using forceps.

2. Freshly isolated monocytes in 1 ml of cytokine milieu (see Chapter 2.2.10) were added to these wells and incubated for 48 h at 37°C/5% CO_2. Classical monocyte-derived DCs and macrophages were grown (see Chapter 2.2.3) with coverslips at the bottom of each well for 6 days. LPS was added to cells if necessary for an additional 24 h.

3. A hooked needle was used to gently lift the coverslips (which contain the cells) out of the wells. The coverslips were then transferred to a clean 24-well plate using a pair of forceps and placed sample side facing up.

4. 2 ml of fixation buffer containing methanol/acetone (1:1) was added to the coverslips in the wells and stored at -20 ℃ overnight. For longer storage, top up of fixation buffer is needed.

5. For immature DCs that do not adhere to coverslips, 200 μl of cells which have been resuspended at 0.5×10^6 cells/ml were loaded onto a microscope slide through a cuvette which had been attached with the paper pad in a metal holder. Cells underwent a cytospin by centrifugation at 800 rpm for 3 min. They were then subsequently fixed with fixation buffer.

6. To proceed with H&E staining, microscope slides or coverslips that were handled with forceps were dipped into the following solutions as follows:

    a. Mayer's Hematoxylin- 5 min

    b. Washed in a jar of tap water until clear

    c. 2% NH4OH- 1 to 2 dips

    d. Washed in a jar of tap water

    e. Alcoholic Y Eosin- 1 min

    f. Washed in a jar of tap water until clear

    g. 70% Ethanol-3 dips

    h. 95% Ethanol (1)- 5 dips

    i. 95% Ethanol (2)- 7 dips

    j. 100% Ethanol (1)- 10 dips

    k. 100% Ethanol (2) – 10 dips

    l. Xylene (1)- 10 dips

    m. Xylene (2)- 10 dips

    n. Xylene (3)- 10 dips

    o. Xylene (4)- 10 dips

7. Coverslips and slides were finally mounted with DPX mountant and left to dry under the fumehood before capture using a Leica DM2000 microscope (Leica Microsystems, Singapore).

Immunofluorescence staining

Monocytes were differentiated on glass coverslips and immunostaining was performed to detect intracellular TNF-α and iNOS. The coverslips were then mounted onto slides and viewed under a fluorescence microscope. This protocol describes the steps taken for immunostaining specific for the detection of intracellular TNF-α and iNOS in monocytes.

1. Round-shaped 10mm glass coverslips were first dipped in 70% ethanol and allowed to dry on a C-fold towel. They were then placed at the bottom of plastic 24-well culture plates using forceps.

2. Freshly isolated monocytes in 1 ml of cytokine milieu (see Chapter 2.2.10) were added to these wells and incubated for 48 h at 37°C/5% CO_2.

3. Next, LPS (100ng/ml) and 1 μl/ml of Golgi stop were added to the cells and incubated for a further 6 h at 37°C/5% CO_2.

4. A hooked needle was used to gently lift the coverslips (which contain the cells) out of the wells. The coverslips were then transferred to a clean 24-well plate using a pair of forceps and placed sample side facing up.

5. 2 ml of fixation buffer containing methanol/acetone (1:1) was added to the coverslips in the wells and stored at -20 ºC overnight. For longer storage, top up of fixation buffer is needed.

6. Following fixation, cells were washed 3 times by adding 1ml of PBS/10mM glycine into each well. Care was taken to add and aspirate buffers gently from the side of each well.

7. 200 μl of Permeabilization/washing buffer was added into each well containing the coverslips and incubate at r.t. for 30 min.

8. 5 μg/ml of purified mouse anti-human iNOS/NOS Type II (clone 6/iNOS/NOS Type II; BD Pharmingen) was added to the buffer in each well. Plates were sealed with parafilm and incubated for 30-45 min at r.t.

9. The buffer in each well was gently aspirated and 1ml of PBS/glycine was added. Washing was carried out 3 times.

10. Next, secondary antibody goat-anti-mouse Alexa Fluor 647 IgG (Molecular Probes, Invitrogen, Singapore) and PE-conjugated mouse-anti-human TNF-α (clone 6401.1111; BD Pharmingen) were diluted to a concentration of 5 μg/ml in Permeabilization/washing buffer and added to each well. Plates were sealed with parafilm and incubated for 20-30 min at r.t.

11. After incubation, the buffer in each well was gently aspirated and 1ml of PBS/glycine was added. Washing was carried out 3 times.

12. 2 μl of DAPI (100 μg/ml) in 2ml of PBS/glycine was added to each well and left for 20 s. Coverslips were then washed with PBS/glycine 3 times.

13. Coverslips were then dipped in milliQ water and excess water droplets dried off by gently tapping it on a C-fold towel.

14. 3-4 μl of ProLong Gold anti-fading mounting medium (Invitrogen) was pipetted onto a glass slide and coverslips were mounted onto the slides with sample side facing downwards.

15. Slides were stored at 4°C overnight in the dark for the mounting medium to set. Secure coverslips by placing nail polish at the coverslip edges. Slides were viewed under a fluorescence microscope (Axio imager.Z1, Axiocam HRM camera; Carl Zeiss Micro Imaging, Inc., Singapore) using AxioVision LE software (Version 4.6).

2.3 Mouse studies

2.3.1 Mice

C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA). IFN-$\gamma^{-/-}$, beta-2-microglobulin (β_2m)$^{-/-}$ and CCR2$^{-/-}$ mice on C57BL/6 background were from the Jackson Laboratory (Bar Harbor, ME). All mice used were 6-10 weeks of age and of the female gender. Mice were maintained under specific pathogen free conditions within National University of Singapore's satellite animal housing unit and used according to institutional guidelines.

2.3.2 Elicitation of Contact Hypersensitivity(CHS)

Chemicals

1-chloro-2,4-di-nitrobenzene (DNCB), trimellitic anhydride (TMA), acetone and olive oil were all purchased from Sigma-Aldrich (Singapore). Chemicals were diluted w/v in vehicle (4:1; acetone; olive oil)

Hapten sensitization and challenge to elicit CHS

Mice were shaved on the flanks one day prior to sensitization with either 1% DNCB or 10% TMA and challenged 5 days later with the same chemical on the ears. CHS responses were measured by the swelling of the ears measured using a micrometer (Mitutoyo, Japan).

1. Flanks of mice were shaved 24 h prior to topical application of 50 μl of chemical allergen on day 0

2. Mice were challenged by giving a topical application of 25 μl of chemical allergen on the ventral and dorsum of both ears on day 5.

3. Mice were culled 18 h after challenge unless specified otherwise.

Depletion of CD4 or CD8 T-cells during CHS

To study the role of CD4 or CD8 T-cells in the elicitation phase of CHS, mice were given 250μg of anti-CD8α (clone: 53-6.7) or anti-CD4 (clone: GK1.5) via the intra-peritoneal route two days before the last challenge.

2.3.3 Isolation of mouse dermal skin cells for flow cytometry

This protocol illustrates the steps taken to remove the epidermis from the dermis of mice ears using a solution of dispase before further digesting the dermis in Collagenase type IV to liberate the dermal cells. Cells were then washed and prepared for analysis by flow cytometry.

1. Ears were carefully excised from euthanized mice. The dorsal and ventral side of each ear was separated by gently pulling them apart with forceps.

2. The dorsal and ventral side of each ear was made to float on 2 ml of Dispase (Gibco) in a 24-well plate with the inner side facing down. The plates were then incubated at 37°C/5% CO_2 for 90 min.

3. After incubation, the dorsal and ventral side of each ear was transferred from the Dispase solution and made to float on a drop of 100 µl of PBS in a petri dish. The epidermis was carefully removed from the dermis using forceps.

4. The dermal layers were pulled into a 24 –well plate (4 pieces per well) and minced into smaller pieces using a pair of scissors. 2 ml of Collagenase type IV containing 1 µl/ml of Golgi-Stop was added to each well and plates were incubated at 37°C/5% CO_2 for 2.5 h.

5. After incubation, the suspension in each well was homogenized by gently aspirating and dispensing with a 3ml syringe attached with a 19G needle to obtain a single cell suspension.

6. The single cell suspension was then filtered through a 61 µm gauze into a 15 ml falcon tube, centrifuged at 1200 rpm for 5 min at 4°C before FACS analysis.

2.3.4 Microscopy

Mice ears were carefully excised and embedded immediately in Tissue-Tek O.C.T compound (Sakura, Qiagen, Singapore) for cryosectioning.

Haematoxylin and eosin staining

Microscope slides with embedded cryosection samples were first fixed in acetone for 5 min before undergoing the same steps as indicated in Chapter 2.2.15. Coverslips and slides were finally mounted with DPX mountant and left to dry under the fumehood before capture using a Leica DM2000 microscope (Leica Microsystems, Singapore).

Immunofluorescence staining

1. Microscope slides with embedded cryosection samples were allowed to thaw by placing them under the fume hood for a minimum of 1 h

2. A Dako pen was used to outline an area around each sample on the slide

3. Slides were fixed in acetone for 5 min and washed twice with PBS in washing jars

4. Slides were then placed in a staining jar containing the blocking buffer for 10 min before washing twice with PBS

5. Excess liquid around each sample was aspirated gently and primary antibodies that have been diluted in staining buffer were added onto each sample. Typically 25-50 ul of antibody solution was added to each sample.

6. Slides were placed in a humid chamber and incubated overnight at 4°C

7. After incubation, slides were dipped into a staining jar containing PBS for 5 min before transferring slides to another jar containing PBS. Washing steps were conducted 3 times.

8. Excess liquid around each sample was aspirated gently and secondary antibodies that have been diluted in staining buffer were added onto each sample. Typically 25-50 ul of antibody solution was added to each sample.

9. Slides were placed in a humid chamber and incubated for 45 min at r.t.

10. After incubation, slides were dipped into a staining jar containing PBS for 5 min before transferring slides to another jar containing PBS. Washing steps were conducted 3 times and in the dark.

11. Slides were placed in a staining jar containing DAPI diluted in PBS for 5 min at r.t.

12. Slides were washed once with PBS.

13. Slides were then mounted with Dako mounting medium and stored at 4°C until ready for viewing under a fluorescence microscope (Axio imager.Z1, Axiocam HRM camera; Carl Zeiss Micro Imaging, Inc., Singapore) and analyzed using AxioVision LE software (Version 4.6).

2.3.5 Isolation of draining lymph nodes for cell culture

This protocol illustrates the procedure for testing cytokines produced from lymph node cells of sensitized mice.

1. Auricular draining lymph nodes from euthanized mice were carefully excised using a pair of forceps

2. Lymph nodes were physically disrupted and passed through a 70μm cell strainer to obtain a single cell suspension

3. Cells were washed twice with MACS buffer, counted using a hemocytometer and resuspended to 4 x 10^6/ml in complete RPMI-1640 medium.

4. Cells (2 x 10^6 cells per well) were placed in 48-well plates that have been pre-coated with 1μg/ml of anti-CD3 antibodies. 1μg/ml of anti-CD28 antibodies was also added into the cell culture.

5. Cells were cultured overnight at 37°C 5 % CO_2 in an incubator and supernatants were harvested the next day for cytokine analysis.

2.3.6   Homogenization of ear tissues

This protocol illustrates the procedure of homogenizing ear tissues for the measurement of cytokine protein concentrations.

1. Ear tissues were excised from euthanized mice and cut into small pieces before placing each pair of ears into a 2ml RNAse/DNAse free tube pre-filled with 1.4mm ceramic beads (Omni International, Singapore)

2. 275μl of RIPA buffer (Sigma-Aldrich) containing protease inhibitor (25 X dilution) (Roche, Singapore) was added into the tube.

3. The tubes containing the ear tissues were homogenized using the Omni Bead Rupter 24 Homogenizer (Omni International, Singapore) at a rotation speed of 4.5 m/s for 45 sec.

4. The tubes were cooled to remove excess heat by placing in ice for 1 min and homogenized again at 4.5 m/s for another 45 sec

5. The supernatant was removed from each tube and cytokines were analyzed using ELISA.

2.3.7 Detection of cytokines

GM-CSF, IFN-γ, IL-4, IL-5, IL-10, IL-13 and IL-17a levels were detected using sandwich ELISA with DuoSet ELISA development kit from R&D systems (Singapore) according to the manufacturer's protocol.

2.4 Statistical Analysis

Results shown were mean ± SD or mean ± SE as indicated. Comparisons between two groups were performed by Student's *t*-test. Comparisons between three or more groups were performed by one-way ANOVA followed by Tukey's post test. Data were analyzed using Prism 5 (GraphPad).

TABLE 2.1 Primary antibodies used in human studies

Target	mAB clone	Isotype	Conjugation	Purpose	Source
CCR1	TG4/CCR1	Mouse IgG2b	AF647	Surface staining	BioLegend
CCR2	TG5/CCR2	Mouse IgG2b	AF647	Surface staining	BioLegend
CCR5	HEK/1/85a	Rat IgG2a	AF647	Surface staining	BioLegend
CCR7	3D12	Rat IgG2a	PE	Surface staining	eBioscience
CD3	UCHT1	Mouse IgG1	PB	Surface staining	BD Pharmingen
CD4	RPA-T4	Mouse IgG1	FITC	Surface staining	BD Pharmingen
CD8	RPA-T8	Mouse IgG1	APC	Surface staining	BD Pharmingen
CD14	MΦP9	Mouse IgG2a	APC	Surface staining	BD Pharmingen
CD40	5C3	Mouse IgG1	FITC	Surface staining	BD Pharmingen
CD40L	TRAP1	Mouse IgG1	FITC	Surface staining	BD Pharmingen
CD80	L307.4	Mouse IgG1	PE	Surface staining	BD Pharmingen

CD83	HB15e	Mouse IgG1	APC	Surface staining	BD Pharmingen
CD86	2331	Mouse IgG1	PE	Surface staining	BD Pharmingen
CX$_3$CR1	2A9-1	Rat IgG2b	AF647	Surface staining	BioLegend
DC-SIGN	9E9A8	Mouse IgG2a	PE	Surface staining	BD Pharmingen
GM-CSF	BVD2-21C11	Rat IgG2a	AF647	Intracellular staining	eBioscience
HLA-A, B, C	W6/32	Mouse IgG2a	PB	Surface staining	BioLegend
HLA-DR	G46-6	Mouse IgG2a	FITC	Surface staining	BD Pharmingen
IFN-γ	25723.11	Mouse IgG1	PE	Intracellular staining	BD Pharmingen
IL-4	MP4-25D2	Rat IgG1	APC	Intracellular staining	BioLegend
iNOS/NOS Type II	Clone 6	Mouse IgG2a	Purified	Intracellular staining & Microscopy	BD Pharmingen
Perforin	dG9	Mouse IgG2b	AF647	Intracellular staining	BioLegend
TLR2	TL2.1	Mouse IgG2a	PE	Surface staining	eBioscience
TLR3	TLR3.7	Mouse IgG1	PE	Surface staining	eBioscience
TLR4	HTA125	Mouse IgG2a	PE	Surface staining	BioLegend
TLR8	44C143	Mouse IgG1	PE	Surface staining	Abcam
TNF-α	6401.1111	Mouse IgG1	PE	Intracellular staining & Microscopy	BD Pharmingen

TABLE 2.2 Primary antibodies used in mice studies

Target	clone	Isotype	Conjugation	Purpose	Source
CD3	145-2C11	Hamster IgG1	Purified	Microscopy	eBioscience
CD4	GK1.5	Rat IgG2b	Purified	Depletion *in vivo*	eBioscience
CD8α	53-6.7	Rat IgG2a	Purified	Depletion *in vivo*	eBioscience
CD11b	M1/70	Rat IgG2b	PE-Cy7	Surface staining	eBioscience
CD11c	HL3	Hamster IgG1	PE	Surface staining	BD Pharmingen
CD31	2H8	Hamster IgG1	Purified	Microscopy	Millipore
CD45	30-F11	Rat IgG2b	APC-Cy7	Surface staining	BD Pharmingen

CD68	FA-11	Rat IgG2a	FITC	Surface staining	Serotec
IA/IE	M5/ 114.15.2	Rat IgG2b	PerCP-Cy5.5	Surface staining	eBioscience
IA/IE	M5/114.15.2	Rat IgG2b	Purified	Surface staining	eBioscience
iNOS/NOS Type II	polyclonal	Rabbit IgG1	Purified	Microscopy	Calbiochem
iNOS/NOS Type II	polyclonal	Rabbit IgG1	Purified	Intracellular staining	BD Pharmigen
Ly6C	HK1.4	Rat IgG2c	PB	Surface staining	BioLegend
Ly6G	1A8	Rat IgG2a	PE	Surface staining	BD Pharmingen
TCRαβ	H57-597	Hamster IgG1	APC	Surface staining	eBioscience
TNF-α	MP6-XT22	Rat IgG1	AF488	Intracellular staining	BioLegend

TABLE 2.3 Secondary antibodies

Target	Host	Conjugation	Source
Anti-Rabbit	Goat	AF647	Invitrogen
Anti-Rabbit	Donkey	Cy3	Jackson laboratories
Anti-Rat	Donkey	Cy2	Jackson laboratories
Anti-Hamster	Donkey	DyLight649	Jackson laboratories

CHAPTER 3: Requirements for human CD8 T-cell mediated DC IL-12p70 production

3.1 Introduction

CD4 T-cells were first shown to demonstrate their helper role by utilizing CD40L to prime DCs for the production of IL-12 (Cella et al., 1996; Koch et al., 1996) However, in contrast to this well established role of CD4 Th1 cells, little was known if CD8 T-cells, which normally relied on signals from DCs to induce their cytotoxicity (Bennett et al., 1998; Jung et al., 2002), could also function as helper cells. Mailliard *et al.* (2002) then showed that naive human CD8 T-cells could co-operate with naive CD4 T-cells to induce IL-12p70 from DCs and this was dependent on the expression of IFN-γ and CD40L by CD8 T-cells and CD4 T-cells respectively. Recent reports using mouse models from our lab have also revealed that activated or memory CD8 T-cells could prime splenic DCs for IL-12p70 in the absence of CD4 T-cells and this was heavily dependent on CD8 T-cells' ability to express CD40L (Wong et al., 2008; Wong et al., 2009). Since CD8 T-cells have been shown to acquire their effector functions and infiltrate into inflamed sites at a much earlier time than CD4 T-cells (Akiba et al., 2002; Battistini et al., 2003), we wondered if human CD8 T-cells were also able to prime DCs for IL-12 in the absence of CD4 T-cells.

In this chapter, we devised an *in vitro* system that allowed us to study the effect of human blood CD8 T-cells on monocyte-derived DCs during a mixed lymphocyte reaction (MLR) and antigen-specific manner using the Flu M1 peptide in the context of HLA-A2.01. We also utilized blocking antibodies against soluble factors and CD40L to determine if these factors were necessary for CD8 T-cells to induce DCs for the production of IL-12. Finally, we also determined if CD8 T-cells could prime DCs for the production of other cytokines and chemokines using a multiplex bioassay.

3.2 Results

3.2.1 Purity of MACS isolated blood CD8 T-cells and Monocytes

Since we would be studying the effect of CD8 T-cells on DCs in an *in vitro* co-culture system, it is important that the purity of isolated CD8 T-cells and monocytes (which would be differentiated into DCs before co-culture) had to be relatively high in order to rule out any confounding factors due to contaminating cells. To examine the purity of these cells, FACS analysis was performed on isolated cells after MACS separation.

CD8 T-cells were first isolated by positively selecting PBMCs using anti-CD8 conjugated microbeads. All viable cells were included to give a more stringent picture of cell purity (Fig.3.1.A). We found that cells which have been positively selected with only anti-CD8 microbeads gave a purity of only 92.3% CD8 T-cells (Fig. 3.1C). As NK cells and NKT cells have been shown to express low levels of CD8, we depleted the cell fraction of these cells by negative selection with anti-CD56 magnetic beads and collecting the fraction of cells which were not bound to the magnetic column. We found that this additional step gave an enriched population of CD8 T-cells with a higher purity of 98.9% (Fig. 3.1D).

Monocytes were isolated from peripheral blood by negatively selecting PBMCs using the Monocyte Isolation Kit II. Non-monocytes were indirectly magnetically labeled using a cocktail of biotin-conjugated antibodies provided in the kit against CD3, CD7, CD16, CD19, CD56, CD123 and Glycophorin A. Isolation of unlabeled monocytes was

achieved by depletion of the magnetically labeled cells. We found that this procedure

gave a percentage of 97.2% of CD14+HLA-DR+ monocytes (Fig. 3.1G).

The purity of CD8 T-cells and monocytes obtained were periodically checked throughout

the whole course of the study, and were found to fall within 95-99% for CD8 T-cells and

96-98% for monocytes.
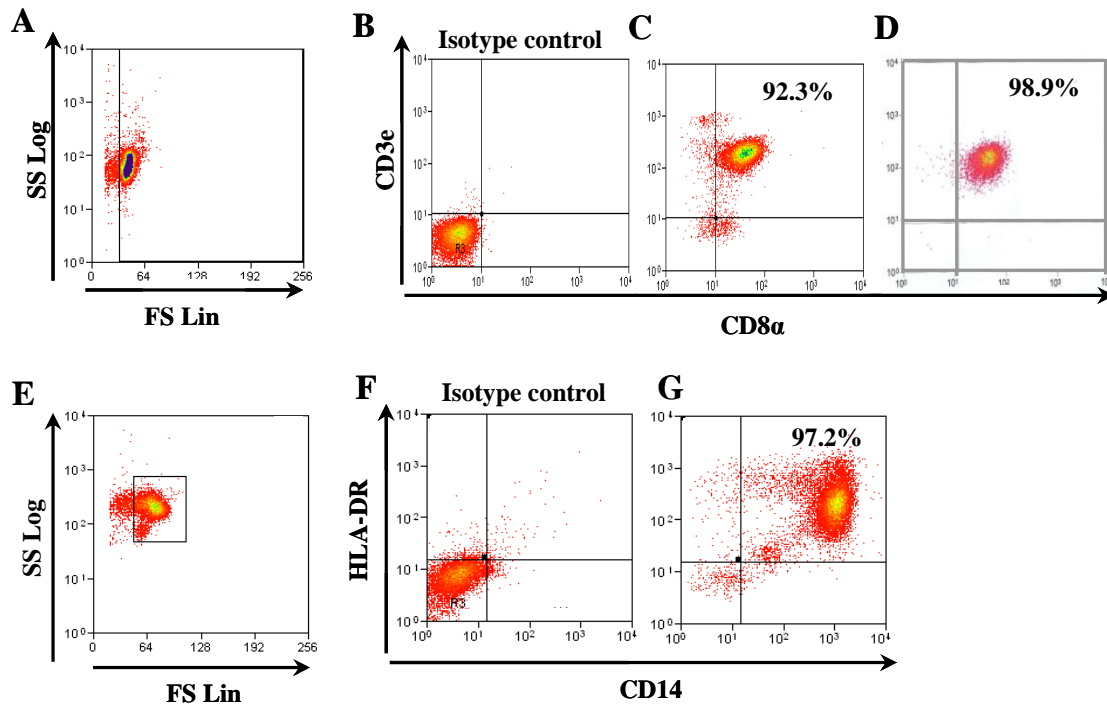
Fig. 3.1 Purity of blood CD8 T-cells and Monocytes isolated by MACS.

PBMCs were selected for CD8 T-cells and monocytes using MACS and analyzed using flow cytometry. (A) Forward and side scatter plot of isolated CD8 T-cells (B) Isolated CD8 T-cells stained with isotype antibodies (C) Isolated CD8 T-cells selected using anti-CD8 positive selection only. Cells were stained with anti-CD3 PB and anti-CD8 APC. (D) Enriched population of CD8 T-cells after depletion of contaminating NK cells with anti-CD56 MACS beads. (E) Forward and side scatter plot of isolated monocytes. (F) Isolated monocytes negatively selected using Monocyte Isolation Kit II and stained with isotype antibodies. (G) Isolated monocytes negatively selected and stained with anti-CD14 APC and anti-HLA-DR FITC antibodies. Results are representative of five experiments.

3.2.2 CD8 T-cells upregulate the surface expression of co-stimulatory molecules on DCs

To investigate the modulating effects of CD8 T-cells on monocyte-derived DCs, we first determined if CD8 T-cells could activate DCs during an allogeneic co-culture. CD8 T-cells isolated from the blood of healthy donors were used either freshly isolated or activated for 48 h using PMA/ionomycin as a stimulus as previously described (Wong et al., 2008). We first determined the activation status of CD8 T-cells after stimulation. We found that activated CD8 T-cells downregulated their expression of CD45RA and CCR7 compared to freshly isolated CD8 T-cells (Fig.3.2A), suggesting that these cells have adopted an effector phenotype (Sallusto et al., 1999). Next, freshly isolated and activated CD8 T-cells were incubated with anti-CD3/CD28 for 6 h in the presence of Golgi-stop^{TM}, fixed and stained intracellularly by flow cytometry. We found that CD8 T-cells that were freshly isolated did not express much intracellular IFN-γ, however upon activation, activated CD8 T-cells expressed a significant higher percentage of intracellular IFN-γ (Fig.3.2B). The percentage of perforin expression was similar between these cells (Fig. 3.2C).
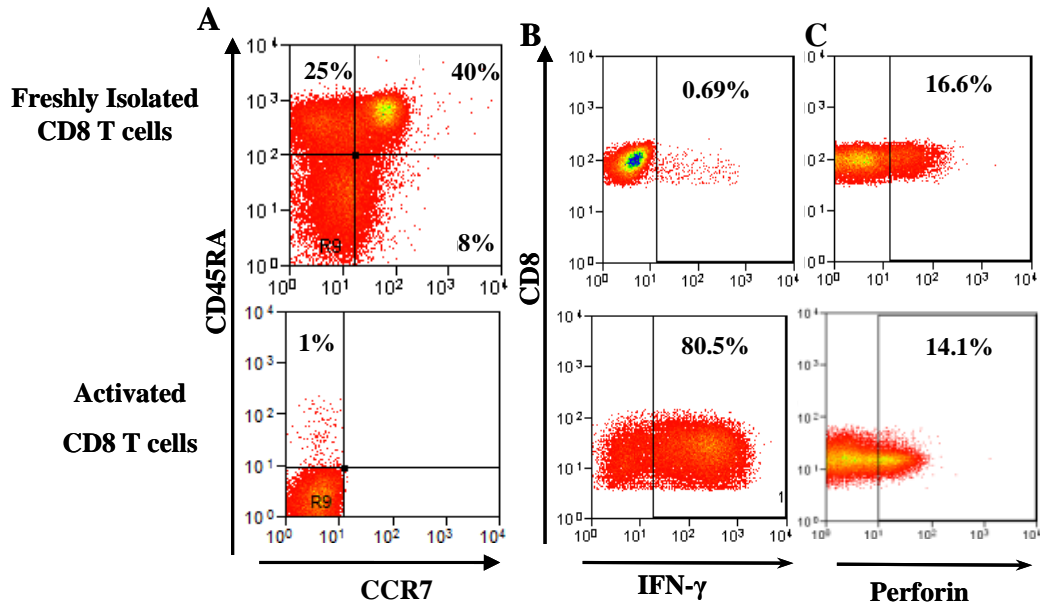
Fig. 3.2 Phenotypic comparison of freshly isolated and activated CD8 T-cells.

CD8 T-cells were analyzed either freshly isolated after MACS selection or after activation with PMA/ionomycin for 48 h. (A) CD8 T-cells were stained with anti-CD45RA APC and anti-CCR7 PE. CD8 T-cells were cultured with anti-CD3/anti-CD28 antibodies for 6 h in the presence of Golgi-Stop^{TM}. Cells were harvested, fixed, permeabilized and stained for intracellular (B) IFN-γ and (C) Perforin. Events shown are gated on CD3$^+$ CD8$^+$ cells. Results are representative of three experiments.

We then co-cultured CD8 T-cells with allogeneic DCs for 18 h and DCs were evaluated by analyzing the expression and/or upregulation of co-stimulatory molecules CD40, CD80 and CD86, as well as the maturation marker CD83. Immature DCs that were unstimulated did not express CD80 and CD83 but expressed intermediate levels of CD86 (Fig. 3.3B) compared to the isotype control (Fig. 3.3A). DCs that were activated with LPS served as a positive control and showed upregulation of CD40, CD80, CD86, as well as the maturation marker, CD83 (Fig. 3.3C). When DCs were co-cultured with CD8 T-cells, we found that freshly isolated CD8 T cells could only upregulate CD40 and CD80

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expression (Fig. 3.3D) and this was to a much lesser extent than the positive control of LPS. In contrast, activated CD8 T cells induced the up-regulation of all the molecules tested (Fig. 3.3E) and was expressed at a higher percentage and MFI than those induced by freshly isolated CD8 T-cells. In addition, the increase in MFI expression of CD80 and CD86 was higher than those induced by the positive control, LPS. Hence, CD8 T-cells are able to activate DCs by inducing the upregulation of these co-stimulatory molecules and this activation was best achieved with the co-culture of activated CD8 T-cells.
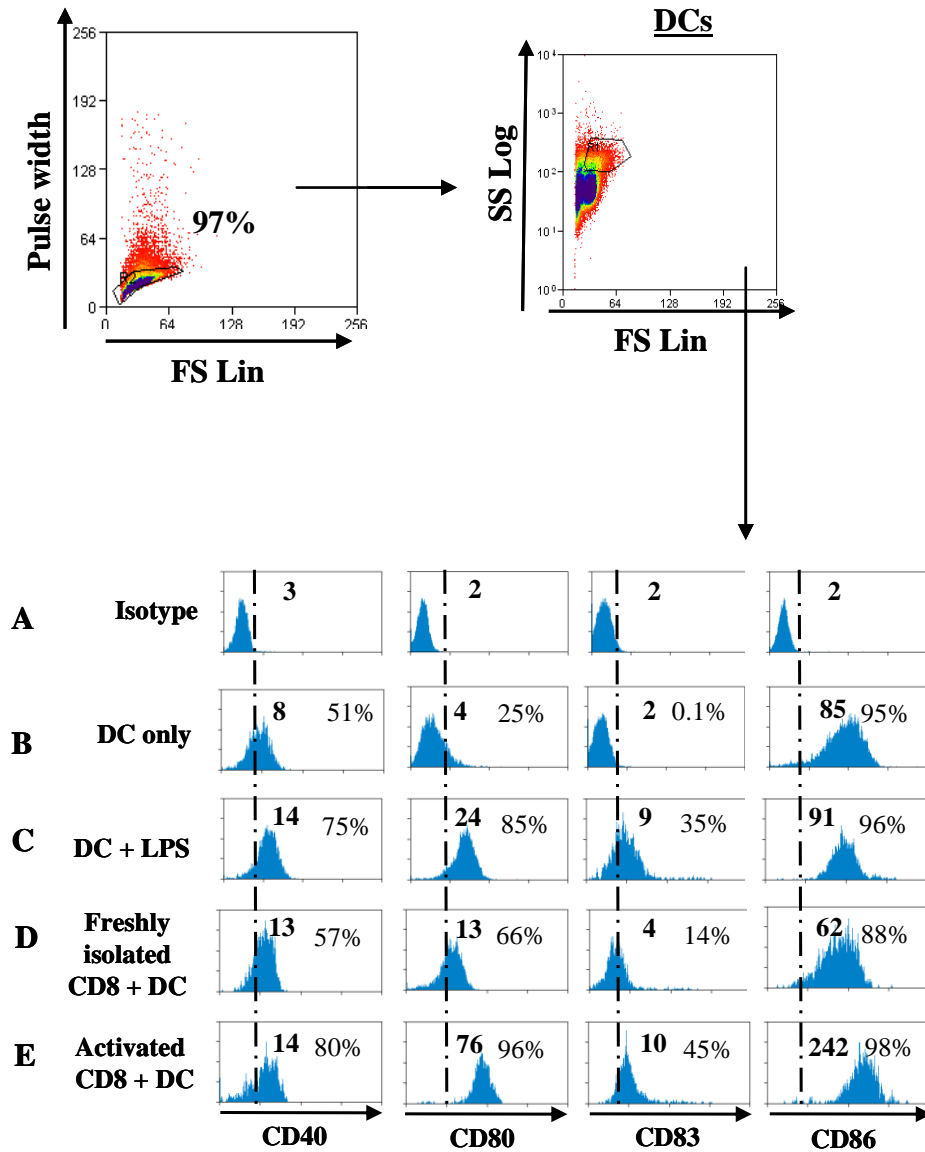
Fig. 3.3 Activated CD8 T-cells induce the up-regulation of co-stimulatory molecules and maturation of DCs.

Cells were first selected for singlets by gating on cells with low pulse width and DCs discriminated by gating on the high side scatter plot. (A) Day 6 immature DCs were stained with isotype control antibodies. (B) Day 6 immature DCs (C) DCs activated with LPS (C) DCs co-cultured with freshly isolated or (D) activated allogeneic CD8 T cells for 18 h were washed and stained for surface expression of DC markers anti-CD40, anti-CD80, anti-CD83 and anti-CD86 using flow cytometry. Bold numbers indicate mean fluorescence index (MFI) and numbers on right indicate the percentage of cells. Results are representative of three experiments.

3.2.3   Pre-activation of CD8 T-cells and LPS were required for DC IL-12p70

We next examined if CD8 T-cells could induce DCs to produce IL-12. We first used DCs that were stimulated with LPS as a readout. DCs that were cultured with LPS for 18 h produced IL-12p40 (Fig. 3.4A) but failed to produce IL-12p70 (Fig. 3.4B). Since previous reports have suggested that the production of IL-12p70 requires at least 2 activating signals (Snijders et al., 1998), we added *rh*IFN-γ together with LPS. DCs stimulated with both LPS and IFN-γ produced increased amounts of IL-12p40 compared to DCs stimulated with LPS alone (Fig. 3.4A). In addition, they produced robust amounts of IL-12p70 (Fig. 3.4B), hence making it a useful positive control. We then analyzed the supernatants that were harvested from CD8 T-cell-DC co-cultures. Both freshly isolated and activated CD8 T-cells failed to induce the production of both IL-12p40 and p70 from DCs alone (Fig. 3.4A and B). We then added LPS to the CD8 T-cell-DC co-cultures to determine if a second signal was needed for the production of IL-12. We found that IL-12p40 was present in supernatants from freshly isolated CD8 T-cells cultured with DCs in the presence of LPS, however this condition was still not enough for the production of IL-12p70 (Fig. 3.4A and B) even at longer co-culture time points of up to 7 days (data not shown). In contrast to the weak modulating activity of freshly isolated CD8 T-cells, activated CD8 T-cells induced a significant increase in the amount of IL-12p40 from DCs in the presence of LPS (Fig. 3.4A). Strikingly, a significant amount of IL-12p70 was also detected from activated CD8 T-cells cultured with DCs and LPS (Fig. 3.4B). Hence, pre-activation of CD8 T-cells was required not only to induce DC maturation, but also to co-operate with LPS as the second signal to prime DCs for IL-12p70 production.
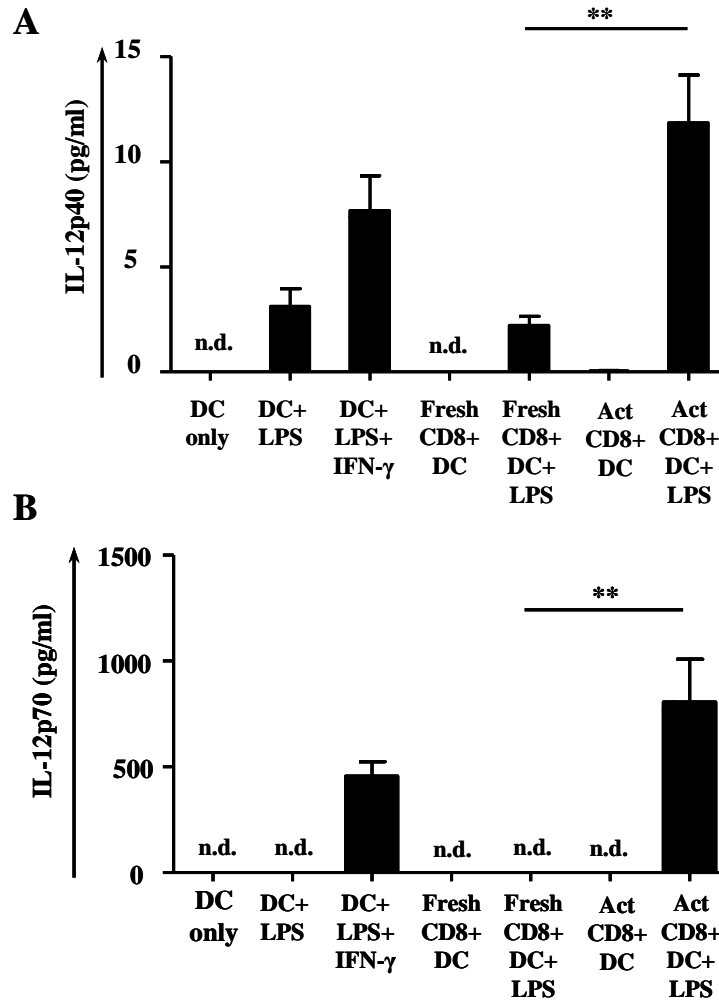
Fig. 3.4 Pre-activation of CD8 T-cells and LPS were required for induction of DC IL-12p70

Freshly isolated or activated CD8 T cells were cultured with allogeneic Day 6 immature DCs for 18 h in the presence or absence of LPS. Supernatants were harvested and analyzed for the presence of (A) IL-12p40 and (B) IL-12p70 using ELISA. **p<0.001 using student's *t*-test. Results are pooled from 3 independent donors.

3.2.4   Priming of DC for IL-12p70 production is antigen specific

To demonstrate the capacity of CD8 T-cells to induce IL-12p70 production by DCs in an antigen-specific manner, we generated CD8 T-cells lines (CTLs) specific for the influenza M1 peptide in the context of HLA.A2.1. These flu-specific CTLs were first isolated from blood through cell-sorting by specific tetramer isolation and kept in culture with addition of IL-2, IL-7 and re-stimulated with peptide-loaded DCs weekly to maintain their activation status. The frequency of tetramer-binding cells was found to be about 85.7% (Fig. 3.5A inset). When these CTLs were cultured with autologous DCs in the presence of an irrelevant peptide EV71, IL-12p70 production was undetectable even when LPS was added to the co-cultures (Fig. 3.5A). Consistent with our findings from the allogeneic co-cultures (Fig. 3.4B), CTLs cultured with the relevant peptide-loaded DCs and LPS produced IL-12p70 while those in the absence of LPS did not (Fig. 3.5A).

We next determined if the production of IL-12p70 was dependent on the amount of peptide used. Autologous DCs were pulsed with increasing doses of Flu M1 peptide for 1 h, washed and co-cultured with CTLs. We found that IL-12p70 production increased in a dose-dependent manner with the concentration of Flu M1 peptide used (Fig. 3.5B). Hence, these results indicate that CD8 T cell induction of IL-12p70 from DCs occurs in an antigen specific system.
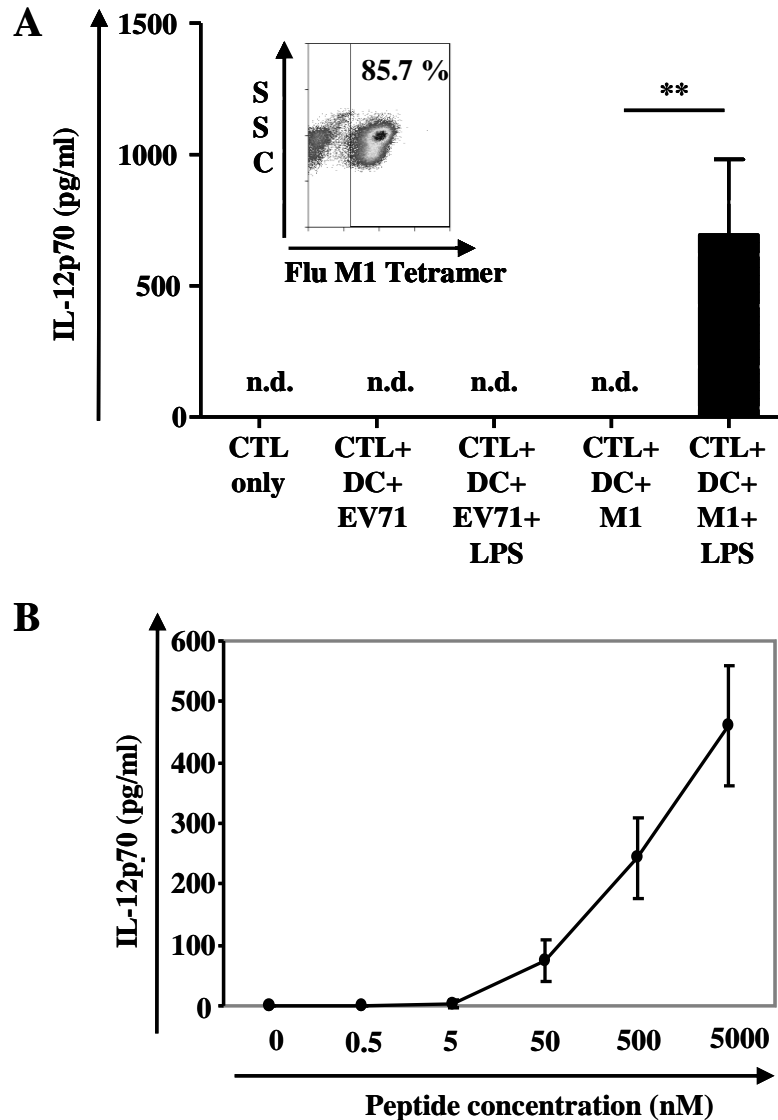
Fig. 3.5 Production of IL-12p70 is peptide specific.

(A) Activated CD8 T cells specific for Flu M1 peptide (percentage of cells specific for Flu M1 tetramer in inset) were co-cultured with autologous Day 6 immature DCs in the presence of 5uM of irrelevant peptide (EV71) or relevant peptide (M1) with or without LPS where indicated. Results are pooled from 3 independent donors. (B) Flu M1 specific CD8 T-cells were co-cultured with autologous Day 6 immature DCs in increasing dose of M1 peptide and LPS for 18 h. Supernatants were harvested and analyzed for IL-12p70 using ELISA. **$p<0.001$ using student's *t*-test. Results are representative of three experiments.

3.2.5 DCs were not killed by CD8 T-cells during co-cultures

We wanted to determine if activated CD8 T-cells may kill DCs during co-cultures, thereby affecting their ability to produce IL-12p70. To investigate this, we co-cultured allogeneic activated CD8 T-cells and DCs with or without LPS for 18 h before staining cells with Annexin-V and 7-AAD. We could not measure DC killing with the ^{51}Cr assay as DCs were unable to hold the ^{51}Cr well and the spontaneous release was very high (data not shown). DCs cultured without CD8 T-cells served as controls. DCs were discriminated from CD8 T-cells in co-cultures using the forward and side scatter and expression of MHC class II. Annexin-V and 7-AAD staining revealed that the proportion of live DCs, which were negative for both Annexin-V and 7-AAD, was not significantly different among all the co-culture groups, with or without CD8 T-cells (Fig. 3.6A). Annexin-V and 7-AAD could accurately pick up dead DCs as we found in a separate experiment that these non-viable DCs cultured in media without AB serum were double positive for Annexin-V and 7-AAD and stained positive for trypan blue as observed by microscopy (data not shown).

We next studied if CD8 T-cells were able to kill non-DC cell types, specifically the T2 cell line. The T2 cell line only expresses HLA-A2, does not express HLA-DR and is MHC class II negative (Cerundolo et al., 1990). T2 cells were pulsed with ^{51}Cr before pulsing with the Flu M1 peptide. They were then cultured with activated CD8 T-cells that were generated from the Flu specific CD8 T-cell line (CTLs) Results from the ^{51}Cr assay revealed that these CTLs were able to kill T2 cells pulsed with the Flu M1 peptide (Fig. 3.6B), hence indicating the antigen specificity of the killing activity. Therefore, we

conclude that these activated CD8 T-cells retained their cytotoxic ability but did not kill

DCs during co-cultures.

A

Annexin-V & 7-AAD staining

B

^{51}Cr release assay
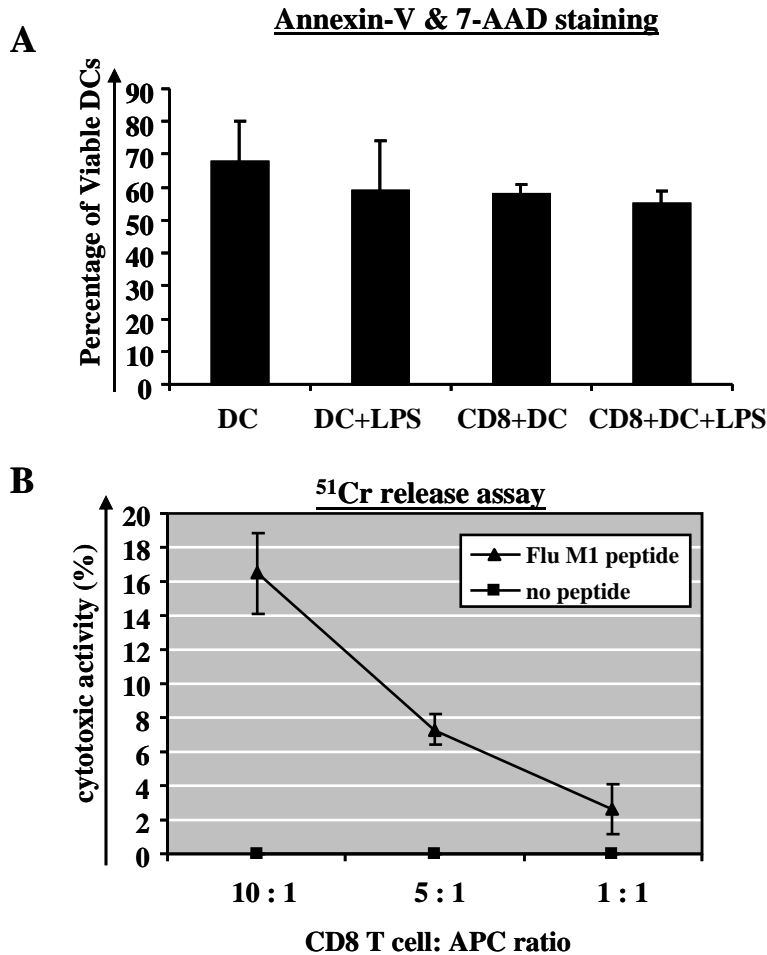
Fig. 3.6 Activated CD8 T-cells did not cause significant killing of DCs during co-culture.

(A) Co-cultures were harvested after 18 h and DCs were gated using MHC class II. Viability of DCs were assessed using flow cytometry for Annexin-V and 7-AAD negative cells (B) T1 cells were used as target cells and were used either without peptide or pulsed with 5μm of Flu M1 peptide. Flu-specific CD8 T-cells were added to target cells in the indicated effector to target ratios for 5 h. Supernatants were harvested and the amount of ^{51}Cr released was detected using a gamma counter. Specific lysis was calculated using the formula (test cpm − spontaneous cpm) / (maximum release cpm - spontaneous cpm) x 100 %. Results are mean ± SD and representative of 3 experiments.

3.2.6   IFN-γ, and not CD40L, is essential for CD8 T-cell mediated IL-12p70 production

Previous studies have indicated that CD40L, IFN-γ and TNF-α are important factors involved in CD8 T-cell stimulation of DC IL-12p70 production (Hochrein et al., 2000; Mailliard et al., 2002; Nakamura et al., 2007; Wong et al., 2008). Hence, we added blocking antibodies to these factors during the co-cultures. We found that IL-12p70 production was completely abrogated when IFN-γ was blocked with a combination of anti-IFN-γ and anti-IFN-γR, while its production was reduced when TNF-α was similarly inhibited (Fig. 3.7A). Surprisingly, we noted that blocking CD40L did not affect the production of IL-12p70 (Fig. 3.7A). This similar trend was also seen with the influenza specific CD8 T cells in our antigen specific model (Fig. 3.7B). As CD40L expressed on mouse CD8 T cells was shown to be an important factor for the production of IL-12p70 (Wong et al., 2008), we further investigated whether CD40L was involved in our model of IL-12p70 production. Using CD4 T cells as a positive control, activated and co-cultured CD8 T cells were shown to express minimal CD40L mRNA in contrast to CD4 T cells (Fig. 3.8A). In addition, surface expression of CD40L was not detected on activated CD8 T cells (Fig. 3.8B). These data indicate that activated human CD8 T cells employ IFN-γ as the principal factor for rapid IL-12p70 production from human DCs.
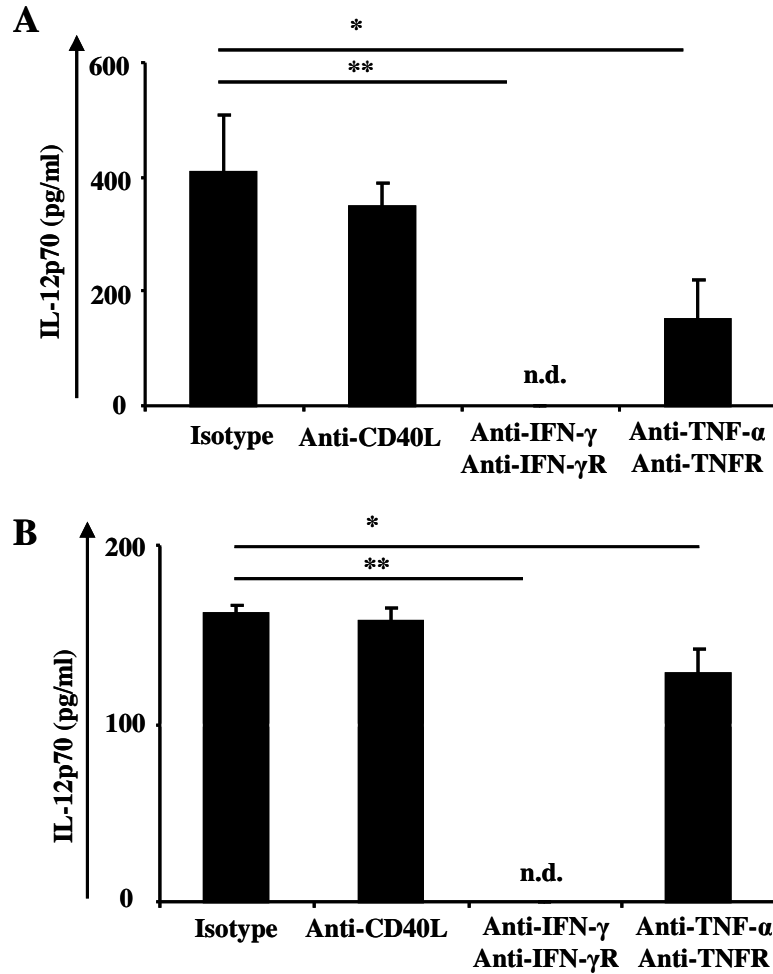
Fig. 3.7 Production of IL-12p70 from DCs is dependent on co-operation of TLR signal and IFN-γ secretion, but not CD40L, from activated CD8 T-cells.

(A) Allogeneic activated CD8 T cells or (B) Flu M1 autologous CD8 T cells were incubated with immature DCs in the presence of LPS with indicated blocking antibodies for 18h. Supernatants were harvested and analyzed for IL-12p70 using ELISA. *p<0.05, **p<0.001 using one way ANOVA. Results are representative of three independent experiments.

A

B
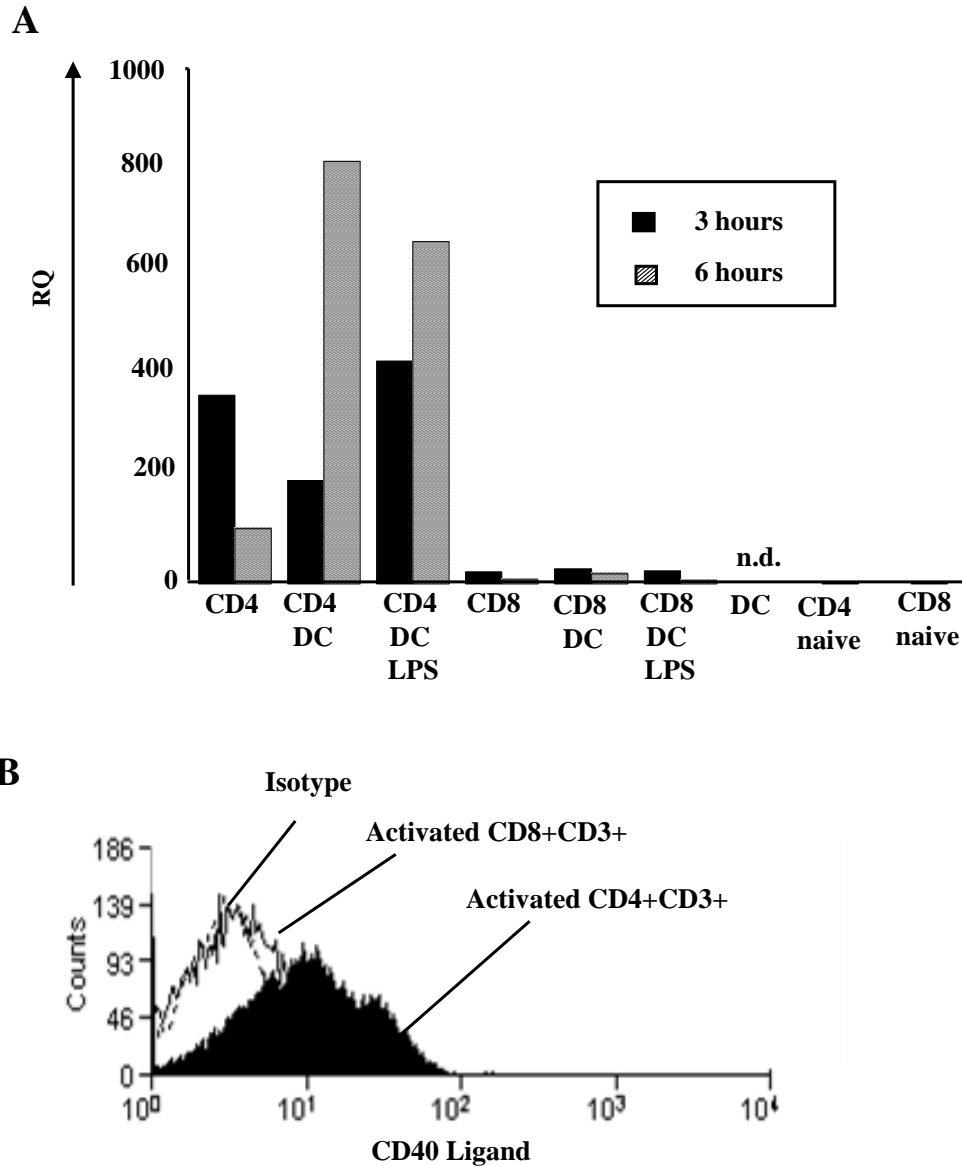
Fig. 3.8 CD40L expression was minimally detected on CD8 T-cells, as compared to CD4 T-cells.

Freshly isolated or activated CD4 and CD8 T cells were co-cultured with allogeneic DCs, in the presence or absence of LPS for 3 and 6 hours. (A) CD40L mRNA levels were determined by quantitative real-time PCR, using 18S levels as endogenous control with DC only as a calibrator. (B) Surface expression of CD40L on activated T cell populations determined by flow cytometry. Results are representative of three independent experiments

3.2.7 Analysis of other cytokines and chemokines produced during co-culture

We next investigated whether the interaction of activated CD8 T-cells and DCs results in the production of other immunomodulatory cytokines and chemokines. DCs stimulated with LPS alone produced inflammatory cytokines IL-6 and TNF-α, with small amounts of GM-CSF and IL-10 (Table 3.1A) while activated CD8 T-cells stimulated with anti-CD3 and CD28 produced GM-CSF, IFN-γ and TNF-α (Table 3.1B). When activated CD8 T cells were cultured with DCs, the levels of IFN-γ and TNF-α were enhanced in the presence of LPS. Notably, IL-1 production by DCs required the presence of both CD8 T-cells and LPS (Table 3.1B). IL-10 was only produced by DCs stimulated by LPS (Table 3.1A) and was absent in the presence of CD8 T-cells (Table 3.1B). The cytokine profile was similar for influenza specific CTLs, with CTLs co-cultured with peptide loaded DCs in the presence of LPS secreting the largest amount of pro-inflammatory cytokines (Table 3.1C). No IL-4, IL-18 and IL-23 were detected in any of the cultures.

We next determined which cell type in the co-cultures was producing these cytokines. Using intracellular staining of co-cultured cells, we first ruled out doublets (Fig. 3.9A), followed by distinguishing CD8 T-cells from DCs using the forward and side scatter (Fig. 3.9B and C) and this was further confirmed by showing that the larger side scatter which contains the DCs expressed a much higher level of MHC class II (Fig. 3.9F). We found that IFN-γ, GM-CSF and TNF-α were expressed by both CD8 T-cells and DCs (Fig.3.9E and F), although IFN-γ was mainly expressed by CD8 T-cells (Fig. 3.9E) while GM-CSF and TNF-α were mainly expressed by DCs (Fig. 3.9F). We had difficulty detecting IL-1α and β through intracellular staining as the quantity expressed was too small.

We also analyzed the production of chemokines by CD8 T-cells and DCs. Activated CD8 T-cells stimulated with anti-CD3/CD28 secreted RANTES, MIP-1α and ß but failed to produce IP-10 or MCP-1 (Table 3.2B) while DCs stimulated with LPS produced large amounts of MCP-1, MIP-1α and ß and low levels of RANTES and IP-10 (Table 3.2A). Strikingly, when CD8 T-cells were co-cultured with DCs, a sharp increase in MCP-1, MIP-1α and ß, IP-10 and RANTES was observed (Table 3.2B). The pattern of chemokines was comparable in influenza specific CTL-DC co-cultures (Table 3.2C). These data suggest that CD8 T-cell-DC interactions result in the production of inflammatory cytokines and chemokines which can potentiate the recruitment and differentiation of other immune effectors.

TABLE 3.1 Cytokine production from co-cultures

Cytokine levels (ng/ml) in supernatant co-cultures[a]									
(A)			**(B) Allogeneic co-cultures**			**(C) Peptide-specific co-cultures**			
	DC only	**DC+LPS**	**CD8 +anti CD3/CD28[b]**	**CD8+DC**	**CD8+DC +LPS**	**CTL only[c]**	**CTL+DC**	**CTL+DC +peptide**	**CTL+DC +peptide +LPS**
IL-1α	ND	ND	ND	ND	0.5 ± 0.05	ND	ND	ND	0.4 ± 0.02
	ND	ND	ND	ND	0.4 ± 0.03	ND	ND	ND	0.2 ± 0.05
	ND	ND	ND	ND	0.1 ± 0.2	ND	ND	ND	0.1 ± 0.003
IL-1ß	ND	ND	ND	ND	0.4 ± 0.05	ND	ND	ND	0.2 ± 0.009
	ND	ND	ND	ND	0.2 ± 0.08	ND	ND	ND	0.1 ± 0.008
	ND	ND	ND	ND	0.2 ± 0.001	ND	ND	ND	0.1 ± 0.03
IL-6	ND	13.5 ± 1.8	ND	0.2 ± 0.07	13.6 ± 1.4	ND	0.3 ± 0.05	0.4 ± 0.05	15.0 ± 1.2
	ND	3.2 ± 0.05	ND	0.2 ± 0.05	4.5 ± 0.2	ND	0.1 ± 0.005	1.3 ± 0.3	30.5 ± 10.3
	ND	10.2 ± 1.4	ND	0.3 ± 0.02	11.4 ± 1.7	ND	0.3 ± 0.002	0.8 ± 0.01	22.4 ± 1.2
IL-10	ND	0.3 ± 0.02	ND	ND	ND	ND	ND	ND	ND
	ND	0.1 ± 0.009	ND	ND	ND	ND	ND	ND	ND
	ND	0.2 ± 0.01	ND	ND	ND	ND	ND	ND	ND

Cytokine levels (ng/ml) in supernatant co-cultures[a]									
	(A)		(B) Allogeneic co-cultures				(C) Peptide-specific co-cultures		
	DC only	DC+LPS	CD8 +anti CD3/CD28[b]	CD8+DC	CD8+DC +LPS	CTL only[c]	CTL+DC	CTL+DC +peptide	CTL+DC +peptide +LPS
GM-CSF	ND	0.2 ± 0.02	0.5 ± 0.08	0.5 ± 0.03	0.6 ± 0.08	0.1 ± 0.01	0.05 ± 0.02	0.2 ± 0.01	0.7 ± 0.05
	ND	0.1 ± 0.01	0.4 ± 0.1	0.6 ± 0.02	0.7 ± 0.04	0.05 ± 0.02	0.8 ± 0.06	0.5 ± 0.09	1.2 ± 0.04
	ND	0.3 ± 0.05	0.4 ± 0.006	0.5 ± 0.02	0.7 ± 0.01	0.2 ± 0.01	0.2 ± 0.03	0.4 ± 0.04	1.7 ± 0.06
IFN-γ	ND	0.1 ± 0.02	5.26 ± 0.7	9.02 ± 2.7	21.7 ± 2.9	5.2 ± 0.09	0.1 ± 0.02	6.5 ± 1.1	10.4 ± 1.1
	ND	0.03 ± 0.004	20.8 ± 0.2	2.1 ± 1.2	18.4 ± 2.8	1.1 ± 0.05	28.6 ± 1.5	0.5 ± 0.06	31.1 ± 0.9
	ND	ND	7.6 ± 0.6	4.4 ± 0.5	19.3 ± 2.7	3.8 ± 0.3	10.4 ± 1.3	4.2 ± 1.6	15.4 ± 0.6
TNF-α	ND	2.9 ± 0.3	6.0 ± 0.9	7.0 ± 0.09	16.4 ± 2.8	1.1 ± 0.04	0.4 ± 0.07	1.1 ± 0.2	28.3 ± 4.0
	ND	2.8 ± 0.7	0.3 ± 0.03	0.4 ± 0.1	3.7 ± 0.4	2.1 ± 0.05	5.9 ± 0.03	17.0 ± 1.7	84.1 ± 16.8
	ND	ND	4.5 ± 0.04	4.2 ± 0.05	10.4 ± 0.02	0.9 ± 0.01	8.7 ± 0.1	4.6 ± 0.4	32.4 ± 1.8

[a]Amount of secreted protein in 18 hour co-cultures of 2 X 10^5 cells per well with a ratio of 2:1 allogeneic activated CD8 T cells to DCs or Autologous Flu M1 specific CTLs to DCs measured by multiplex bead arrays. Results are mean ± SD of triplicates and listed as 3 independent donors.

[b]Activated CD8 T cells were re-stimulated with anti-CD3 (10ng/ml) and anti-CD28 (400ng/ml)

[c]Autologous Flu specific CTLs were re-stimulated with peptide loaded autologous DCs one week before harvesting for culture

ND indicates not detected

TABLE 3.2 Chemokine production from co-cultures

Chemokine levels (ng/ml) in supernatant co-cultures[a]									
	(A)		(B) Allogeneic co-cultures			(C) Peptide-specific co-cultures			
	DC only	DC+LPS	CD8 +anti-CD3/CD28[b]	CD8+DC	CD8+DC +LPS	CTL only[c]	CTL+DC	CTL+DC +peptide	CTL+DC+peptide +LPS
IP-10 (CXCL10)	ND	1.1 ± 0.3	ND	124 ± 8.0	246 ± 2.5	0.4 ± 0.002	10.3 ± 3.5	15.0 ± 0.4	46.4 ± 6.8
	ND	4.8 ± 0.5	ND	114 ± 25	118.4 ± 11.9	0.2 ± 0.001	15.2 ± 1.4	12.3 ± 0.1	50.2 ± 7.1
	ND	2.2 ± 0.1	ND	133 ± 4.8	184 ± 10.3	0.4 ± 0.01	8.4 ± 2.0	19.7 ± 0.09	65.1 ± 9.7
RANTES (CCL5)	ND	0.3 ± 0.02	11.2 ± 4.0	8.4 ± 0.2	13.9 ± 2.8	1.4 ± 0.4	1.7 ± 0.3	5.7 ± 0.6	8.7 ± 2.3
	ND	1.0 ± 0.05	16.5 ± 1.7	9.3 ± 0.1	19.4 ± 2.3	2.8 ± 1.9	2.2 ± 1.8	7.9 ± 0.9	12.7 ± 1.8
	ND	0.2 ± 0.07	14.3 ± 5.5	12.2 ± 0.9	20.2 ± 1.9	3.7 ± 2.7	3.9 ± 4.2	3.4 ± 0.4	18.2 ± 2.4
MCP-1 (CCL2)	4.8 ± 1.2	12.9 ± 2.2	ND	42.9 ± 5.4	21.3 ± 3.5	ND	28.2 ± 2.1	48.3 ± 1.0	9.5 ± 1.8
	6.2 ± 2.0	23.1 ± 0.5	ND	50.3 ± 7.2	47.6 ± 15.2	ND	11.7 ± 1.2	40.4 ± 2.6	4.4 ± 0.7
	7.3 ± 1.4	45.4 ± 3.2	ND	105.8 ± 2.3	78.2 ± 23.5	ND	19.5 ± 5.1	55.6 ± 3.6	7.7 ± 1.2
MIP-1α (CCL3)	1.4 ± 0.5	2.0 ± 1.0	2.6 ± 0.3	8.0 ± 1.9	13.1 ± 2.1	1.3 ± 0.5	3.5 ± 0.9	12.7 ± 1.3	90.6 ± 21.7
	ND	4.2 ± 0.3	5.9 ± 0.7	5.6 ± 0.7	19.2 ± 3.8	1.2 ± 0.4	7.3 ± 0.2	11.5 ± 1.1	23.0 ± 0.5
	0.4 ± 0.002	3.7 ± 0.2	2.2 ± 0.009	9.4 ± 0.4	21.4 ± 2.2	0.9 ± 0.03	2.4 ± 0.1	10.9 ± 3.4	44.3 ± 0.8

Chemokine levels (ng/ml) in supernatant co-cultures[a]									
	(A)		**(B) Allogeneic co-cultures**			**(C) Peptide-specific co-cultures**			
	DC only	**DC+LPS**	**CD8 +anti-CD3/CD28[b]**	**CD8+DC**	**CD8+DC +LPS**	**CTL only[c]**	**CTL+DC**	**CTL+DC +peptide**	**CTL+DC+peptide +LPS**
MIP-1ß (CCL4)	19.4 ± 7.8	48.5 ± 8.7	20.7 ± 2.7	53.4 ± 10.9	84.1 ± 6.4	15.1 ± 1.2	7.7 ± 0.6	11.2 ± 1.0	78.9 ± 5.0
	0.9 ± 0.07	22.8 ± 2.3	24.3 ± 5.7	32.8 ± 2.1	70.3 ± 12.5	20.2 ± 1.4	4.5 ± 0.8	12.7 ± 2.8	85.2 ± 6.7
	9.4 ± 0.5	33.4 ± 5.3	17.2 ± 0.08	21.7 ± 1.2	89.2 ± 9.3	12.3 ± 1.0	9.0 ± 1.2	19.9 ± 3.4	90.5 ± 10.4

[a]Amount of secreted protein in 18 hour co-cultures of 2 X 10^5 cells per well with a ratio of 2:1 allogeneic activated CD8 T cells to DCs or Autologous Flu M1 specific CTLs to DCs measured by multiplex bead arrays. Results are mean ± SD of triplicates and listed as 3 independent donors.

[b]Activated CD8 T cells were re-stimulated with anti-CD3 (10ng/ml) and anti-CD28 (400ng/ml)

[c]Autologous Flu specific CTLs were re-stimulated with peptide loaded autologous DCs one week before harvesting for culture

ND indicates not detected
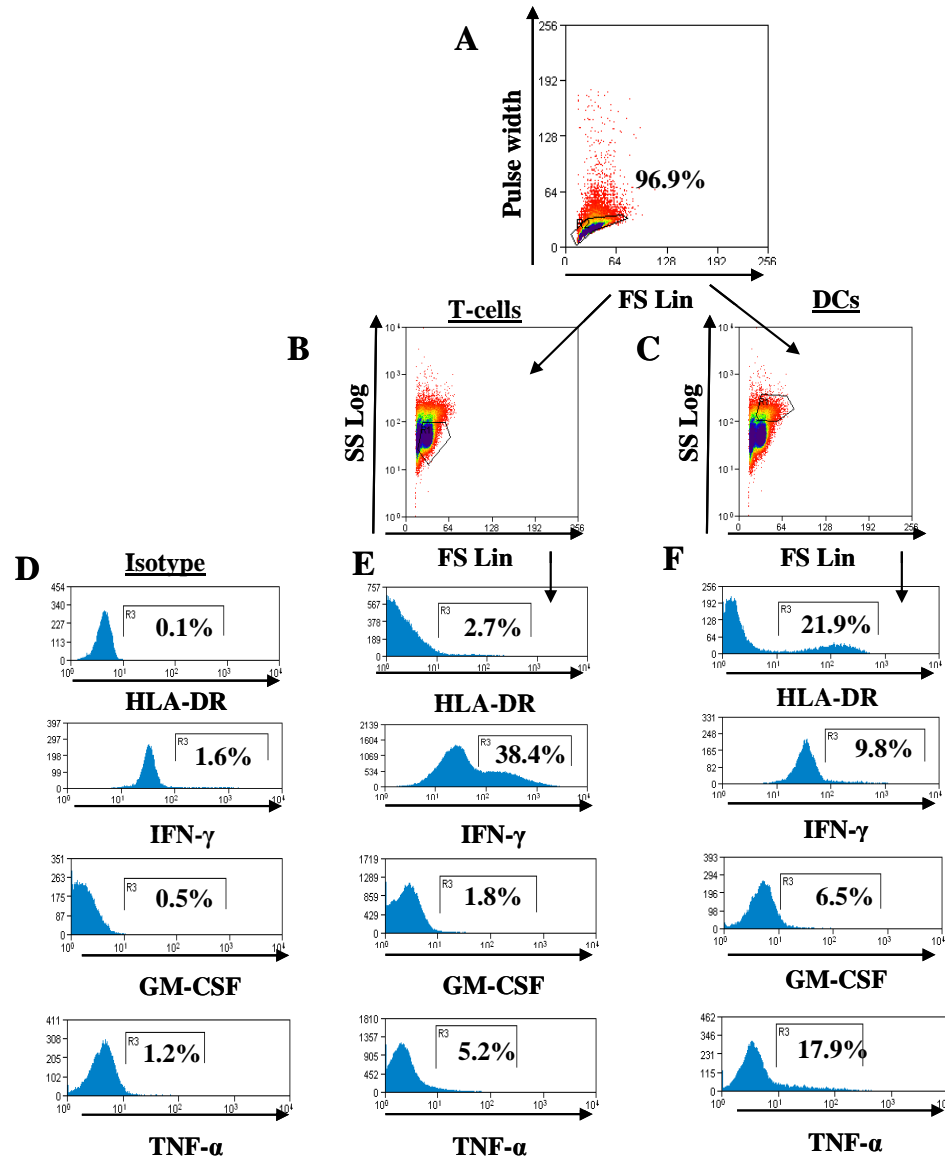
Fig. 3.9. Activated CD8 T-cells expressed high levels of IFN-γ while DCs expressed higher levels of TNF-α and GM-CSF.

Activated CD8 T-cells were co-cultured with immature DCs in the presence of 100ng/ml of LPS and 1μl/ml of Golgi-Stop^{TM} for 6h and cells harvested, washed and stained for HLA-DR and permeabilized before staining for intracellular cytokines. Cells were first selected for singlets by gating for cells with (A) low pulse width.(B) CD8 T-cells and (C) DCs were then discriminated by gating on the low and high side scatter plots respectively. Expression of HLA-DR and production of intracellular cytokines IFN-γ, GM-CSF and TNF-α with (D) isotype controls by (E) CD8 T-cells and (F) DCs are shown. Numbers represent percentages of positive cells. Data shown are representative of four experiments.

3.3 Discussion

To understand how human CD8 T-cells could prime DCs for IL-12p70 production, we first established an *in vitro* system using both allogeneic and antigen-specific models. We found that, in contrast to freshly isolated CD8 T-cells that had weak DC-modulating activities, activated human peripheral blood CD8 T-cells were able to induce DC activation by up-regulating co-stimulatory molecules and priming DCs for IL-12p70. In addition, we noted that the ability of activated CD8 T-cells to modulate DCs, in comparison to freshly isolated CD8 T-cells, was due to their propensity to secrete IFN-γ and TNF-α rapidly. We also found that LPS was essential to co-operate with activated CD8 T-cells for efficient priming of IL-12p70 from DCs.

The inability of freshly isolated CD8 T-cells to prime DCs for IL-12p70 in our model is in line with Kalinski's studies demonstrating that naive CD8 T cells require the presence of CD40L-expressing CD4 T cells and pre-activated DCs for concomitant production of IL-12p70 (Mailliard et al., 2002). In this study, DCs were pre-activated with TNF-α for 24 h before exposing them to naive CD8 T-cells and CD4 T-cells. In contrast, our study did not utilize pre-activated DCs but adopted immature DCs co-cultured concurrently with freshly isolated CD8 T-cells and LPS for 18 h. These results may also explain why activated CD8 T-cells were superior to freshly isolated CD8 T-cells because they could secrete IFN-γ and TNF-α rapidly during interaction which may facilitate the maturation of the DCs. However, while our studies suggested that CD8 T-cell's production of IFN-γ was the principal factor involved, Kalinski's studies demonstrated CD8 T-cell's

production of TNF-α to play a more significant role instead (Mailliard et al., 2002; Watchmaker et al., 2008). This disparity could be due to the usage of different models as we adopted LPS while they chose CD40L as the second signal instead for IL-12p70 production. In addition, the time course of the co-cultures was also markedly different. While Kalinski's group used a total co-culture period of 72 hours by priming DCs with CD8 T cells for 48 hours before re-stimulating them with CD40L-expressing cells for another 24 hours, we show that CD8 T cell- induction of DC IL-12p70 in the presence of LPS could occur within 18 hours of their interaction. A detailed study of the kinetics of CD8 induced IL-12p70 production from DCs will resolve this issue.

Notably, we found that LPS was also necessary to prime DCs for IL-12p70 during co-cultures with activated CD8 T-cells. This notion is consistent with several previous studies that demonstrate the two-signal requirement for efficient production of IL-12p70 (Schulz et al., 2000; Snijders et al., 1998; Trinchieri, 2003). In these studies, they demonstrate that the first signal, usually IFN-γ or CD40L, is provided by T-cells while the second signal can be provided by microbial compounds such as LPS. In addition, the two-signal requirement proved unique for the production of IL-12, since either CD40 engagement or LPS was sufficient for the efficient production of other pro-inflammatory cytokines such as TNF-α, IL-8 and the p40 subunit of IL-12, and may be considered as a safety mechanism for optimal control of potentially harmful Th1 responses (Snijders et al., 1998).

Our human CD8-DC co-culture studies are also supportive of our previous findings in mice (Wong et al., 2008; Wong et al., 2009). However, while CD40L was expressed on mouse CD8 T cells and was important for IL-12p70 priming, CD40L was not detected on peripheral blood human CD8 T cells. The absence of CD40L on human CD8 T cells can be explained by a previous study whereby CD8 T cells were shown to have a strong bias towards Th1 immunity with 80-90% of human CD8 T cells differentiating into IFN-γ-secreting Tc1 cells upon activation and 10-13% expressing CD40L only in the presence of Th2 cytokines (Vukmanovic-Stejic et al., 2000). It is possible that these CD40L-expressing Tc2 CD8 cells exist in significant numbers only in certain disease states or are confined to particular immune compartments (Birkhofer et al., 1996; Maggi et al., 1994; Salgame et al., 1991; Till et al., 1995). In addition, while studies in mice showed that both CD40L and IFN-γ, together with LPS, were necessary for CD8 T-cell induction of DC IL-12p70, our study showed that IFN-γ alone was enough to prime DCs for IL-12p70 in the presence of LPS. This discrepancy could be due to differences in mice and human studies, and also due to the fact that different DC subsets were adopted for both studies. Studies in mice used steady state resident lymphoid splenic DCs while ours adopted IL-4 and GM-CSF generated DCs which were thought to represent inflammatory DCs instead (Naik et al., 2006; Shortman and Naik, 2007).

Interestingly, we noticed that activated CD8 T-cells did not seem to kill DCs during their interaction, although they were able to kill peptide-pulsed T2 cells. Studies have shown that DCs could be protected from CTL killing by upregulating the expression of the granzyme B inhibitor serine protease inhibitor (SPI-6) when stimulated with LPS or

CD40L expressed on Th1 cells (Medema et al., 2001). In addition, memory CD8 T cells that release the DC-activating factor TNF-α before the release of cytotoxic granules were showed to induce DC expression of an endogenous granzyme B inhibitor PI-9 that protected DCs from CTL killing with similar efficacy as CD4 T cells (Watchmaker et al., 2008). Hence, DCs could be protected by CD8 T-cells from killing by a similar mechanism during their interactions in our study.

Finally, we determined if other soluble factors were produced during CD8 T-cell-DC co-cultures. We found increased production of cytokines GM-CSF, IFN-γ, TNF-α and chemokines MCP-1, MIP-1α and ß, RANTES and IP-10. These results suggest that the interaction of CD8 T-cells and DCs might have important implications in the creation of a pro-inflammatory environment that could play an essential role in protective immunity. Given that many different immune cell types such as T-cells, NK cells, monocytes, DCs, eosinophils and neutrophils are also responsive to these chemokines, it is possible that CD8 T-cell-DC interactions could initiate the recruitment of other immune effector cell types to the site of CD8 T-cell-DC interactions, for example at peripheral inflammatory sites or secondary lymphoid organs that would support the ongoing inflammatory process.

CHAPTER 4: Phenotypic characterization of human monocyte-derived cells (TNF/iNOS-producing dendritic cells) differentiated in the presence of CD8 T-cell-DC cytokine milieu

4.1 Introduction

In the previous chapter, we showed that CD8 T-cells could prime DCs for IL-12, an important cytokine for Th1 responses. However in addition to IL-12, we noticed that this CD8 T-cell-DC interaction also resulted in an array of inflammatory cytokines and chemokines being produced. We were curious if these inflammatory mediators could play a role in amplifying the Th1 response by influencing the differentiation of other immune cells. In particular, monocytes are known for their plasticity (Geissmann et al., 2003) and ability to differentiate into antigen-presenting cells according to the microenvironment they encounter (Auffray et al., 2009). This confers the ability to either magnify or dampen the immune signals they encounter. Indeed, a previous study showed that NK cells can induce the differentiation of monocytes into cells representing DCs that prime CD4 T-cells for Th1 responses (Zhang et al., 2007). These monocyte-derived cells were characterized by elongated cell bodies and a stellate-like morphology. They also displayed DC functional properties and have increased surface expression of CD40, CD80, CD86 and DC-SIGN while down-regulating their surface expression of CD14. In addition, another study noted that monocytes cultured with activated CD8 T-cells upregulated DC associated markers such as HLA-DR, CD1a, CD80, CD83, CD86

(Wirths et al., 2002) . However, further analysis of these cells with regards to morphological and functional properties were not conducted. Thereafter, similar morphological and phenotypic observations of monocytes differentiating into cells representing DCs by CD4 T-cells (Mariotti et al., 2008) and γδ T-cells (Eberl et al., 2009) were also reported. However, it remains unknown if the differentiation of monocytes into APCs can occur without direct contact with other immune cells, and whether soluble factors produced by CD8 T-cell-DC interactions can directly influence monocyte differentiation in a bystander manner to sustain the immune response.

To determine how CD8 T-cells may sustain the immune response during their interaction with DCs, we investigated how the cytokine milieu engendered by CD8 T-cell-DC interactions would affect the outcome of monocyte differentiation. In this chapter, we first studied the morphology and phenotype of these monocytes after differentiation with supernatants harvested from CD8 T-cell-DC co-cultures and compared these differentiated cells to classically generated monocyte-derived DCs and macrophages generated with IL-4-/GM-CSF and M-CSF respectively. In particular, we investigated changes in morphology through H&E staining and studying phenotypic changes by characterizing their expression of MHC class I, HLA-DR, co-stimulatory molecules CD14, CD40, CD80, CD86, DC-SIGN and the DC maturation marker, CD83. We would also further characterize these cells by studying their chemokine and toll-like receptor repertoire.

4.2 Results

4.2.1 CD8 T-cell-DC cytokine milieu differentiates monocytes into cells with distinct morphologies

We first investigated how the cytokine milieu engendered by CD8 T-cell-DC interactions modifies the morphology of monocytes. Supernatants were harvested from CD8 T-cell-DC co-cultures after 18 h. These supernatants were then added to freshly isolated monocytes and incubated for 48 h (see Chapter 2.2.10 for methods and Table 4.1 for explanation). Incubation of monocytes with supernatants for longer time periods (up to 5 days) did not yield any significant changes in cell morphology (data not shown). The presence of LPS in supernatants was minimized by washing DCs after 3 hours of LPS pulsing thoroughly before addition of CD8 T cells. Residual LPS in the supernatants were tested using an endotoxin test kit (CL-1000® Chromogenic LAL Endpoint Assay kit (Cambrex)) and 6 EU/ml was found to be present in these supernatants while those that had no DCs pulsed with LPS had no endotoxin detected. Hence, the minimization of LPS in these supernatants was to ensure that any effect seen in the differentiated monocytes was caused as much as possible by immune cell-derived cytokines.

TABLE 4.1 Brief explanation of co-culture supernatants

Name of supernatant	Cells in co-culture	Abbreviation
DC	DCs only	Supernatant$_{DC}$
DCLPS	DCs stimulated with LPS	Supernatant$_{DCLPS}$
CD8DC	Activated CD8T-cells with allogeneic DCs	Supernatant$_{CD8DC}$
CD8DCLPS	Activated CD8T-cells with DCs that have been stimulated with LPS	Supernatant$_{CD8DCLPS}$
CD8	Activated CD8 T-cells re-stimulated with anti-CD3/CD28	Supernatant$_{CD8}$

We found that monocytes cultured with DC or DCLPS supernatants (Supernatant$_{DC \, or \, DCLPS}$) were round and adherent (Fig. 4.1 B and C) and exhibited similar morphology to monocytes cultured with medium alone (Fig. 4.1A). In contrast, monocytes exposed to CD8DC or CD8DCLPS supernatants (Supernatant$_{CD8DC \, or \, CD8DCLPS}$) were also adherent but developed distinct long, spindle dendrites (Fig. 4.1D and E). We also noted that monocytes cultured with supernatants that were harvested from activated CD8 T-cells alone (Supernatant$_{CD8}$) had some cells that displayed long, spindle dendrites (Fig. 4.1F), although this was much lesser than those induced by Supernatant$_{CD8DC \, or \, CD8DCLPS}$ (Fig.4.1D and E). In addition, we also noted that cells differentiated with Supernatant$_{CD8}$ were not very viable as about 40-50% of these cells stained positive for trypan blue when viewed under the microscope.

We next compared the morphology of these cells with classically generated monocyte derived DCs and macrophages. We found that monocytes differentiated with Supernatant$_{CD8DC \, or \, CD8DCLPS}$ (Fig. 4.1D and E) displayed a closer morphology to mature classical IL-4/GM-CSF derived DCs (Fig. 4.1H) with the presence of adherent long spindle dendrites rather than immature classical IL-4/GM-CSF derived DCs that were non-adherent (Fig. 4.1G) Monocytes differentiated with Supernatant$_{CD8DC \, or \, CD8DCLPS}$ (Fig. 4.1D and E) were also clearly distinct from immature M-CSF derived macrophages (Fig. 4.1I) which were round and mature M-CSF derived macrophages which exhibited vacuoles instead (Fig 4.1J). Hence, these results suggest that monocytes differentiated with CD8 T-cell-DC cytokine milieus (Supernatant$_{CD8DC \, or \, CD8DCLPS}$) developed distinct morphologies that could resemble mature DCs.

A No cytokines

B Supernatant$_{DC}$

C Supernatant$_{DCLPS}$

D Supernatant$_{CD8DC}$

E Supernatant$_{CD8DCLPS}$

F Supernatant$_{CD8}$

G Immature IL-4/GM-CSF DCs

H Mature IL-4/GM-CSF DCs

I Immature M-CSF macrophages

J Mature M-CSF macrophages
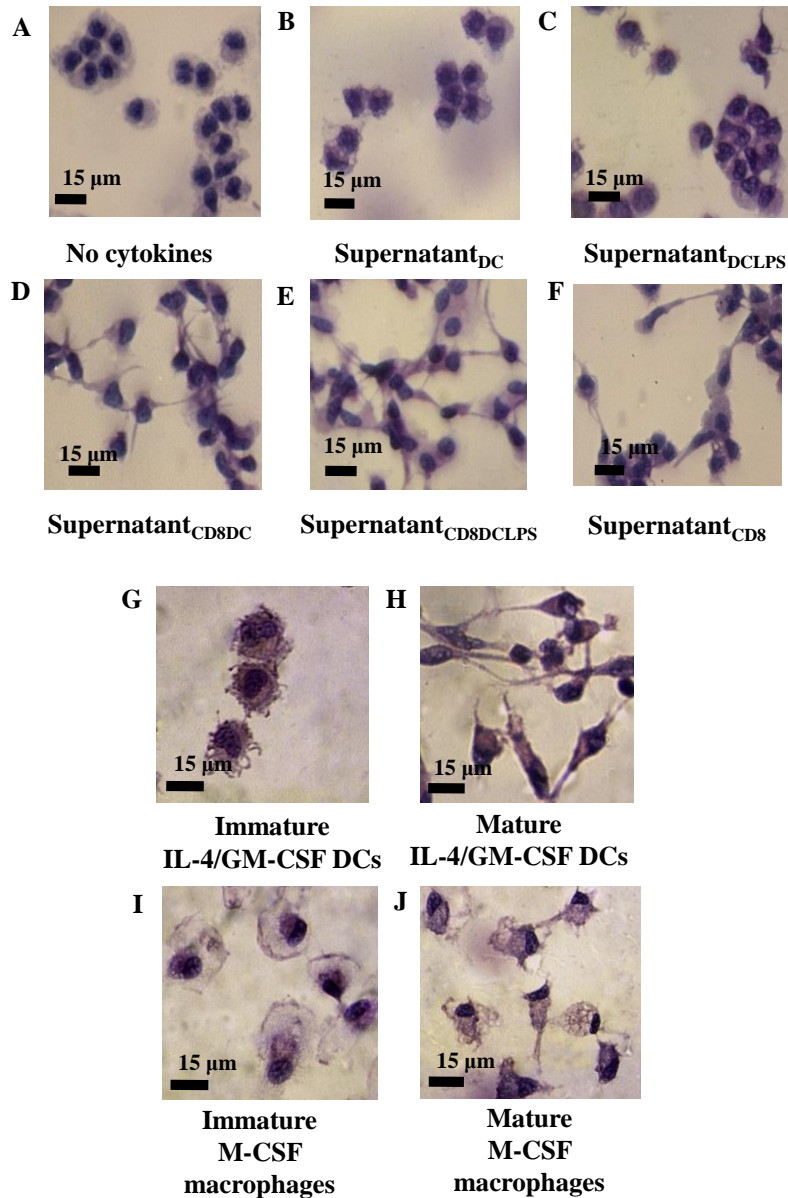
Fig. 4.1 Monocytes exposed to CD8 T-cell-DC cytokine milieu differentiate into cells with distinct morphologies.

Monocytes were cultured with (A) culture medium alone (no cytokines) or supernatants harvested from cultures of (B) DC alone, (C) DCLPS, (D) CD8DC or (E) CD8DCLPS for 48 h. (G) Classical DCs and (I) macrophages were generated from monocytes in the presence of IL-4/GM-CSF and M-CSF respectively for 6 days. (H, J) LPS was added for a further 24h to induce their maturation. Cells were stained with hematoxylin-and-eosin (H&E) and captured using a Leica DM2000 microscope (Leica Microsystems, Singapore). Original magnification was x 630 for all panels (scale bar: 15 mm). Data shown are representatives of six experiments.

4.2.2 Expression of MHC class I and HLA-DR by monocytes differentiated with CD8 T-cell-DC cytokine milieu

We further investigated the phenotype of these differentiated monocytes by flow cytometry. Monocytes differentiated with the respective supernatants were analyzed for their surface expression of MHC class I and II (HLA-DR). We also compared the expression of these molecules to classically generated activated IL-4/GM-CSF DCs and M-CSF macrophages. Monocytes differentiated in the presence of Supernatant$_{DC \text{ or } DCLPS}$ (Fig. 4.2B and C) had similar MHC class I and HLA-DR levels compared to untreated monocytes (Fig. 4.2A). In contrast, monocytes differentiated with Supernatant$_{CD8DC \text{ or } CD8DCLPS}$ (Fig. 4.2D and E) expressed higher levels of both MHC class I and HLA-DR compared to monocytes differentiated in the presence of Supernatant$_{DC \text{ or } DCLPS}$ (Fig. 4.2B and C). While monocytes differentiated with Supernatant$_{CD8DC \text{ or } CD8DCLPS}$ (Fig. 4.2D and E) expressed lower levels of MHC class I than IL-4/GM-CSF DCs (Fig. 4.2F), they exhibited a higher expression of HLA-DR compared to these cells. Notably, monocytes differentiated with Supernatant$_{CD8DC \text{ or } CD8DCLPS}$ (Fig. 4.2D and E) also expressed much higher levels of both MHC class I and HLA-DR compared to M-CSF derived macrophages (Fig. 4.2G). Thus, these data suggest that monocytes differentiated with Supernatant$_{CD8DC \text{ or } CD8DCLPS}$ were able to upregulate antigen presenting molecules.
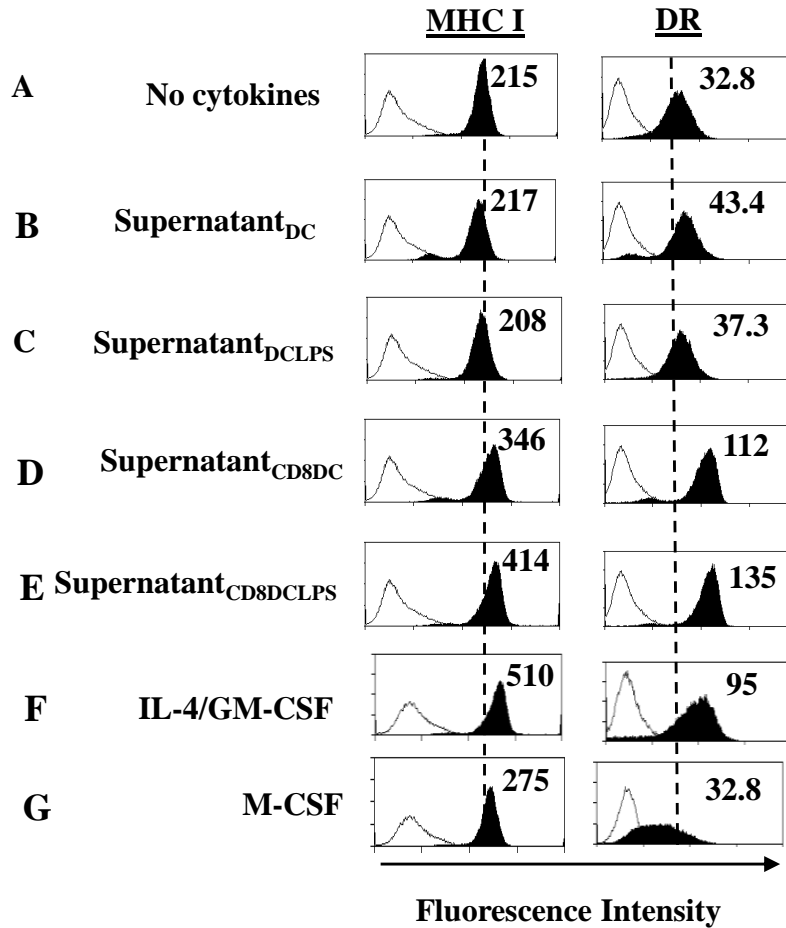
Fig. 4.2 Monocytes exposed to CD8 T-cell-DC cytokine milieu upregulate MHC class I and HLA-DR.

Monocytes differentiated with (A) no cytokines (B) Supernatant$_{DC}$ (C) Supernatant$_{DCLPS}$ (D) Supernatant$_{CD8DC}$ (E) Supernatant$_{CD8DCLPS}$ or (F) mature IL-4/GM-CSF DCs and (G) mature M-CSF macrophages were analyzed by flow cytometry for the expression of MHC class I and HLA-DR. Numbers indicate relative difference in MFI between surface antigen expression and isotype control. Open histograms represent cells stained with isotype control and closed histograms represent cells stained with indicated antibody. Results are representatives of four independent experiments with comparable results.

4.2.3   Monocytes differentiated with CD8 T-cell-DC cytokine milieu upregulate co-stimulatory molecules

We next determined if monocytes differentiated with CD8 T-cell DC cytokine milieus could upregulate co-stimulatory molecules. We found that compared to untreated monocytes (Fig. 4.3A), CD14 expression was increased on monocytes cultured with Supernatant$_{DC \text{ or } DCLPS}$ (Fig. 4.3B and C) but not on monocytes cultured with Supernatant$_{CD8DC \text{ or } CD8DCLPS}$ (Fig. 4.3D and E). Remarkably, monocytes cultured with Supernatant$_{CD8DC \text{ or } CD8DCLPS}$ (Fig. 4.3D and E) showed substantial increase in co-stimulatory molecules CD40, CD80 andCD86, which was minimally detected on monocytes cultured with Supernatant$_{DC \text{ or } DCLPS}$ (Fig. 4.3B and C). In addition, monocytes cultured with Supernatant$_{CD8DC \text{ or } CD8DCLPS}$ (Fig 4.3Dand E) also expressed CD209 (DC-SIGN).

Next, we compared the expression of these molecules on monocytes differentiated with Supernatant$_{CD8DC \text{ or } CD8DCLPS}$ to IL-4/GM-CSF DCs and M-CSF macrophages. IL-4/GM-CSF DCs expressed high levels of CD40, CD80, CD86 and DC-SIGN and downregulated CD14 expression (Fig. 4.3F). On the other hand, M-CSF macrophages upregulated CD14 expression but expressed low levels of CD40, CD80, CD86 and DC-SIGN (Fig. 4.3G). We found that with the exception of CD14, monocytes differentiated with Supernatant$_{CD8DC \text{ or } CD8DCLPS}$ (Fig. 4.3D and E) had a much higher expression of CD40, CD80, CD86 and DC-SIGN compared to M-CSF macrophages (Fig. 4.3G). In addition, monocytes differentiated with Supernatant$_{CD8DC \text{ or } CD8DCLPS}$ (Fig. 4.3D and E) also expressed similar levels of CD40 to IL-4/GM-CSF DCs (Fig. 4.3F), although

CD80,CD86 and DC-SIGN was expressed lower than that of IL-4/GM-CSF DCs. However, the overall data suggest that CD8 T-cell-DC cytokine milieus differentiate monocytes into cells which were more similar to IL-4/GM-CSF DCs than M-CSF macrophages.
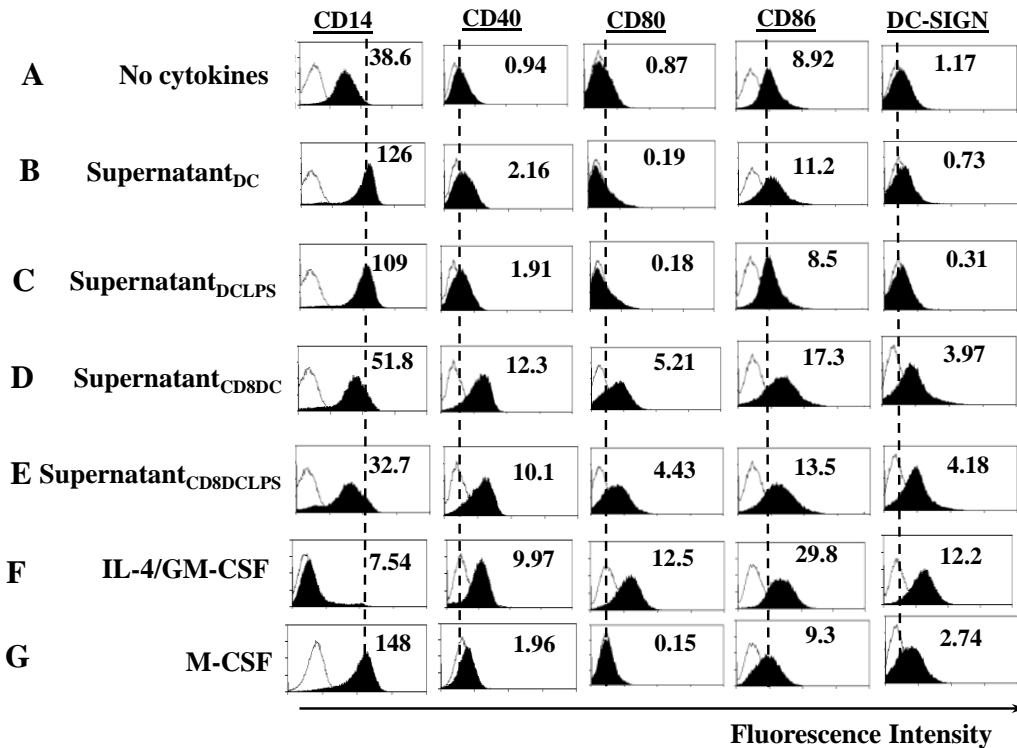
Fig. 4.3 Monocytes exposed to CD8 T-cell-DC cytokine milieu upregulate distinct surface markers.

Monocytes differentiated with (A) no cytokines (B) Supernatant$_{DC}$ (C) Supernatant$_{DCLPS}$ (D) Supernatant$_{CD8DC}$ (E) Supernatant$_{CD8DCLPS}$ or (F) mature IL-4/GM-CSF DCs and (G) mature M-CSF macrophages were analyzed by flow cytometry for the expression of CD14, CD40, CD80, CD86 and DC-SIGN. Open histograms represent cells stained with isotype control and closed histograms represent cells stained with indicated antibody. Numbers indicate relative difference in MFI between surface antigen expression and isotype control. Results are representatives of four independent experiments with comparable results.

4.2.4 Upregulation of chemokine receptors by monocytes differentiated with CD8 T-cell-DC cytokine milieu

We further investigated the expression of chemokine receptors on these differentiated monocytes. We found that monocytes differentiated with Supernatant$_{DC \ or \ DCLPS}$ (Fig. 4.4B and C) downregulated the expression of CCR1, CCR2 and CCR5 but had similar expression levels of CCR7 and CX$_3$CR1 compared to untreated monocytes (Fig. 4.4A). In contrast, monocytes that were differentiated with Supernatant$_{CD8DC \ or \ CD8DCLPS}$ (Fig 4.4Dand E) had a significant increase in the expression of CCR1 and CX$_3$CR1, but had lower expression levels of CCR2 and CCR5 as compared to monocytes that were differentiated with Supernatant$_{DC \ or \ DCLPS}$ (Fig. 4.4B and C). In contrast to monocytes differentiated with Supernatant$_{DC \ or \ DCLPS}$ (Fig. 4.4B and C), monocytes differentiated with Supernatant$_{CD8DC \ or \ CD8DCLPS}$ (Fig 4.4Dand E) also expressed a slight upregulation of CCR7.

In addition, we found that IL-4/GM-CSF DCs (Fig. 4.4F) and M-CSF macrophages (Fig. 4.4G) expressed low levels of CCR1, CCR2 and intermediate levels of CX$_3$CR1. In comparison to untreated monocytes (Fig. 4.4A), IL-4/GM-CSF DCs up-regulated CCR7 while M-CSF macrophages maintained its expression of CCR5 (Fig. 4.4F and G). Strikingly, we found that monocytes exposed to Supernatant$_{CD8DC \ or \ CD8DCLPS}$ (Fig 4.4Dand E) had higher levels of CCR1, CCR2, CX$_3$CR1 and similar levels of CCR5 as IL-4/GM-CSF DCs (Fig. 4.4F). In contrast, monocytes exposed to Supernatant$_{DC \ or \ DCLPS}$ (Fig. 4.4B and C) resembled M-CSF macrophages through higher expression of CCR5 and had no upregulation of CCR7 (Fig. 4.4G). These data suggest that CD8 T-cell-DC

cytokine milieus differentiate monocytes into cells which differ from the other differentiated monocytes through a distinct chemokine repertoire that may influence their migrating patterns into inflamed sites.

		CCR1	CCR2	CCR5	CCR7	CX$_3$CR1
A	**No cytokines**	32.8	75.8	296	0.23	5.56
B	**Supernatant$_{DC}$**	10.8	55.6	203	0.09	8.35
C	**Supernatant$_{DCLPS}$**	6.25	49.8	219	0.19	7.67
D	**Supernatant$_{CD8DC}$**	102	32.9	116	0.82	23.1
E	**Supernatant$_{CD8DCLPS}$**	118	29.9	117	1.41	23.2
F	**IL-4/GM-CSF**	19.7	19.9	114	7.86	15.8
G	**M-CSF**	21.2	23.9	299	0.29	17.2

Fluorescence Intensity

Fig. 4.4 Monocytes exposed to CD8 T-cell-DC cytokine milieu upregulate distinct chemokine receptors.

Monocytes differentiated with (A) no cytokines (B) Supernatant$_{DC}$ (C) Supernatant$_{DCLPS}$ (D) Supernatant$_{CD8DC}$ (E) Supernatant$_{CD8DCLPS}$ or (F) mature IL-4/GM-CSF DCs and (G) mature M-CSF macrophages were analyzed by flow cytometry for the expression of CCR1, CCR2, CCR5. CCR7 and CX$_3$CR1. Open histograms represent cells stained with isotype control and closed histograms represent cells stained with indicated antibody. Numbers indicate relative difference in MFI between surface antigen expression and isotype control. Results are representatives of four independent experiments with comparable results.

4.2.5   Upregulation of toll-like receptors on monocytes differentiated with CD8 T-cell-DC cytokine milieu

We also examined the expression of toll-like receptors (TLRs) on differentiated monocytes. We found that monocytes differentiated with Supernatant$_{DC \ or \ DCLPS}$ (Fig. 4.5B and C) upregulated only TLR2 and TLR8 when compared to untreated monocytes (Fig. 4.5A). In contrast, monocytes differentiated with Supernatant$_{CD8DC \ or \ CD8DCLPS}$ (Fig. 4.5D and E) upregulated all TLRs tested (namely TLR2, TLR3, TLR4 and TLR8) compared to monocytes differentiated with Supernatant$_{DC \ or \ DCLPS}$ (Fig. 4.5B and C) as well as untreated monocytes (Fig.4.5A).

We next compared the expression of TLRs on IL-4/GM-CSF DCs and M-CSF macrophages to these differentiated monocytes. Both IL-4/GM-CSF DCs (Fig. 4.5F) and M-CSF macrophages (Fig. 4.5G) showed upregulated expression of TLR2, TLR4 and TLR8 compared to untreated monocytes (Fig. 4.5A) and were of similar levels to those expressed by monocytes differentiated with Supernatant$_{CD8DC \ or \ CD8DCLPS}$ (Fig. 4.5D and E). Importantly, we found that TLR3 was only upregulated by IL-4/GM-CSF DCs (Fig.4.5F) and monocytes differentiated with Supernatant$_{CD8DC \ or \ CD8DCLPS}$ (Fig.4.5D and E) and this upregulation was not detected on M-CSF macrophages (Fig.4.5G) nor monocytes differentiated with Supernatant$_{DC \ or \ DCLPS}$ (Fig. 4.5B and C). Hence, these data suggest that monocytes differentiated with CD8 T-cell-DC cytokine milieus upregulate important TLRs which may be useful during inflammation and may resemble DCs more closely through their expression of TLR3.

		TLR2	TLR3	TLR4	TLR8
A	**No cytokines**	7.65	0.99	4.32	2.92
B	**Supernatant$_{DC}$**	13.65	0.18	3.15	3.54
C	**Supernatant$_{DCLPS}$**	18.69	1.02	3.82	3.81
D	**Supernatant$_{CD8DC}$**	35.2	3.25	6.90	5.25
E	**Supernatant$_{CD8DCLPS}$**	40.7	4.21	9.99	5.53
F	**IL-4/GM-CSF**	32.4	5.18	6.12	7.01
G	**M-CSF**	37.8	1.32	7.05	6.23

Fluorescence Intensity

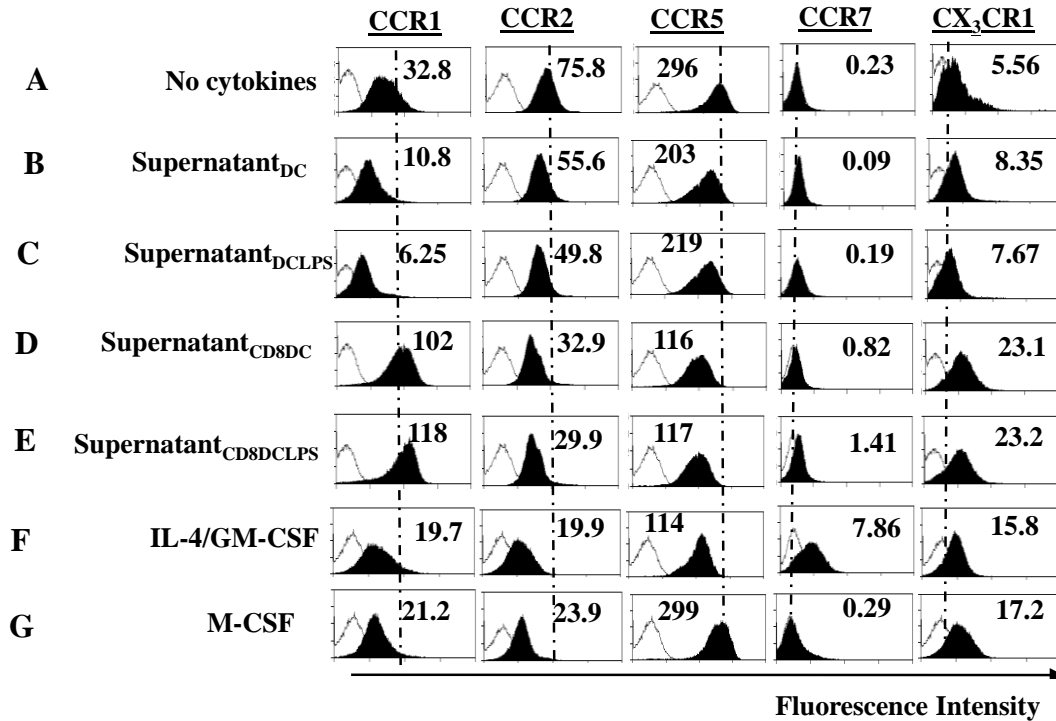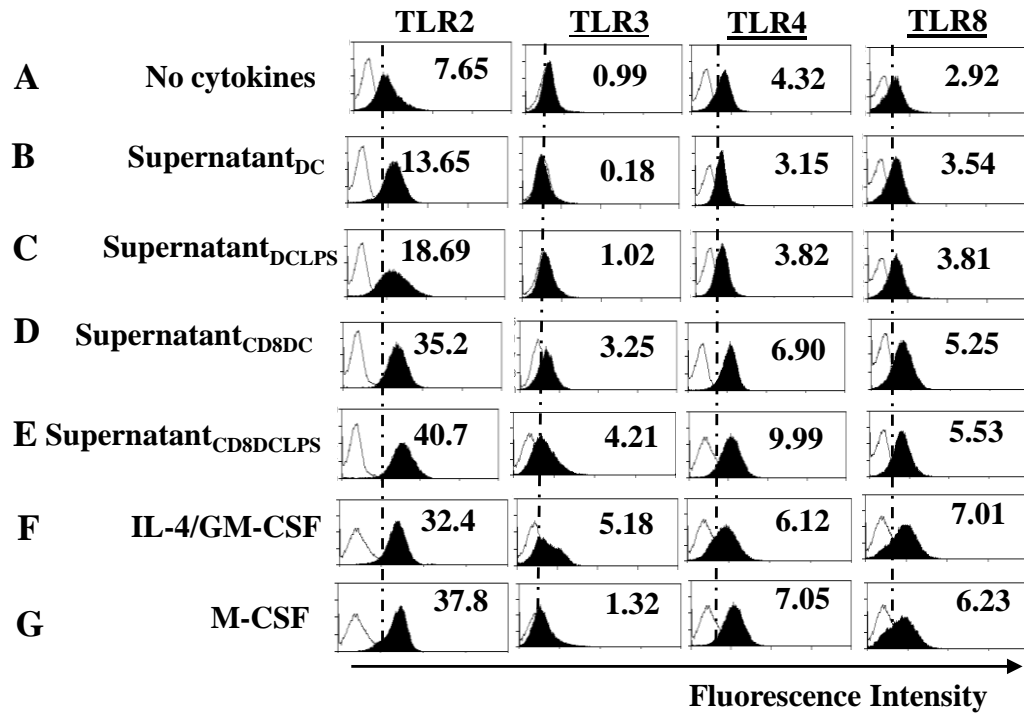**Fig. 4.5 Monocytes exposed to CD8 T-cell-DC cytokine milieu upregulate distinct Toll-Like receptors.**

Monocytes differentiated with (A) no cytokines (B) Supernatant$_{DC}$ (C) Supernatant$_{DCLPS}$ (D) Supernatant$_{CD8DC}$ (E) Supernatant$_{CD8DCLPS}$ or (F) mature IL-4/GM-CSF DCs and (G) mature M-CSF macrophages were analyzed by flow cytometry for the expression of TLR2, TLR3, TLR4 and TLR8. Open histograms represent cells stained with isotype control and closed histograms represent cells stained with indicated antibody. Numbers indicate relative difference in MFI between surface antigen expression and isotype control. Results are representatives of four independent experiments with comparable results.

4.2.6   Monocytes differentiated with CD8 T-cell-DC cytokine milieu expressed CD83

We next determined if these differentiated monocytes expressed CD83, a classical maturation marker expressed on human DCs (Cao et al., 2005). We found that CD83 was expressed on mature IL-4/GM-CSF DCs and neither on untreated monocytes nor mature M-CSF macrophages (Fig. 4.6). Strikingly, monocytes that were differentiated with Supernatant$_{CD8DC \text{ or } CD8DCLPS}$ showed a slight upregulation of CD83 and this expression was further enhanced upon stimulation with LPS for an additional 24 h (Fig. 4.6) Notably, monocytes differentiated with Supernatant$_{DC \text{ or } DCLPS}$ did not express CD83 even after subsequent stimulation with LPS. Overall, these results suggest that monocytes that have been differentiated in the presence of CD8-T-cell DC cytokine milieus closely resemble DCs through their ability to upregulate the expression of CD83.
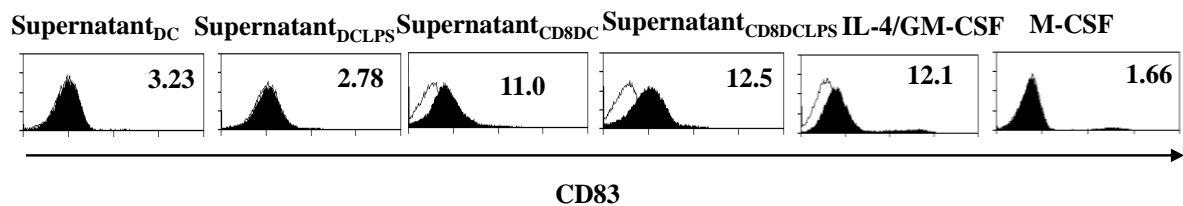
Fig. 4.6 Monocytes exposed to CD8 T-cell-DC cytokine milieu upregulate CD83.

Monocytes differentiated with various supernatants and activated overnight with LPS or mature IL-4/GM-CSF DCs or M-CSF macrophages were analyzed by flow cytometry for the expression of CD83. Open histograms represent cells stained with isotype control and closed histograms represent cells stained with anti-CD83. Numbers indicate relative difference in MFI between surface antigen expression and isotype control. Results are representatives of three independent experiments with comparable results.

4.2.7 Differentiation efficiency of monocytes exposed to cytokine milieus

To determine the percentage of DCs that have differentiated from monocytes in cytokine milieus, we calculated the percentages of viable cells that express HLA-DR and CD86. We found that we could obtain a differentiation efficiency of about 60-75% for monocytes that differentiate into DCs with Supernatant$_{CD8DCLPS}$ while we only had a differentiation efficiency of 15-26% with Supernatant$_{DCLPS}$. Fig. 4.7 shows a representative result of the gating strategy and calculations used for assessing the differentiation efficiency and Table 4.2 shows the results obtained from five independent experiments.
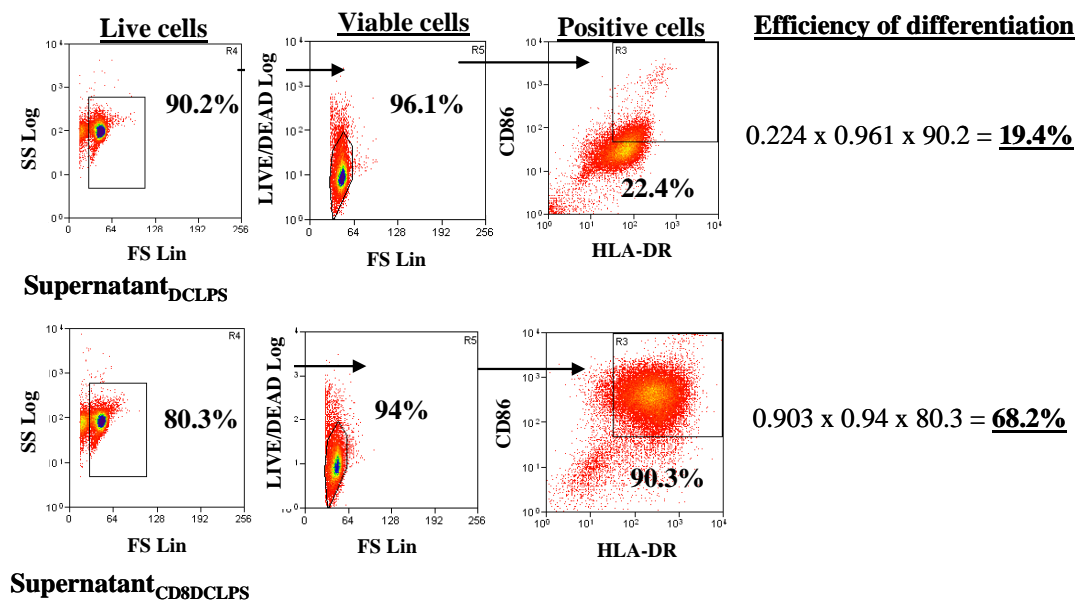
Fig. 4.7 Differentiation efficiency of monocytes exposed to supernatants

Monocytes differentiated with Supernatant$_{DCLPS}$ or Supernatant$_{CD8DCLPS}$ were stained and analyzed via flow cytometry. The differentiation efficiency of monocytes were calculated by first gating on live cells, followed by viable cells (gated as cells that stain negative for the LIVE/DEAD[®] fixable violet dead cell stain) and finally on CD86 and HLA-DR positive cells. Results are representative of four experiments.

TABLE 4.2 Differentiation efficiency of monocytes exposed to supernatants

Experiment	Supernatant$_{DCLPS}$	Supernatant$_{CD8DCLPS}$
1	0.224 x 0.961 x 90.2 = **19.4%**	0.903 x 0.94 x 80.3 = **68.2%**
2	0.197 x 0.95 x 89.2 = **16.7%**	0.885 x 0.91 x 82.5 = **66.4%**
3	0.258 x 0.92 x 92.5 = **21.9%**	0.952 x 0.92 x 85.7 = **75.1%**
4	0.32 x 0.895 x 91.3 = **26.1 %**	0.85 x 0.89 x 79.9 = **60.4%**
5	0.21 x 0.942 x 89.9 = **17.8%**	0.91 x 0.93 x 84.2 = **71.3%**

4.3 Discussion

In this chapter, we show that supernatants derived from CD8 T-cell-DC co-cultures were able to induce the differentiation of human monocytes into cells with morphological and phenotypic characteristics of DCs. These monocyte-derived cells were adherent and exhibited long spindle dendrites, resembling classically activated IL-4/GM-CSF DCs rather than macrophages which displayed distinct vacuoles instead. In addition, these cells upregulated MHC class I and HLA-DR, as well as co-stimulatory molecules CD40, CD80, CD86 and DC-SIGN. They also downregulated CD14, a macrophage associated marker, and expressed CD83, an important marker associated with human mature DCs. Notably, these monocyte derived cells also upregulated chemokine receptors CCR1 and CX$_3$CR1 as well as TLR2, TLR3, TLR4 and TLR8. These results indicate that CD8 T-cells have the potential to modulate the immune response during their interaction with DCs, by producing cytokines that differentiate monocytes into cells with distinct DC features.

We found that monocytes differentiated in the absence of cytokines acquired a macrophage phenotype, suggesting that the differentiation of monocytes into macrophages seem to represent a default differentiation program of monocytes on extravasations (Lewis et al., 1999). Moreover, the addition of supernatants derived from DC co-cultures did not interfere with this process. Conversely, the addition of supernatants derived from CD8 T-cell-DC co-cultures to monocytes resulted in distinct

changes associated with DCs such as the elongation of cell bodies and presence of characteristic fine cytoplasmic processes instead of the broader pseudopodia of macrophages. This is in line with previous studies suggesting the need for a well-defined cytokine cocktail for monocytes to differentiate into DCs (Comes et al., 2002; Mohamadzadeh et al., 2001; Santini et al., 2000; Zou and Tam, 2002) . In addition, several cell types such as CD4 T-cells (Mariotti et al., 2008), CD8 T-cells (Wirths et al., 2002), NK cells (Zhang et al., 2007) and γδ T-cells (Eberl et al., 2009) have been indicated as possible sources of these cytokines. Notably, these cells were shown to differentiate monocytes into cells with similar DC phenotypic characteristics such as high expression of MHC class I, HLA-DR, upregulation of CD40, CD80, CD86 and DC-SIGN as well as the expression of CD83.

Although we noticed that monocytes differentiated with CD8 T-cell-DC derived supernatants expressed most markers associated with DCs, we were unable to detect the expression of CD1 molecules, such as CD1a, which belong to a family of glycoproteins involved in the presentation of lipid antigens to T cells (Porcelli, 1995). However, previous studies have suggested that the usage of FCS versus human serum in the culture media affects the expression of CD1 molecules on DCs but not their antigen-presenting capacity (Duperrier et al., 2000; Smed-Sörensen et al., 2008; Triozzi and Aldrich, 1997) Since our cells were all cultured with 10% human serum, this could account for the lack of expression of CD1 molecules as we also did not detect CD1a expression on classical monocyte-derived DCs in our cultures.

Chemokines are significantly involved in inflammatory and immunological responses via their ability to recruit selective leukocyte subsets (Rollins, 1997). Studies have also shown that expression of chemokine receptors are heavily implicated in DC trafficking to tissues and lymph nodes (Dieu et al., 1998; Sozzani et al., 1999). We found that monocytes differentiated with CD8 T-cell-DC supernatants expressed substantial levels of CCR1, CCR2 and CCR5 which is in line with previous studies documenting the expression of these chemokine receptors on monocyte-derived DCs (Sato et al., 1999; Sozzani et al., 1999). Interestingly, monocytes differentiated with CD8 T-cell-DC supernatants expressed increased levels of CX_3CR1 which was shown to be expressed by non-lymphoid tissue resident DCs (Geissmann et al., 2010) and implicated in diseases (Liu et al., 2008; Niess and Reinecker, 2005). Altogether, the expression of these chemokine receptors by monocytes differentiated with CD8 T-cell-DC supernatants suggest that these monocyte-derived cells may reside in inflamed tissues. However, although these cells do not express high levels of CCR7, we cannot exclude the fact that they may still be found in lymph nodes where they prime T-cells as monocytes can migrate directly into lymph nodes through CCR2 to differentiate into DCs (Nakano et al., 2009).

The expression of TLRs on monocyte-derived cells is important for the recognition of molecular patterns specific to microbial pathogens (Aderem and Ulevitch, 2000; Medzhitov and Janeway, 2000; Medzhitov et al., 1997). This is critical as signaling

through TLRs strongly activates DCs to upregulate co-stimulatory molecules and to produce pro-inflammatory cytokines (Akira et al., 2001; Hertz et al., 2001). We found that monocytes differentiated with CD8 T-cell-DC supernatants upregulated TLR2, TLR3, TLR4 and TLR8. The upregulation of TLR2 and TLR4 suggests that differentiated monocytes become more sensitive to bacterial products such as LPS while the upregulation of TLR8 indicate increased responsiveness to single stranded RNA present on viruses during inflammation. Interestingly, studies have shown that the expression of TLR3 is restricted only to DCs among monocyte-derived cells (Muzio et al., 2000). Upon recognition of double stranded RNA, TLR3 induces the activation of NF-κB to increase production of type I interferons (Alexopoulou et al., 2001). Its presence have also been shown to be involved in DC cross-presentation, CTL responses, and antiviral protection (Jelinek et al., 2011). Thus, the presence of TLR3 on monocytes differentiated with CD8 T-cell-DC supernatants indicates that these monocyte-derived cells may acquire DC characteristics associated with the functions of TLR3.

While, the current morphological and phenotypic characterization of these monocyte-derived cells suggest that monocytes differentiated with CD8 T-cell-DC supernatants may represent DCs, DCs are a heterogeneous population with no single surface marker that unequivocally identifies them (Shortman and Liu, 2002). Hence, further characterization of these cells would rely on the functional abilities which would be addressed in the next few chapters.

CHAPTER 5: Functional characterization of human monocyte-derived cells (TNF/iNOS-producing dendritic cells) differentiated in the presence of CD8 T-cell-DC cytokine milieu

5.1   Introduction

Despite the availability of different markers to classify monocyte-derived cells as DC and macrophages, it still remains a challenge to separate these two cell populations. This is largely attributed to the many phenotypic similarities showed by these cells, including similar surface marker expression such as CD11c and F4/80 and the requirement for analogous growth factors such as M-CSF in mice (Hume, 2008; MacDonald et al., 2002). In human studies, the phenotypic properties of monocyte-derived cells are often compared to the gold standard DCs generated by culturing monocytes with IL-4 and GM-CSF (Sallusto and Lanzavecchia, 1994; Schuler et al., 2003). In turn, macrophages are believed to exhibit properties that mimic monocytes that have been differentiated with M-CSF (Brugger et al., 1991). However, others have also shown that various other stimuli (Chomarat et al., 2000; Mohamadzadeh et al., 2001; Santini et al., 2000; Zou and Tam, 2002) can induce the differentiation of monocytes into DCs or macrophages which may exhibit different phenotypic properties from these gold standard protocols. Therefore, although results in our previous chapter have suggested that monocytes differentiated with CD8-T-cell-DC cytokine milieus exhibit phenotypic characteristics of DCs, it is still

important to investigate their functional properties as the defining characteristic of a DC is unarguably, its ability to activate naive T-cells (Banchereau and Steinman, 1998)

To further understand these monocyte-derived cells, recent studies have focused on the role of these cells during inflammation. Notably, Serbina *et al.* (2003) discovered that a subset of inflammatory DCs, known as TNF/iNOS-producing DCs (Tip-DCs) were crucial for the clearance of *Listeria monocytogenes* bacteria in mice. These Tip-DCs expressed high levels of TNF-α and iNOS and unlike macrophages present in the spleen, these cells were not infected with *Listeria monocytoges*. In addition, these cells were also capable of priming naive T-cells for proliferation. Subsequently, Tip-DCs in other disease models such as influenza (Aldridge et al., 2009), psoriasis (Lowes et al., 2005) and parasitic infections (Bosschaerts et al., 2010) were documented. Therefore, the ability of monocyte-derived cells to express high levels of TNF-α and iNOS, in addition to their ability to prime naive T-cells, served as an important functional characteristic in classifying monocyte-derived cells as Tip-DCs.

In this chapter, we investigated the functional properties of cells that were differentiated from monocytes with CD8 T-cell-DC cytokine milieus. We first studied their ability to express TNF-α, iNOS as well as other pro-inflammatory cytokines before determining their ability to exhibit phagocytosis, endocytosis and naive T-cell priming. Finally we analyzed their ability to polarize T-cells through intracellular cytokine analysis.

5.2 Results

5.2.1 Monocytes differentiated with CD8 T-cell-DC cytokine milieu expressed increased amounts of TNF-α and iNOS

We first investigated the ability of differentiated monocytes to expressed pro-inflammatory mediators such as TNF-α and iNOS. Monocytes were cultured with the respective supernatants for 48 h and stimulated for an additional 6 h in the presence of LPS and Golgistop^{TM}. We found that more monocytes differentiated with Supernatant$_{DCLPS}$ (Fig. 5.1B) expressed intracellular TNF-α and iNOS than monocytes differentiated with Supernatant$_{DC}$ (Fig. 5.1A). However, monocytes differentiated with Supernatant$_{CD8DC \text{ or } CD8DCLPS}$ (Fig, 5.1C and D) expressed not only the highest percentage of cells with TNF-α and iNOS, but also expressed these inflammatory mediators at a much higher level, as indicated by the larger MFI, than monocytes differentiated with Supernatant$_{DC \text{ or } DCLPS}$ (Fig. 5.1A and B). We next compared the intracellular levels of TNF-α and iNOS of these differentiated monocytes to IL-4/GM-CSF DCs and M-CSF macrophages. In contrast to IL-4/GM-CSF DCs that expressed moderate levels of TNF-α and low levels of iNOS (Fig. 5.1E), M-CSF macrophages expressed low levels of TNF-α and high levels of iNOS instead (Fig. 5.1F). Notably, monocytes differentiated with Supernatant$_{CD8DC \text{ or } CD8DCLPS}$ (Fig. 5.1C and D) expressed similar levels of iNOS as M-CSF macrophages but expressed higher levels of TNF-α than both IL-4/GM-CSF DCs and M-CSF macrophages (Fig. 5.1E and F).
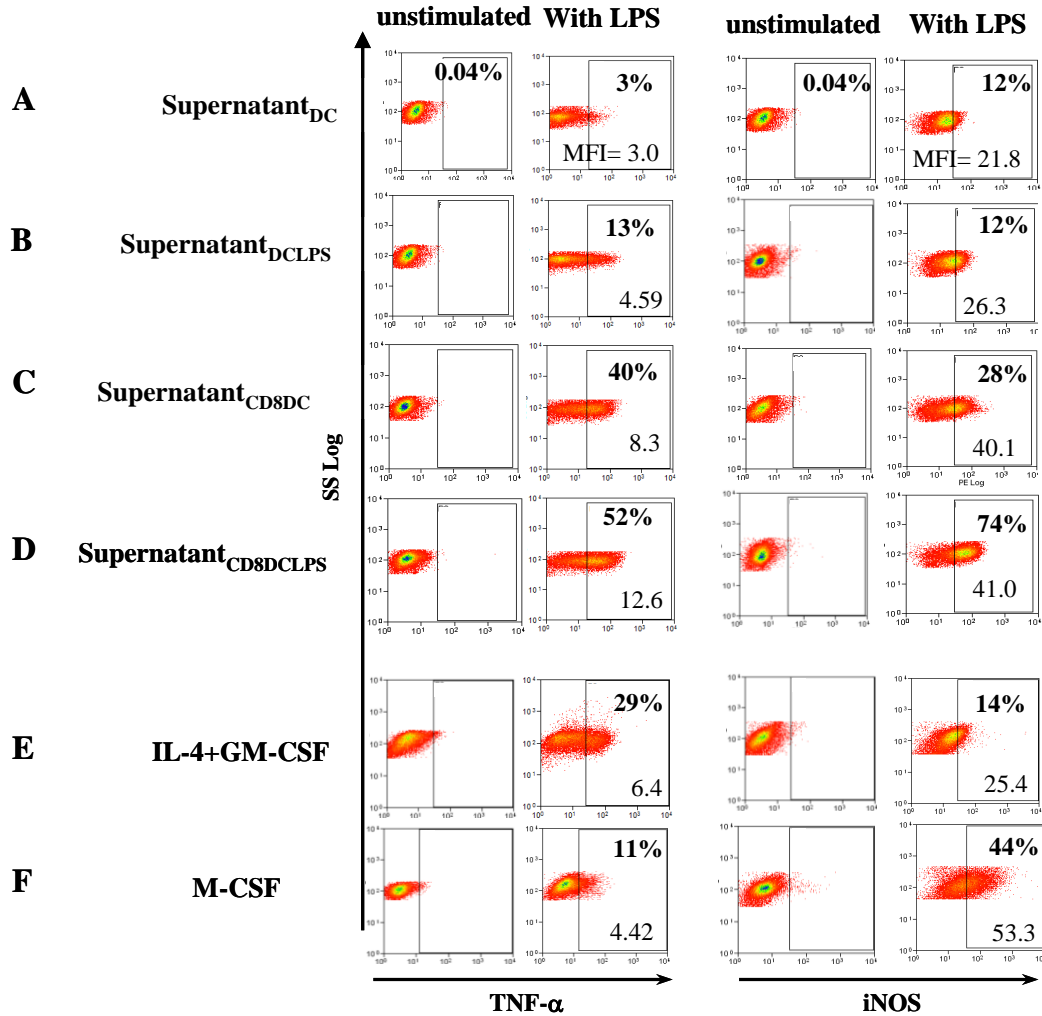
Fig. 5.1 Monocytes exposed to CD8 T-cell-DC cytokine milieu expressed increased amounts of TNF-α and iNOS.

Monocytes were cultured with (A) Supernatant$_{DC}$ (B) Supernatant$_{DCLPS}$ (C) Supernatant$_{CD8DC}$ (D) Supernatant$_{CD8DCLPS}$ (E) IL-4 and GM-CSF (F) M-CSF. Differentiated monocytes were washed and unstimulated (left) or stimulated with 100 ng/ml of LPS (right) together with 1ul/ml of Golgi-stop^{TM} in fresh medium for 6 h to detect intracellular TNF-α and 12 h to detect iNOS. All cells were subsequently analyzed by flow cytometry. Top left numbers represent percentages of positive cells and bottom numbers indicate the mean fluorescence index (MFI). Results are representative of four independent experiments with comparable results.

We further confirmed the expression levels of TNF-α and iNOS expressed by these differentiated monocytes. We found that the increased levels of TNF-α and iNOS expressed by monocytes differentiated with Supernatant$_{CD8DC\ or\ CD8DCLPS}$ compared to monocytes differentiated with Supernatant$_{DC\ or\ DCLPS}$ were statistically significant (Fig. 5.2). In addition, the number of nitric oxide (NO) producing monocytes differentiated with Supernatant$_{CD8DC\ or\ CD8DCLPS}$ were significantly higher than monocytes differentiated with Supernatant$_{DC\ or\ DCLPS}$ (Fig. 5.3). Finally, immunostaining further confirmed the expression of both intracellular TNF-α and iNOS by monocytes differentiated with Supernatant$_{CD8DC\ or\ CD8DCLPS}$ (Fig. 5.4A and B).
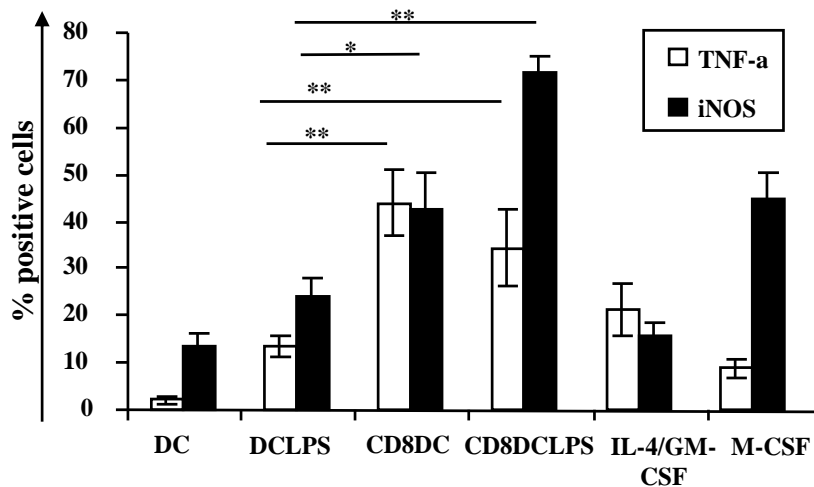
Fig. 5.2 TNF-α and iNOS expression by monocytes exposed to CD8 T-cell-DC cytokine milieu was significant.

Monocytes were differentiated with indicated supernatants or conditions, washed and re-cultured with 100 ng/ml of LPS together with 1ul/ml of Golgi-stop in fresh medium for 6 h to detect intracellular TNF-α and 12 h to detect iNOS. All cells were subsequently analyzed by flow cytometry. Graph represents average expression (mean ± SD) of TNF-a (white bars) and iNOS (black bars) produced from differentiated monocytes from five donors. *p<0.05; **p<0.005 using one-way ANOVA.
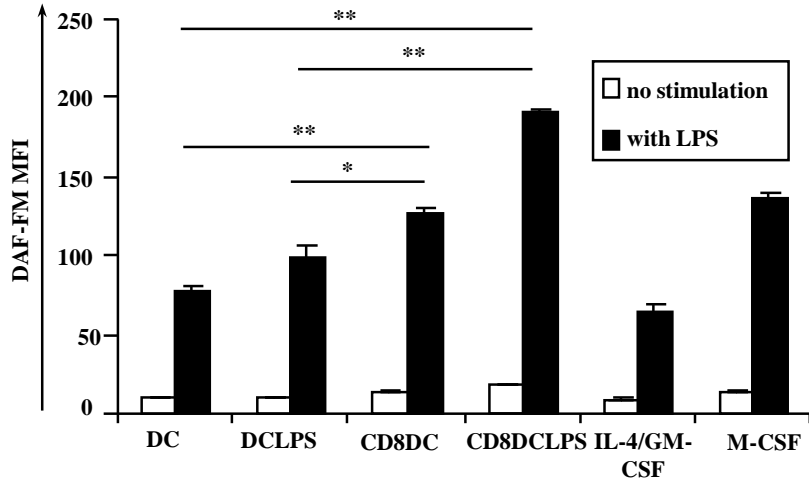
Fig. 5.3 Monocytes exposed to CD8 T-cell-DC cytokine milieu expressed significant amounts of NO.

Monocytes were differentiated with indicated supernatants or conditions, washed and re-cultured with or without 100 ng/ml of LPS together with 1ul/ml of Golgi-stop in fresh medium for 6 h to detect intracellular NO. Graph represents Nitric Oxide (NO) production (mean $\pm$ SD) from 3 donors detected using DAF-FM diacetate and analyzed by flow cytometry. *p<0.05; **p<0.005 using one-way ANOVA.
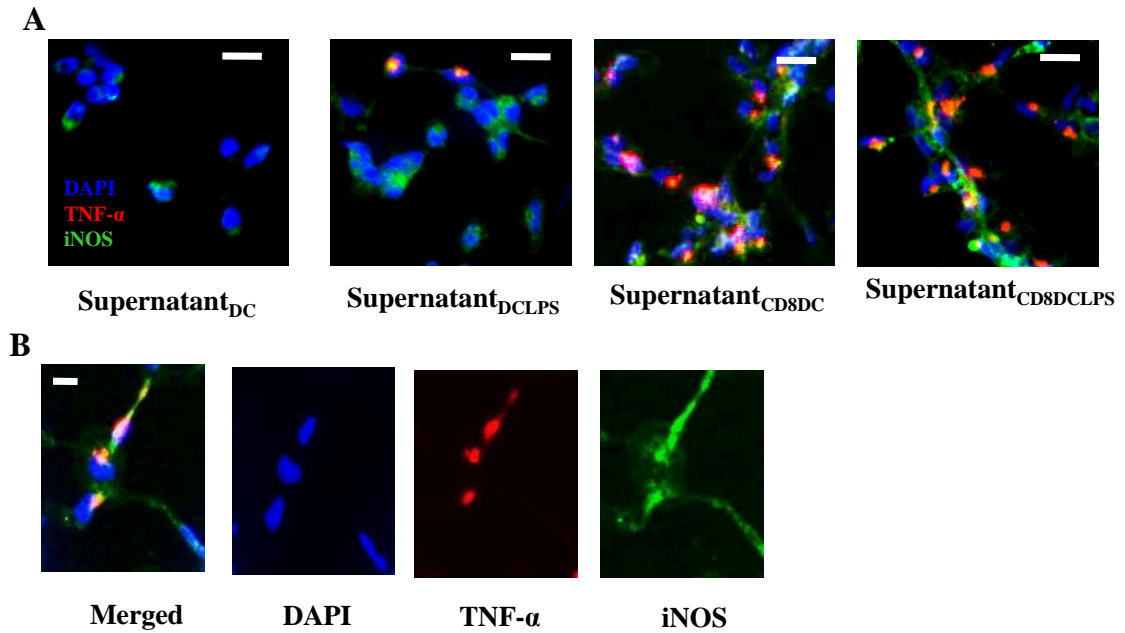
Fig. 5.4 Monocytes exposed to CD8 T-cell-DC cytokine milieu expressed increased amounts of TNF-α and iNOS.

(A) Monocytes were differentiated on glass coverslips with indicated supernatants, stimulated with LPS for 6h in the presence of Golgi Stop^{TM} before staining with DAPI, anti-iNOS and anti-TNF-α and viewed by fluorescence microscopy. (Blue=DAPI, Red=TNF-α, Green=iNOS). Original magnification was x400 for all panels. (Scale bar: 20 μm). (B) Higher magnification of cells differentiated with Supernatant$_{CD8DCLPS}$. Original magnification was ×630 for all panels. (Scale bar: 12μm) Results are representative of three experiments.

5.2.2 Monocytes differentiated with CD8 T-cell-DC cytokine milieu secrete pro-inflammatory cytokines

To further verify the inflammatory nature of these differentiated monocytes, we analyzed their ability to secrete extracellular pro-inflammatory cytokine proteins. Monocytes cultured for 48 hours with the respective supernatants were washed thoroughly and re-plated in fresh culture medium and LPS for an additional 24 hours. Differentiated monocytes cultured without LPS served as a control. We noticed that monocytes differentiated with Supernatant$_{CD8DC \, or \, CD8DCLPS}$ resulted in a significant increase in LPS-stimulated production of IL-1α (Fig. 5.5A), IL-1ß (Fig. 5.5B), IL-6 (Fig. 5.5C) and TNF-α (Fig. 5.5F) relative to stimulated monocytes that had no prior exposure to these supernatants. In addition, LPS-stimulated monocytes differentiated with Supernatant$_{CD8DC \, or \, CD8DCLPS}$ also produced higher levels of IL-12p40 (Fig. 5.5D) and GM-CSF (Fig. 5.5E) compared to LPS-stimulated monocytes differentiated with Supernatant$_{DC \, or \, DCLPS.}$ Notably, the levels of these cytokines produced by LPS-stimulated monocytes differentiated with Supernatant$_{CD8C \, or \, CD8DCLPS}$ were above the un-stimulated controls (Fig. 5.5A-F), suggesting that the cytokines detected were not a result of residual transfer from supernatant cultures. The levels of extracellular TNF-α (Fig. 5.5F) also supported our intracellular flow cytometry (Fig. 5.1A-D) and immunostaining results (Fig. 5.4A). Overall, these results suggest that monocytes differentiated with Supernatant$_{CD8DC \, or \, CD8DCLPS}$ are pro-inflammatory and since they also expressed CD83 (Fig. 4.6), TNF-α and iNOS, they could resemble Tip-DCs previously described in mice (Aldridge et al., 2009; Bosschaerts et al., 2010; Serbina et al., 2003; Tezuka et al., 2007) and humans (Lowes et al., 2005).
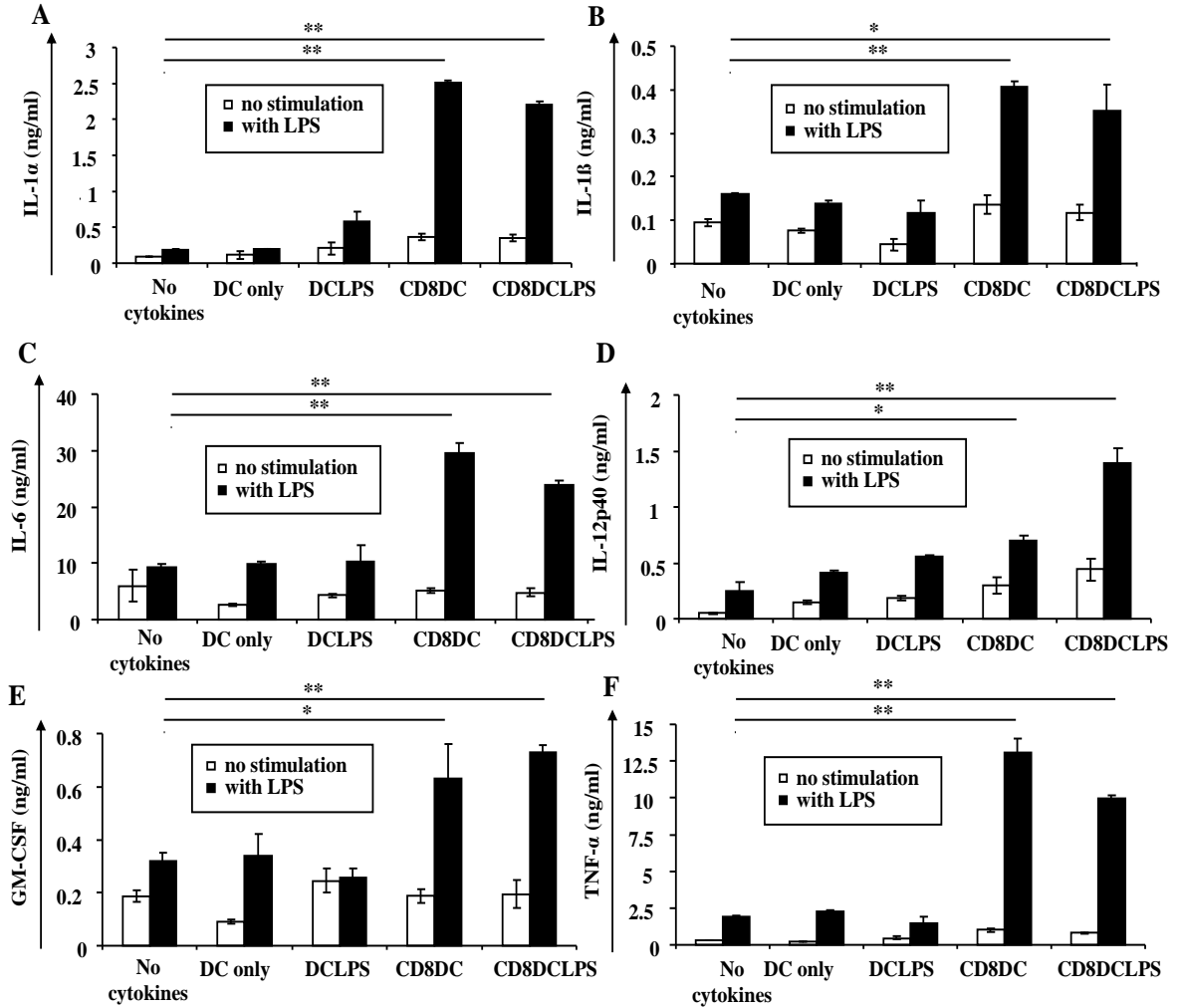
Fig. 5.5 Monocytes differentiated with CD8 T-cell-DC cytokine milieu are highly inflammatory.

Freshly isolated CD14^{+} monocytes were cultured with indicated supernatants or culture medium for 48 h. Cells were then harvested, washed 3 times with DMEM and re-plated in fresh medium with (black bars) or without (white bars) 100ng/ml of LPS. After 18 h, supernatants were harvested and the cytokines (A) IL-1α (B) IL-1ß (C) IL-6 (D) IL-12p40 (E) GM-CSF and (F) TNF-α measured using multiplex bead arrays. Results are representative of three experiments

5.2.3 Monocytes differentiated with CD8 T-cell-DC cytokine milieu down-regulate endocytosis and phagocytosis

We next investigated the functional properties of these differentiated monocytes by first analyzing their ability to endocytose. Monocytes differentiated with Supernatant$_{DC \text{ or } DCLPS}$ displayed a high level of endocytic activity with FITC dextran which were of comparable levels to those displayed by mature M-CSF macrophages (Fig. 5.6). In contrast, mature IL-4/GM-CSF DCs and monocytes differentiated with Supernatant$_{CD8DC \text{ or } CD8DCLPS}$ showed a reduced capacity to endocytose the same particles (Fig. 5.6).
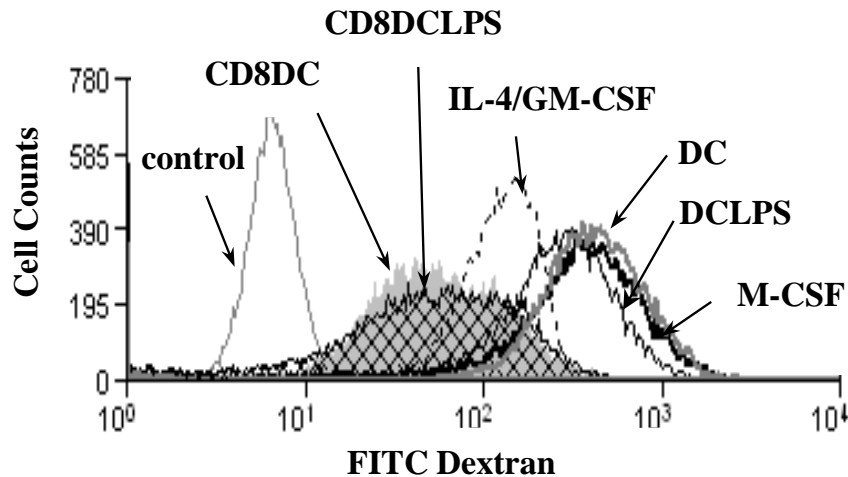
Fig. 5.6 Monocytes differentiated with CD8 T-cell-DC cytokine milieu downregulated endocytosis.

Mature DCs, mature macrophages and monocytes differentiated with supernatants from indicated cultures were harvested and analyzed for endocytosis by incubating with FITC-labeled dextran, washed and analyzed via flow cytometry. Results are representative of three independent experiments.

We subsequently analyzed the ability of these differentiated monocytes to phagocytose *Escherichia coli* AF488 conjugate bioparticles. Monocytes differentiated with Supernatant$_{DC\ or\ DCLPS}$ exhibited robust phagocytic activities which were similar to those displayed by mature M-CSF macrophages (Fig. 5.7). Strikingly, mature IL-4/GM-CSF DCs and monocytes differentiated with Supernatant$_{CD8DC\ or\ CD8DCLPS}$ showed reduced phagocytic activity as measured by the percentage of cells positive for the fluorescent bioparticles (Fig. 5.7). Hence these results suggest that monocytes differentiated with Supernatant$_{DC\ or\ DCLPS}$ display phagocytic and endocytic properties similar to macrophages while monocytes differentiated with Supernatant$_{CD8DC\ or\ CD8DCLPS}$ exhibited more mature DC-like properties through reduced phagocytic and endocytic activities.
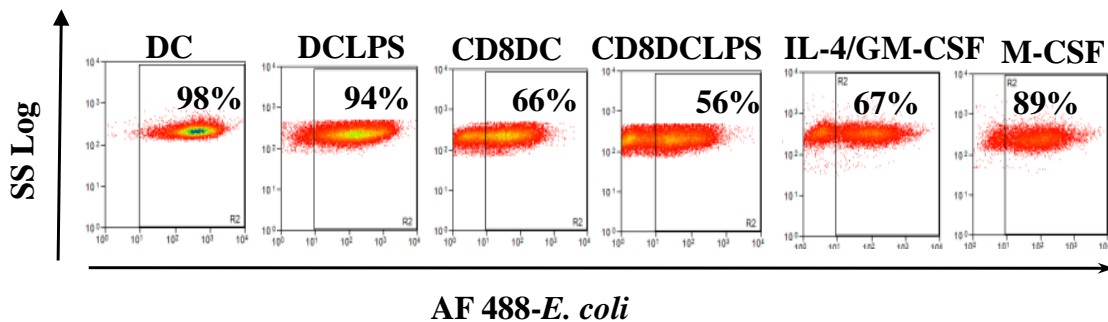
Fig. 5.7 Monocytes differentiated with CD8 T-cell-DC cytokine milieu downregulated phagocytosis.

Mature DCs, mature macrophages and monocytes differentiated with supernatants from indicated cultures were harvested and analyzed for phagocytosis by incubating with FITC-labeled dextran, washed and analyzed via flow cytometry. Results are representative of three independent experiments.

5.2.4 Monocytes differentiated with CD8 T-cell-DC cytokine milieu prime naive CD4 T-cells for proliferation

We further analyzed the ability of these differentiated monocytes to induce the proliferation of allogeneic naive CD45RA$^+$ T-cells which is a defining function of DCs (Banchereau and Steinman, 1998). Differentiated monocytes were co-cultured with naive CD4 T-cells for 6 days and the proliferative response of the T-cells measured by ^{3}H-thymidine incorporation. We found that monocytes that were differentiated with Supernatant$_{DC \text{ or } DCLPS}$ resembled M-CSF macrophages as these cells exhibited minimal T-cell priming ability and did not induce a significant proliferative response of CD4 T-cells compared to untreated monocytes (Fig. 5.8A). In contrast, monocytes that were differentiated with Supernatant$_{CD8DC \text{ or } CD8DCLPS}$ induced a significant increase in proliferating T-cells and this response was also slightly higher than that induced by IL-4/GM-CSF DCs when the ratio of T-cells to APCs was 10:1 (Fig. 5.8A).

We next investigated if this proliferative trend was similar when differentiated monocytes were further activated with LPS. We found that monocytes that were differentiated with Supernatant$_{DC \text{ or } DCLPS}$ still resembled M-CSF macrophages as these cells exhibited minimal T-cell priming ability and did not induce a significant proliferative response of CD4 T-cells compared to untreated monocytes (Fig. 5.8B). Strikingly, monocytes that were differentiated with Supernatant$_{CD8DC \text{ or } CD8DCLPS}$ were still able to induce a significant increase in proliferating T-cells although the response this time was not higher than that induced by IL-4/GM-CSF DCs (Fig. 5.8B). Notably, while IL-4/GM-CSF DCs had significant differences in their stimulating capacities before and after exposure to LPS,

monocytes differentiated with Supernatant$_{CD8DC \text{ or } CD8DCLPS}$ had minimal differences (Fig. 5.8A and B). Hence, these results confirm the DC properties of monocytes differentiated with Supernatant$_{CD8DC \text{ or } CD8DCLPS}$ and their resemblance as Tip-DCs.

A
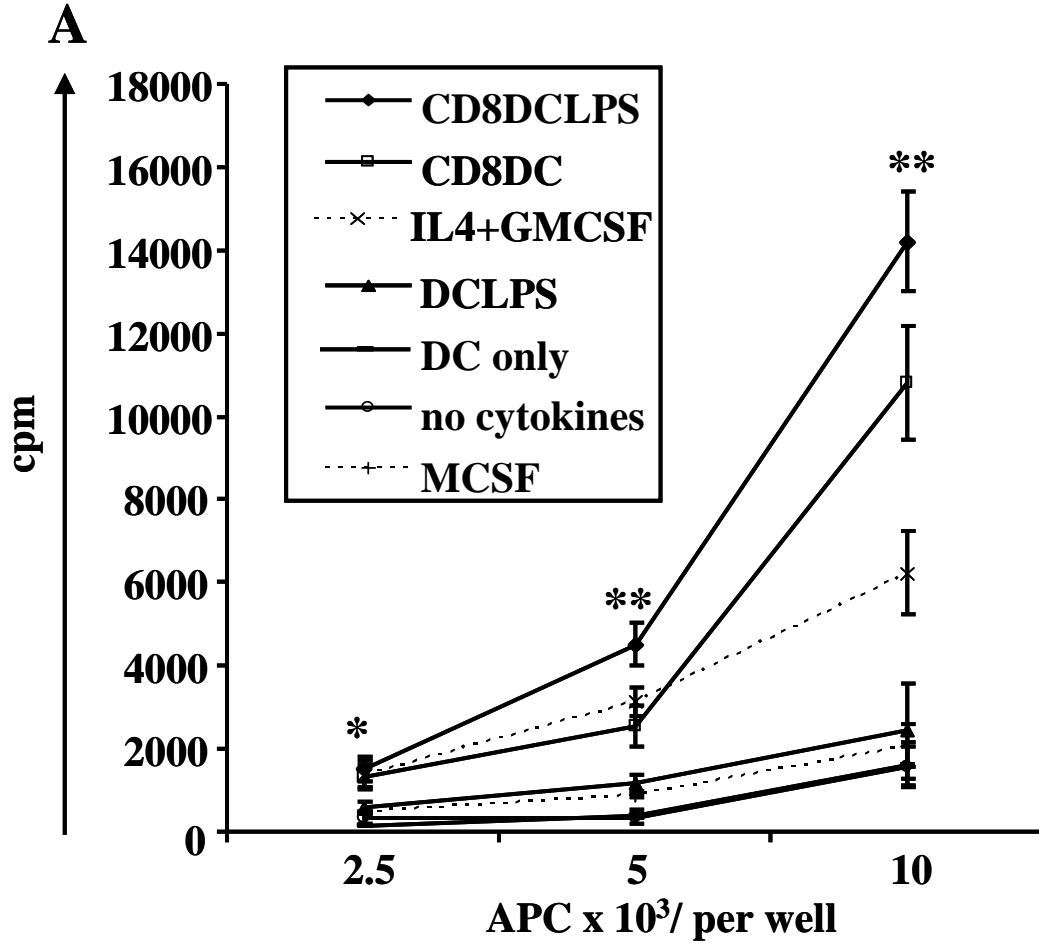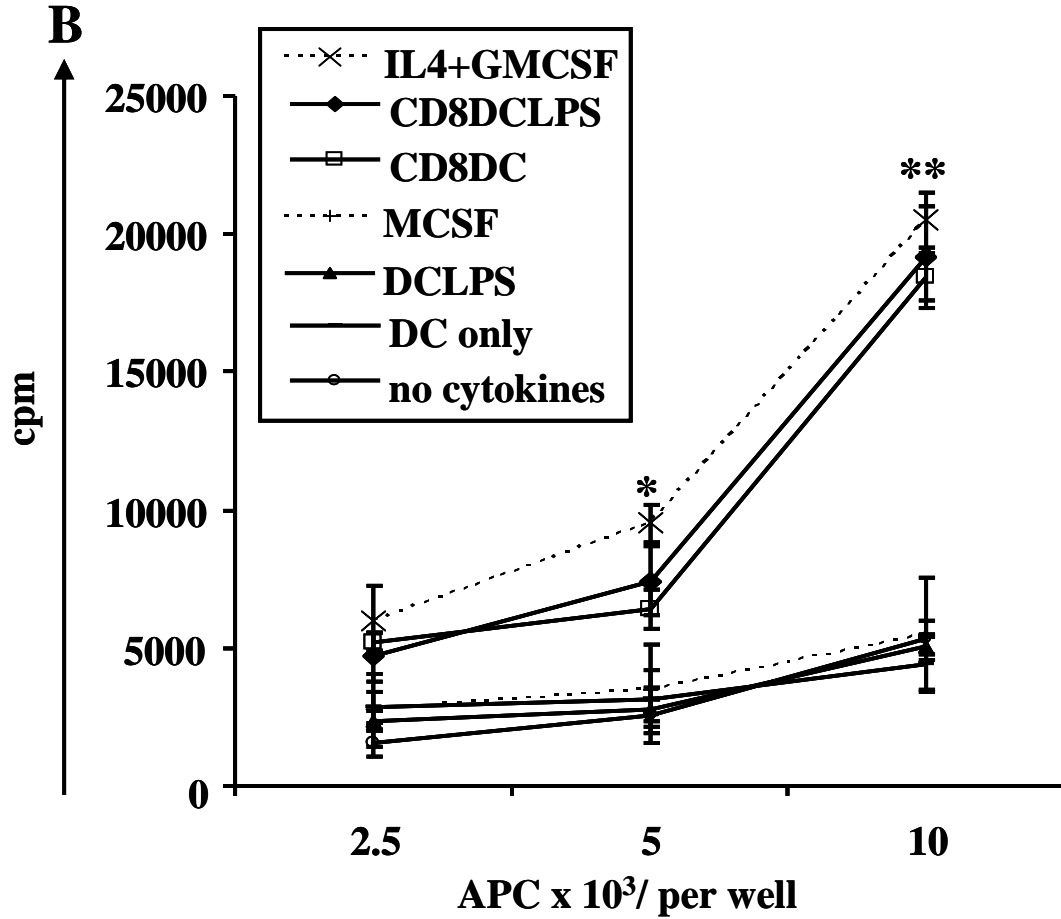
Fig. 5.8 Monocytes differentiated with CD8 T-cell-DC cytokine milieu prime naive CD4 T-cells for proliferation.

To test for CD4 T cell proliferation, monocytes differentiated with indicated supernatants or conditions were harvested, washed and irradiated before being used as APCs to stimulate freshly isolated allogeneic naive CD4+CD45RA+ T cells in the (A) absence or (B) presence of 100ng/ml of LPS. The CD4 T-cell proliferative response was measured after 6 days by ^{3}H-thymidine incorporation; results are expressed as mean counts per minute (cpm) ± SEM. 1x10^5 T cells and varying numbers of APCs were added per well. *p<0.05, **p<0.005 comparing (♦) or (□) versus (▲) or (▬) using one-way ANOVA. Results are representative of three independent experiments.

5.2.5 Monocytes differentiated with CD8 T-cell-DC cytokine milieu prime CD4 T-cells for IFN-γ production

We next studied whether these differentiated monocytes could prime CD4 T-cells towards a specific cytokine producing phenotype. To this end, we tested for the presence of intracellular IFN-γ, IL-4, IL-17 and IL-10 in these CD4 T-cells after co-culture with differentiated monocytes. While there were significant differences in the amount of IL-4 and IFN-γ expressed (Fig. 5.9A), we did not detect any significant differences in the expression of IL-17 and IL-10 in these CD4 T-cells (data not shown). Hence, we focused on the expression of IL-4 and IFN-γ in these set of experiments. We found that M-CSF macrophages and monocytes that were differentiated with Supernatant$_{DC\ or\ DCLPS}$ induced a low percentage of IFN-γ expressing CD4 T-cells (Fig. 5.9A). In contrast, monocytes differentiated with Supernatant$_{CD8DC\ or\ CD8DCLPS}$ generated a similar percentage of IFN-γ expressing CD4 T-cells as IL-4/GM-CSF DCs and this was three-fold more than that induced by monocytes differentiated with Supernatant$_{DC\ or\ DCLPS}$ and M-CSF macrophages (Fig. 5.9A). In addition, the percentage of IFN-γ expressing CD4 T-cells by monocytes differentiated with Supernatant$_{CD8DC\ or\ CD8DCLPS}$ was statistically significant compared to those induced by monocytes differentiated with Supernatant$_{DC\ or\ DCLPS}$ (Fig. 5.9B). Interestingly, M-CSF macrophages and monocytes differentiated with Supernatant$_{DC\ or\ DCLPS}$ were shown to express a slight increase in percentage of IL-4-producing CD4 T-cells compared to those induced by IL-4/GM-CSF DCs and monocytes differentiated with Supernatant$_{CD8DC\ or\ CD8DCLPS}$ (Fig. 5.9A). Hence, these results suggest that monocytes differentiated with Supernatant$_{CD8DC\ or\ CD8DCLPS}$ favour the polarization of CD4 T-cells to a Th1 phenotype by increasing the percentage of IFN-γ expressing CD4 T-cells
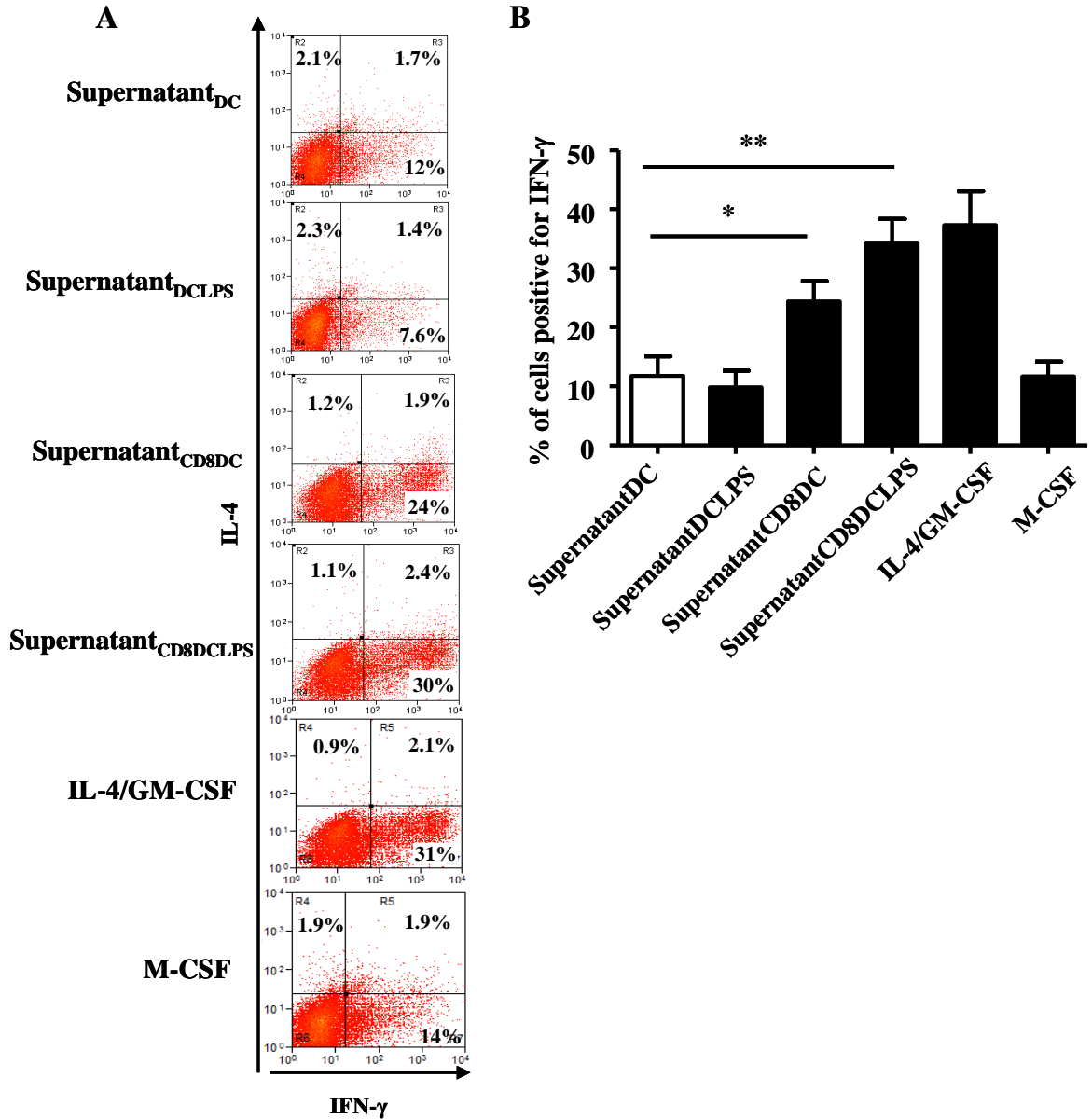
Fig. 5.9 Monocytes differentiated with CD8 T-cell-DC cytokine milieu prime CD4 T-cells for Th1 responses.

Monocytes differentiated with indicated supernatants or conditions were harvested and used as APCs to stimulate allogeneic naive $CD4^+CD45RA^+$ T-cells in the presence of LPS (100ng/ml). Cells were harvested, fixed, stained intracellularly for IL-4 and IFN-γ and analyzed by flow cytometry. Cells were gated on $CD3^+$ cells to discriminate T-cells from APCs. Numbers represent percentages of positive cells. (A) Results are representative of four independent experiments. (B) Results are pooled from 3 independent donors. *p<0.05, **p<0.005 using one-way ANOVA test followed by Tukey's post test

5.2.6   CD4 Th1 responses is dependent on the expression of TNF-α and iNOS of monocyte-derived cells

Although we were able to detect the presence of IL-12p40 in supernatants of differentiated monocytes stimulated with LPS (Fig. 5.5D), we were unable to detect significant levels of IL-12p70. Hence, we investigated if other factors produced by monocytes differentiated with Supernatant$_{CD8DC \text{ or } CD8DCLPS}$ could be responsible for the increased induction of IFN-γ producing CD4 T-cells. Since monocytes differentiated with Supernatant$_{CD8DC \text{ or } CD8DCLPS}$ showed significant expression of TNF-α and iNOS, we thus determined if these 2 factors were responsible for the increased polarization of Th1 cells. To this end, we co-cultured naive CD4 T-cells with monocytes that have been differentiated with Supernatant$_{CD8DC \text{ or } CD8DCLPS}$ in the presence of blocking antibodies. To block the presence of TNF-α, naive CD4 T-cells were first blocked with 10μg/ml of anti-TNF-α receptor antibodies for 1 h prior to co-culture. 10μg/ml of anti-TNF-α blocking antibodies was also simultaneously added to the co-cultures. To block the effects of NO, monocytes that were differentiated with Supernatant$_{CD8DC \text{ or } CD8DCLPS}$ were treated with 10mM of $_L$-NAME for 1 h prior to co-culture. Differentiated monocytes and CD4 T-cells that were blocked with the relevant isotype-blocking antibodies served as a control. We found that the polarization of IFN-γ producing CD4 T-cells by monocytes differentiated with Supernatant$_{CD8DC \text{ or } CD8DCLPS}$ was partially dependent on their expression of TNF-α and iNOS as blocking these factors resulted in a reduction in IFN-γ producing CD4 T-cells (Fig. 5.10). Hence, these results suggest that the increased expression of TNF-α and iNOS seen in monocytes differentiated with Supernatant$_{CD8DC \text{ or } CD8DCLPS}$ is partially responsible for the increased polarization of Th1 cells.
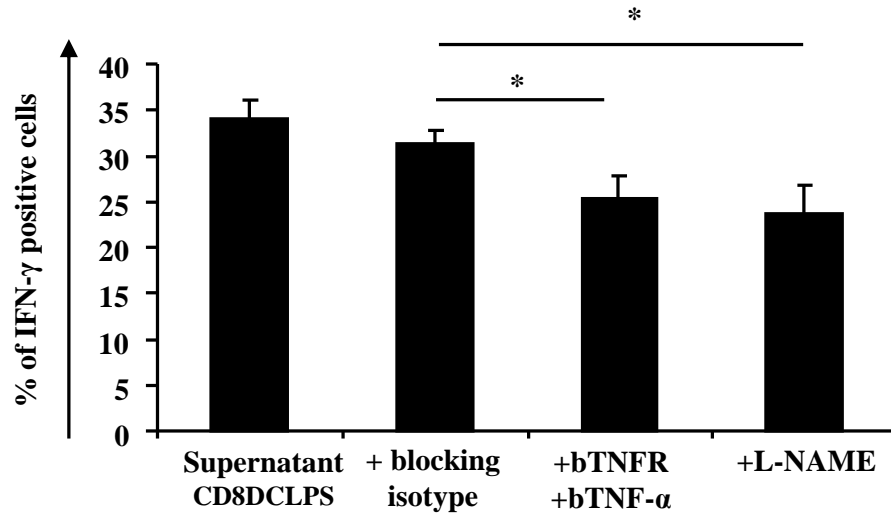
Fig. 5.10 CD4 Th1 responses is dependent on the expression of TNF-α and iNOS by monocytes differentiated with CD8 T-cell-DC cytokine milieu.

Percentage of IFN-γ positive CD4 T-cells from 3 donors detected using flow cytometry. To determine if TNF-α or NO produced by monocytes differentiated with Supernatant$_{CD8DCLPS}$ had an effect on the polarization of CD4 T-cells, differentiated monocytes were cultured with CD4 T-cells that were blocked with anti-TNFR (bTNFR) antibodies (10µg/ml) 1h prior to co-culture as indicated. To further block the effect of TNF-α on CD4 T-cells, anti-TNF-α blocking (bTNF-α) antibodies (10µg/ml) were also added simultaneously to the indicated co-culture. Alternatively, differentiated monocytes were treated with 10mM of $_L$-NAME to block the effects of NO for 1h at 37ºC, washed and used as APCs to stimulate naive CD4 T-cells. Differentiated monocytes and CD4 T-cells that were blocked with relevant isotype blocking antibodies served as a control. *p<0.05 using one-way ANOVA.

5.3 Discussion

In the previous chapter, we showed that soluble factors from human CD8 T-cell-DC co-cultures could differentiate monocytes into cells with phenotypic and morphological characteristics of mature DCs such as the appearance of long spindle dendrites, high expression of MHC class I, HLA-DR, co-stimulatory molecules and upregulation of CD83. In this chapter, we show that these monocyte-derived cells also display functional properties that were similar to DCs, rather than macrophages, as they could induce an allogeneic MLR response and exhibited reduced phagocytic and endocytic activities. Interestingly, these cells also expressed high levels of TNF-α and iNOS which resemble a subset of inflammatory DCs known as Tip-DCs. We also showed that these Tip-DCs were able to prime naive CD4 T-cells for IFN-γ and this was partially dependent on their production of TNF-α and NO. The differentiation of monocytes into Th1-inducing Tip-DCs by human CD8 T-cells thus highlights a novel role similar to other bystander mediated cross-talk seen in infectious models (Remoli et al., 2010; Salazar-Gonzalez et al., 2007) which allows the transmission of CD8 T-cell mediated Th1-inducing signals without the need for physical interaction with infiltrating monocytes.

We found that these Tip-DCs (monocytes differentiated with CD8 T-cell-DC cytokine milieus) were able to induce naive T-cell proliferation, an important hallmark of DCs, similarly to classically generated IL-4/GM-CSF DCs. On the other hand, monocytes that were differentiated with DC-derived cytokine milieus behaved like macrophages and

were unable to induce significant T-cell proliferation. Interestingly, while, classically generated IL-4/GM-CSF DCs had significant differences in their stimulating capacities before and after exposure to LPS, we found that further stimulation of Tip-DCs did not increase their capacity to stimulate T-cell proliferation, suggesting that the latter may already be semi-mature. Indeed, other studies have shown that certain stimuli such as CD137 ligand signaling (Kwajah M M and Schwarz, 2010) can not only induce the differentiation of monocytes into DCs, but were also partially able to induce their maturation. Their semi-functional mature status induced by exposure to soluble factors from CD8 T-cell-DC cytokine milieus during the differentiation process may also be explained by the fact that Tip-DCs also expressed high levels of co-stimulatory molecules even before LPS exposure. Moreover, Tip-DCs had reduced endocytic activity which is an established hallmark of maturation of DCs (Austyn, 1996; Garrett et al., 2000; Sallusto et al., 1995; Steinman, 1991). Downregulation of endocytosis ensures the longevity of peptide–MHC class II complexes at the cell surface (Cella et al., 1997) and by synchronizing the cessation of endocytosis with the activation of peptide loading onto MHC class II molecules, DCs limit their presentation activity to those antigens encountered prior to or concomitant with an inflammatory stimulus. In addition, we also found that Tip-DCs had reduced phagocytic activities compared to classical macrophages. This important functional characteristic further enforces the capacity of Tip-DCs to stimulate T-cell proliferation and that their role may not lie in the ability to phagocytose pathogens like macrophages.

TNF-α is a pivotal mediator of inflammation that activates leukocytes, enhances adherence of neutrophils and monocytes to endothelium, promotes migration of inflammatory cells into the intercellular matrix and triggers local production of other pro-inflammatory cytokines (Tracey and Cerami, 1994). On the other hand, the production of NO upon iNOS expression is an important mediator for immune defense by directly inhibiting pathogen replication (Lowenstein and Padalko, 2004). Both TNF-α and iNOS have been shown to be expressed mainly by macrophages, however, there is increasing evidence that these two mediators can also be expressed by DCs such as Langerhans cells (Larrick et al., 1989; Qureshi et al., 1996) and inflammatory DCs (Lowes et al., 2005; Serbina et al., 2003). Therefore, the high expression of TNF-α and iNOS seen in Tip-DCs generated from monocytes exposed to CD8 T-cell-DC cytokine milieus suggest that DCs may also utilize the production of TNF-α and NO for immunomodulation by interacting with other immune cells. Indeed, a previous study has shown that Tip-DCs occur naturally in mouse mucosa-associated lymphoid tissues (MALT) and the expression of iNOS by these Tip-DCs is crucial for IgA class-switch recombination by plasma cells (Tezuka et al., 2007). In addition, Tip-DCs have also been shown to be important for supporting the proliferation and effector function of influenza specific CD8 T-cells in the lung (Aldridge et al., 2009).

Finally, we observed that Tip-DCs obtained in our *in vitro* cultures were also able to prime naive CD4 T-cells for increased expression of IFN-γ. This was a surprising finding as we were unable to detect any significant IL-12p70 in supernatants of Tip-DCs and CD4 T-cell co-cultures (data not shown). However, we showed that blocking the

expression of TNF-α and iNOS partially reduced the ability of Tip-DCs to polarize Th1 cells. Hence, the ability of TNF-α and iNOS to partially mediate Th1 polarization may thus help to explain why residual Th1 responses can still occur in the absence of IL-12p70 in other disease models (Brombacher et al., 1999; Verhagen et al., 2000). Moreover, others have also shown that NO can contribute to the selective differentiation of Th1 cells (Koncz et al., 2007; Lee et al., 2011; Niedbala et al., 1999) and TNF-α has been shown to drive the *in situ* proliferation of T-cells by upregulating the expression of IL-2 (Agostini et al., 1995) as well as favour Th1 polarization through IL-12 independent mechanisms (Brown et al., 2003). Interestingly, a recent studies have also shown that NO acts to inhibit Th17 responses while favouring Th1 responses (Lee et al., 2011), and this may also explain why we were unable to detect any IL-17 expression (data not shown) in CD4 T-cells that have been polarized by Tip-DCs. Hence, the ability of CD8 T-cells to mediate Th1 responses by differentiating monocytes into Th1 inducing Tip-DCs highlights a previously undescribed mechanism and may also explain the importance of CD8 T-cells in microbial infections (Gurunathan et al., 2000; Peterson et al., 2002; Sud et al., 2006; Uzonna et al., 2004) since TNF-α and NO production by Tip-DCs are key effectors in pathogen clearance (Serbina et al., 2003)

CHAPTER 6: Differentiation mechanism of human TNF/iNOS-producing (Tip) dendritic cells

6.1 Introduction

In the previous chapter, we found that monocytes differentiated with CD8 T-cell-DC cytokine milieus exhibited phenotypic and functional characteristics of Tip-DCs. However, it remains unclear which cytokines played a key role in the differentiation of these cells. Previous studies have found that certain cytokines had the propensity to skew the differentiation of monocytes into DCs or macrophages. For example, IL-6 (Chomarat et al., 2000) has been shown to favour the differentiation of monocytes into macrophages while cytokines IL-2 (Sanarico et al., 2006), IL-15 (Mohamadzadeh et al., 2001), TNF-α (Chomarat et al., 2003; Iwamoto et al., 2007) and IFN-α (Mohty et al., 2003) were demonstrated to differentiate monocytes into DCs with their own characteristic properties. IFN-γ, an important cytokine for the polarization of Th1 responses, demonstrated conflicting results in monocyte differentiation studies. Although an early report by Delneste *et al.* (2002) showed that IFN-γ favoured the differentiation of monocytes towards macrophages, a recent report by Eljaafari *et al.* (2009) argued that IFN-γ differentiates monocytes into tolerogenic DCs that prime naive T-cells to express Foxp3 instead.

In addition, the differentiation of Tip-DCs in mouse models has also been documented to rely heavily on MyD88 signaling (Bosschaerts et al., 2010; Jia et al., 2008). In particular, Bosscharets *et al.* (2010) demonstrated that the conversion of CD11b^{+}Ly6C^{+} monocytes into Tip-DCs in the liver of *T. brucei* infected mice consists of a three-step process including (i) a CCR2-dependent but CCR5- and Mif-independent step crucial for emigration of CD11b^{+}Ly6C^{+} monocytic cells from the bone marrow (ii) a differentiation step of liver CD11b^{+}Ly6C^{+}monocytic cells to immature inflammatory DCs (CD11c^{+} but CD80/CD86/MHC-II^{low}) which is IFN-γ and MyD88 signalling independent; and (iii) a maturation step of inflammatory DCs to functional (CD80/CD86/MHC-II[high]) TNF-α and NO producing Tip-DCs which is IFN-γ and MyD88 signalling dependent. Since IFN-γ showed conflicting monocyte differentiation results and its role in the differentiation of human Tip-DCs *in vitro* remains unknown, it would be interesting to determine if IFN-γ is indeed an important factor for the differentiation of Tip-DCs in our model.

In this chapter, we determine the factors involved in the differentiation of monocytes into Tip-DCs by using blocking antibodies against the cytokine receptors on monocytes. We then investigated how the blocking of these cytokine receptors could affect their differentiation and acquisition of the functional properties of Tip-DCs.

6.2   Results

6.2.1   TNF-α, in CD8-DC cytokine milieu, did not play a significant role in the differentiation of Tip-DCs

We investigated the factors involved in the differentiation of Tip-DCs. We noted that Supernatant$_{CD8DCLPS}$ contained increased amounts of IFN-γ and TNF-α compared to Supernatant$_{DC \text{ or } DCLPS}$ (Table 3.1). Hence, we first determined if TNF-α was implicated in Tip-DC differentiation by treating monocytes with a TNF-α receptor blocking antibody prior to culture with Supernatant$_{CD8DCLPS}$. These cells were harvested after 48 h and subsequently analyzed by flow cytometry. We found that blocking the TNF-α receptor in increasing doses did not result in a dose dependent decrease in the percentage and MFI expression of CD40, CD83, Class I and HLA-DR (Fig. 6.1). In addition, we also did not detect any dose dependent decrease in the expression of TNF-α and iNOS when increasing doses of TNF-α receptor blocking antibodies were added to monocytes (Fig. 6.2). Hence, these results suggest that the presence of TNF-α in the CD8-DC cytokine milieu does not play a significant role in the expression of these markers in the differentiation of Tip-DCs.
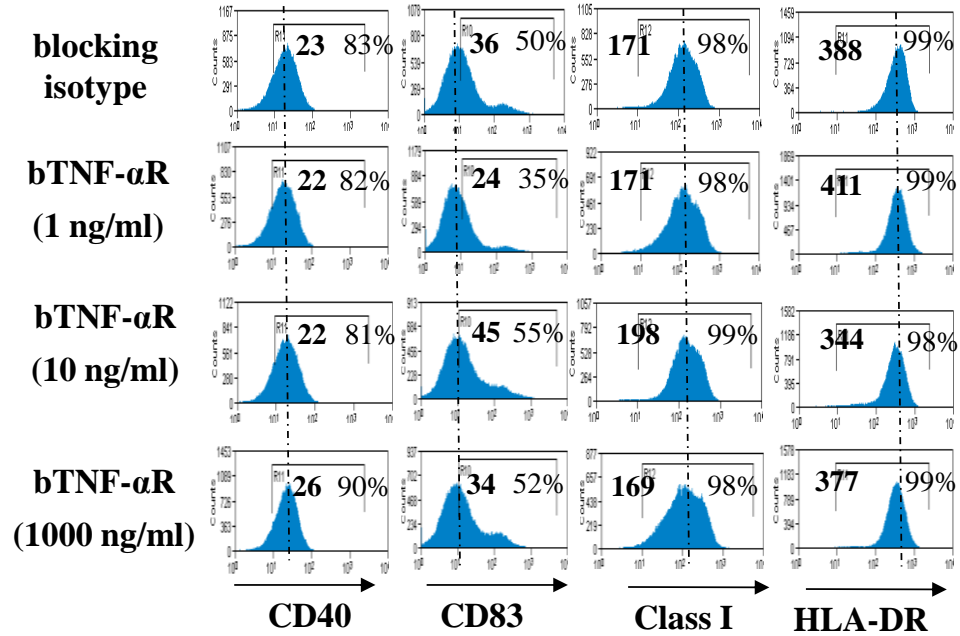
Fig. 6.1 Differentiation of monocytes into Tip-DCs is not dependent on TNF-α.

Monocytes were cultured with an isotype blocking antibody (10μg/ml) or indicated concentrations of anti-TNF receptor blocking antibodies (bTNF-αR) for 1h before adding Supernatant$_{CD8DCLPS}$. Cells were stained for (A) CD40, CD83 Class I and HLA-DR Numbers on right represent percentages of positive cells. Bold numbers represent mean fluorescence intensity (MFI). Results are representative of three independent experiments.
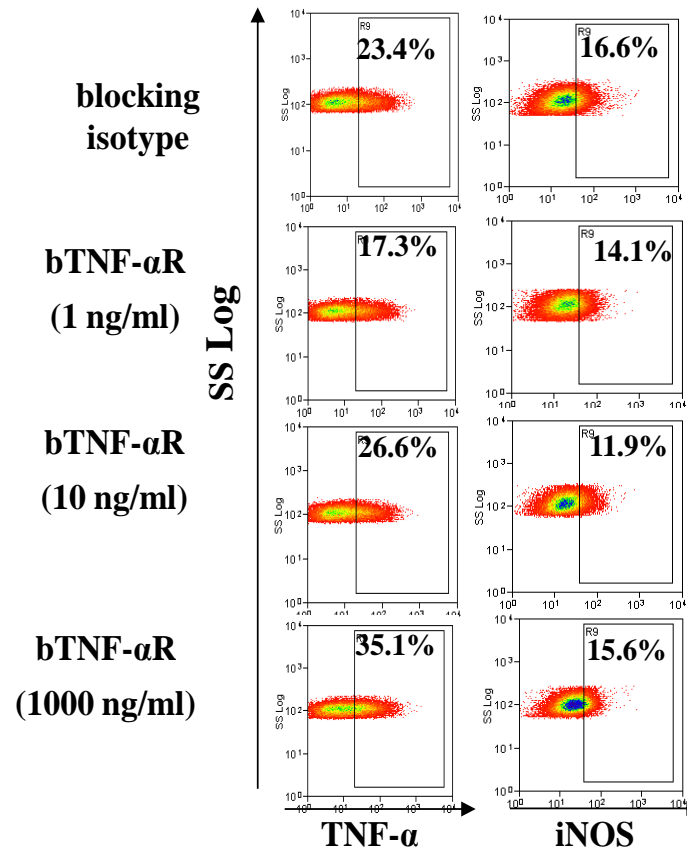
Fig. 6.2 Differentiation of monocytes into Tip-DCs is not dependent on TNF-α.

Monocytes were cultured with an isotype blocking antibody (10μg/ml) or indicated concentrations of anti-TNF receptor blocking antibodies (bTNF-αR) for 1h before adding Supernatant$_{CD8DCLPS}$. Cells were stained for intracellular TNF-α and iNOS by flow cytometry. Numbers represent percentages of positive cells. Results are representative of three independent experiments.

6.2.2 IFN-γ, in CD8 T-cell-DC cytokine milieu, is important for the upregulation of CD40, HLA-DR, CD83 and expression of TNF-α by Tip-DCs

We next determined if IFN-γ may be implicated in Tip-DC differentiation. Monocytes were treated with increasing doses of IFN-γ receptor blocking antibodies before culture with Supernatant$_{CD8DCLPS}$. These cells were harvested after 48 h and subsequently analyzed by flow cytometry. We found that blocking the IFN-γ receptor in increasing doses resulted in a dose dependent decrease in both the percentage and MFI expression of CD40, CD83 and HLA-DR (Fig. 6.3). We also noticed that although there were no significant changes in the percentage expression of MHC class I on these cells, the MFI expression of MHC class I decreased in a dose dependent manner with increasing doses of blocking antibodies (Fig. 6.3). We further investigated if IFN-γ was important for the expression of TNF-α and iNOS in Tip-DCs. We found that blocking the IFN-γ receptor in increasing doses resulted in a dose dependent decrease in the percentage expression of TNF-α, although there was no significant trend for the expression of iNOS when these blocking antibodies were added (Fig. 6.4). Overall, these results indicate that IFN-γ is an important factor for the differentiation of monocytes into Tip-DCs, especially for the upregulation of CD40, CD83, MHC class II and the expression of TNF-α.

A

B
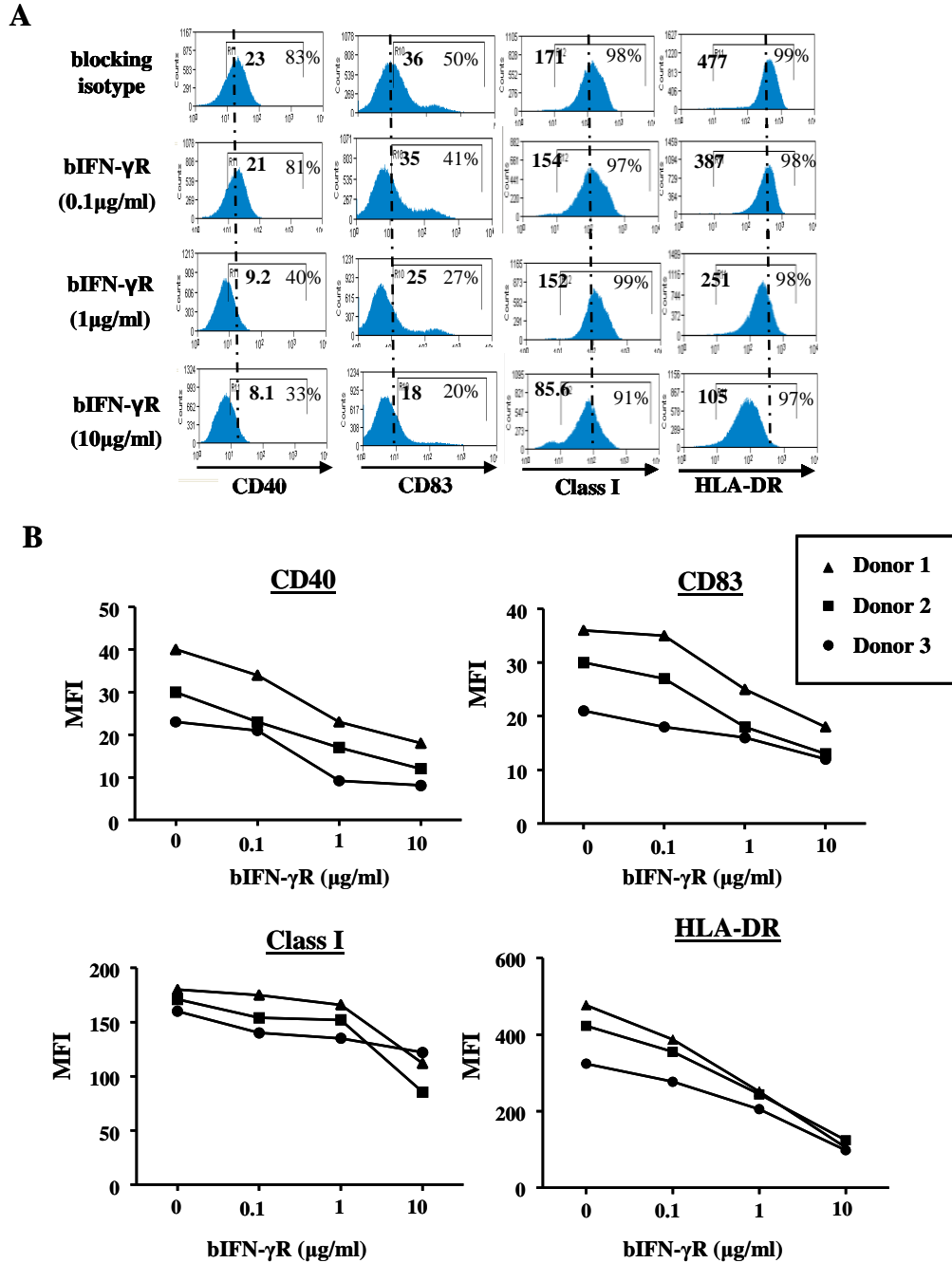
Fig. 6.3 Differentiation of monocytes into Tip-DCs is partially dependent on IFN-γ.

Monocytes were cultured with an isotype blocking antibody (10μg/ml) or indicated concentrations of anti-IFN-γ receptor blocking antibodies (bIFN-γR) for 1h before adding Supernatant$_{CD8DCLPS}$. Cells were stained for CD40, CD83, Class I and HLA-DR. Numbers on right represent percentages of positive cells. Bold numbers represent mean fluorescence intensity (MFI). (A) Results are representative of three independent experiments. (B) MFI results of molecules on Tip-DCs of 3 independent donors with indicated amounts of bIFN-γR.
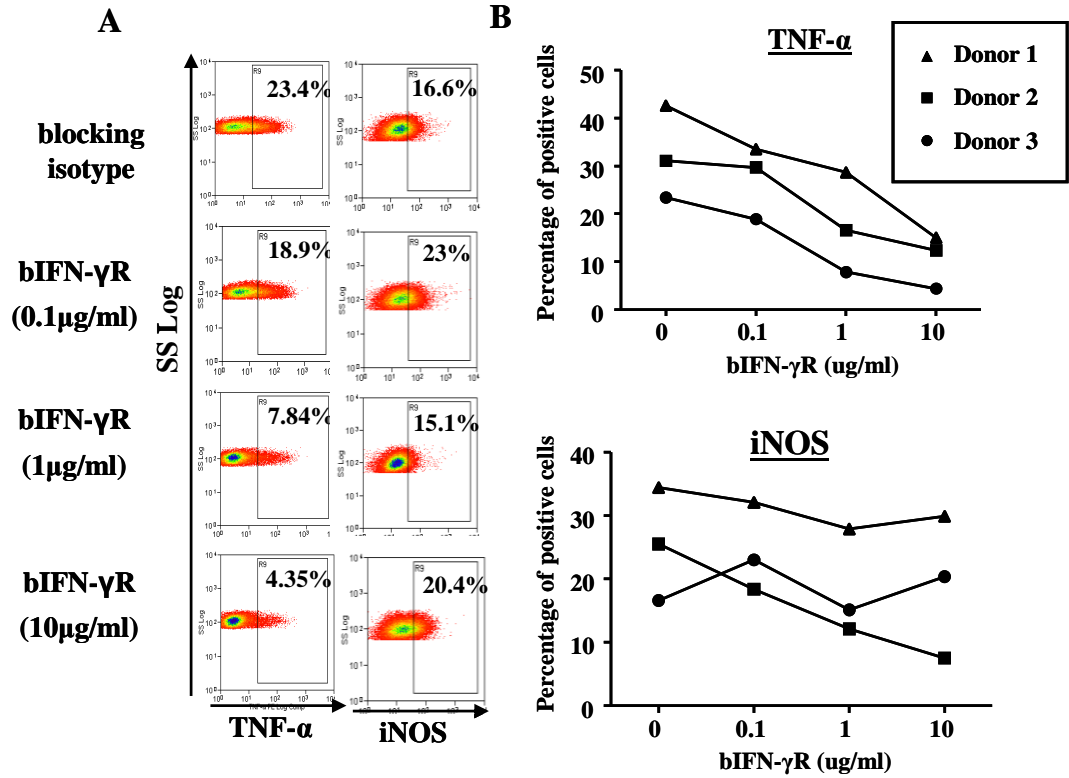
Fig. 6.4 Differentiation of monocytes into Tip-DCs is partially dependent on IFN-γ.

Monocytes were cultured with an isotype blocking antibody (10μg/ml) or indicated concentrations of anti-IFN-γ receptor blocking antibodies (bIFN-γR) for 1h before adding Supernatant$_{CD8DCLPS}$. Cells were stained for intracellular TNF-α and iNOS by flow cytometry. Numbers represent percentages of positive cells. (A) Results are representative of one out of three independent experiments. (B) Percentage of TNF-α and iNOS expressed by Tip-DCs of 3 independent donors with indicated amounts of bIFN-γR.

6.2.3 Monocytes differentiated with recombinant human IFN-γ displayed morphology distinct from Tip-DCs

We next determined whether IFN-γ alone was sufficient to differentiate monocytes into Tip-DCs. To this end, we added 500 U/ml of recombinant human IFN-γ to monocytes for 48 h and analyzed their morphology by H&E staining. We found that monocytes differentiated with IFN-γ alone formed less extensive dendrites (Fig. 6.5A) compared to Tip-DCs which were differentiated from monocytes with Supernatant$_{CD8DCLPS}$ (Fig. 6.5B). In addition, monocytes differentiated with IFN-γ exhibited vacuoles which were similar to activated M-CSF macrophages (Fig. 4.1J) and these structures were not seen in Tip-DCs (Fig. 6.5B). Hence, these results suggest that IFN-γ alone was not sufficient to differentiate monocytes with similar morphologies as Tip-DCs.

A   15 μm   **B**   15 μm

IFN-γ differentiated monocytes   **Supernatant$_{CD8DCLPS}$**
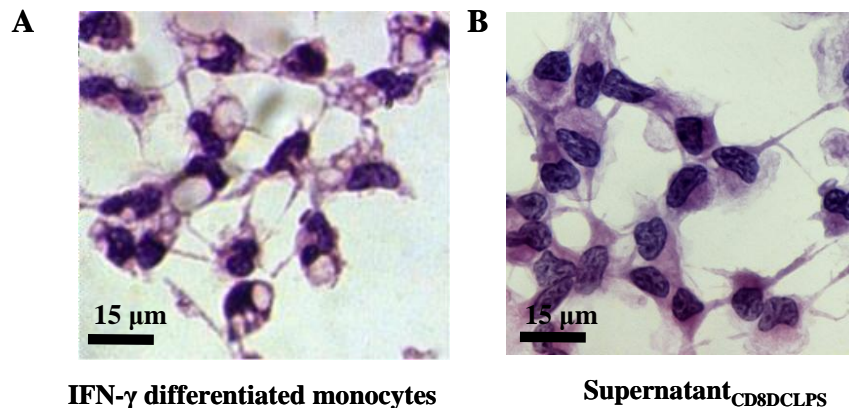
Fig. 6.5 Morphological comparison of IFN-γ differentiated monocytes versus Tip-DCs (Monocytes differentiated with Supernatant$_{CD8DCLPS}$).

Monocytes were differentiated with (A) 500U/ml of *rh*IFN-γ or (B) Supernatant$_{CD8DCLPS}$ for 48h. All cells were subsequently stained with H&E and captured using a Leica DM2000 microscope (Leica Microsystems Pte Ltd., Singapore). Original magnification was ×630 (scale bar: 15μm). Results are representative of four independent experiments.

6.2.4 IFN-γ alone is insufficient to differentiate monocytes into cells with similar expression of TNF-α and iNOS as Tip-DCs

We further investigated if IFN-γ was the sole contributing factor for the expression of TNF-α and iNOS in Tip-DCs. To study this, we added increasing amounts of *rh*IFN-γ to monocytes and cultured them for 48 h before harvesting these cells for intracellular cytokine analysis by flow cytometry. We found that these differentiated monocytes expressed a dose dependent increase in both TNF-α and iNOS (Fig. 6.6A and B) when increasing amounts of *rh*IFN-γ was added to the cultures. However, the expression levels of TNF-α and iNOS by monocytes differentiated with *rh*IFN-γ alone was still significantly lesser than those expressed by Tip-DCs, even at saturating levels (5000 U/ml) of IFN-γ (Fig. 6.6A and B). These results further indicate that while the expression of TNF-α and iNOS is dependent on the presence of IFN-γ, it is not the sole contributing factor in the CD8-DCcytokine milieu for the high expression levels of TNF-α and iNOS observed in Tip-DCs.
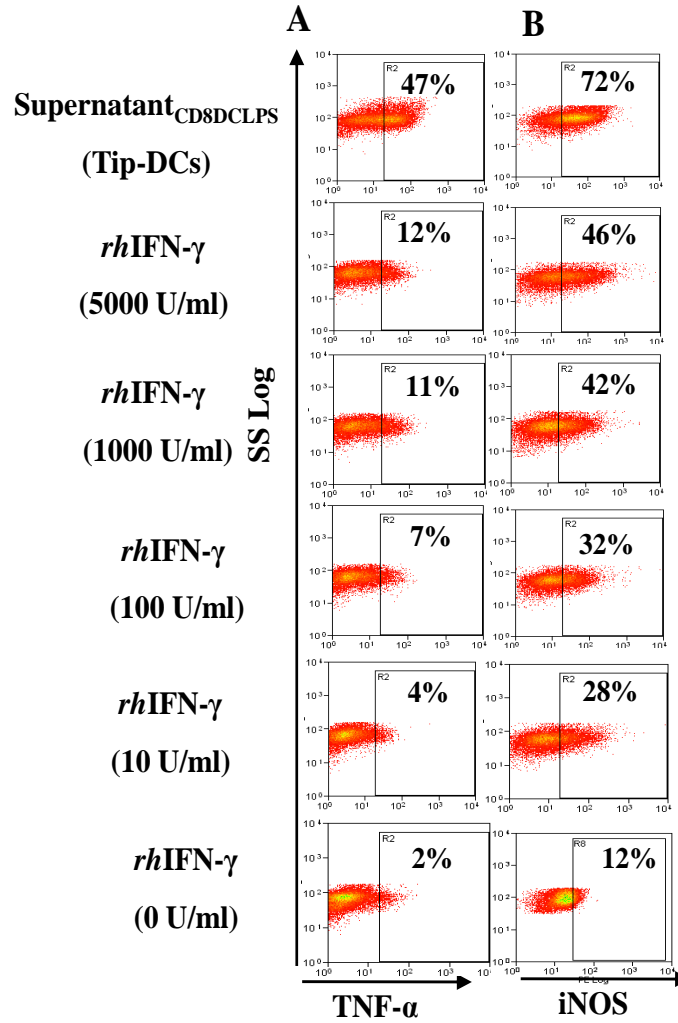
Fig. 6.6 IFN-γ alone is insufficient to differentiate monocytes into cells that express similar amounts of TNF-α and iNOS as Tip-DCs.

Monocytes were cultured with Supernatant$_{CD8DCLPS}$ or indicated amounts of *rh*IFN-γ for 48 h. Cells were harvested, fixed and stained intracellularly for TNF-α and iNOS Numbers represent percentages of positive cells. Results are representative of three independent experiments.

### 6.2.5 IFN-γ alone is insufficient to differentiate monocytes into cells with similar priming ability as Tip-DCs

We also analyzed if IFN-γ was important during the differentiation process to allow differentiated monocytes to acquire T-cell priming abilities. To this end, we treated monocytes with 10 μg/ml of IFN-γ receptor or relevant isotype blocking antibodies before culture with Supernatant$_{CD8DCLPS}$ for 48 h. In addition, we also wanted to determine if IFN-γ alone was enough to differentiate monocytes to prime naive T-cells for proliferation. Hence, monocytes were differentiated with increasing concentrations of *rh*IFN-γ for 48 h. These differentiated monocytes were subsequently studied for their priming ability by using them as APCs to stimulate naive CD4 T-cells for 6 days. The proliferation of naive CD4 T-cells was then quantified by ^{3}H-thymidine incorporation. We found that compared to untreated monocytes, monocytes differentiated with *rh*IFN-γ induced the proliferation of naive CD4 T-cells in a dose dependent manner (Fig. 6.7). However, the level of naive CD4 T-cell proliferation was still significantly lower than those induced by Tip-DCs even at the highest dose of *rh*IFN-γ (1000 U/ml) (Fig 6.7). In addition, blocking the IFN-γ receptor on monocytes before exposing them to Supernatant$_{CD8DCLPS}$ significantly reduced but did not abolish the proliferation of naive CD4 T-cells (Fig. 6.7). Overall, these results indicate that although the presence of IFN-γ during the differentiation of monocytes may be implicated for their subsequent ability to prime naive T-cells, it is not the only factor involved in differentiating monocytes into cells with similar priming ability as Tip-DCs.
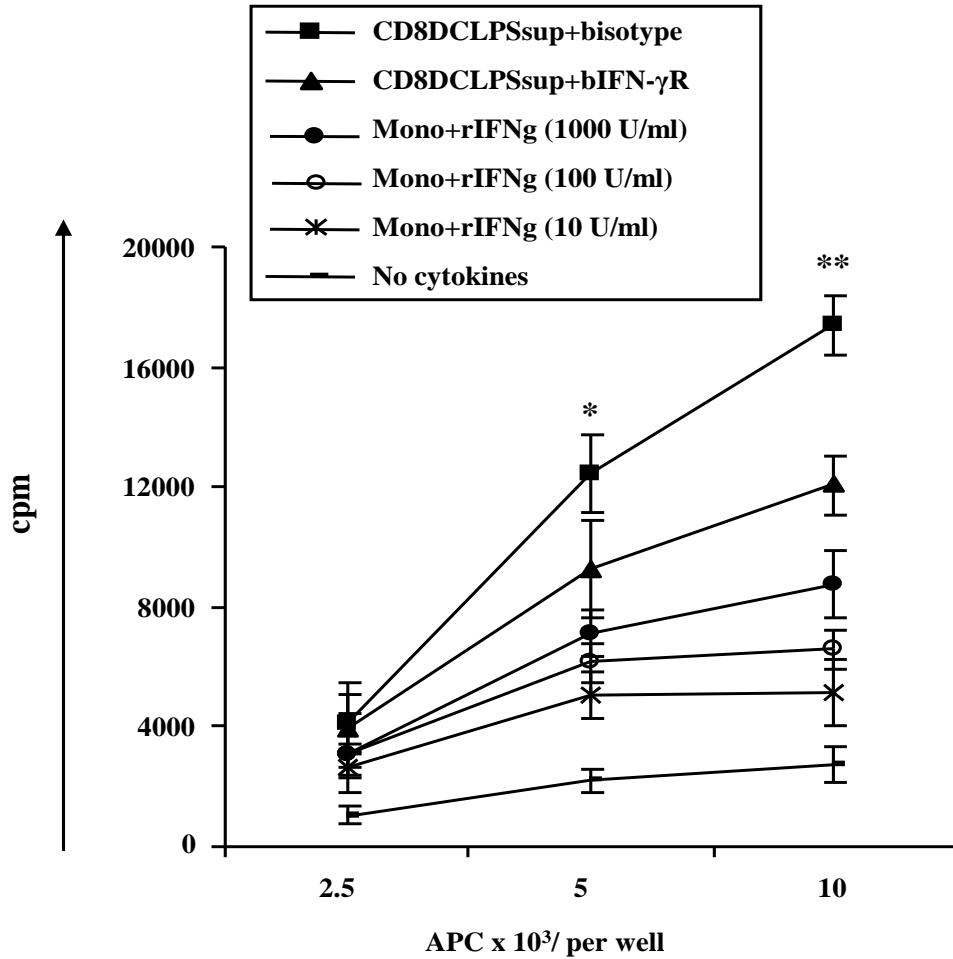
Fig. 6.7 IFN-γ alone is insufficient to differentiate monocytes into cells with similar priming abilities as Tip-DCs.

Monocytes were incubated with isotype blocking (bisotype) or anti-IFN-γR blocking antibodies (bIFN-γR) (both 10μg/ml) before differentiation with Supernatant$_{CD8DCLPS}$ (CD8DCLPSsup). Their ability to induce proliferation of naive CD4 T-cells was compared to monocytes that have been differentiated in increasing concentrations of rhIFN-γ. Proliferation of naïve CD4 T-cells were quantified via ^{3}H-thymidine incorporation. **p<0.005 comparing CD8DCLPSsup+bisotype versus rest of the groups. *p<0.05 comparing CD8DCLPSsup+bisotype versus rest of groups except CD8DCLPSsup+bIFN-γR using one-way ANOVA. Results are representative of three independent experiments.

6.2.6 IFN-γ alone is insufficient to generate viable monocyte-derived cells

While analyzing the phenotype of IFN-γ differentiated monocytes by flow cytometry, we noticed that the viability of these cells were significantly lower, as seen through the forward and side scatter plots, after stimulation with LPS. We found that with the exception of untreated monocytes, monocytes that were differentiated with co-culture supernatants maintained a relatively high viability even after stimulation with LPS (Fig. 6.8A). On the other hand, monocytes that were differentiated with *rh*IFN-γ had a lesser percentage of viable cells after stimulation with LPS (Fig. 6.8B) as compared to cells that have been differentiated with co-culture supernatants (Fig. 6.8A).

To further investigate this, we determined the viability of these differentiated monocytes by comparing their viability before and after 6 h of LPS stimulation using the LIVE/DEAD® fixable violet dead cell stain. Cells that are viable will stain negative for this dye and results were analyzed by flow cytometry. We found that monocytes that were differentiated with co-culture supernatants had no significant changes in viability before and after stimulation with LPS (Fig. 6.9). In contrast, monocytes that were untreated or differentiated with *rh*IFN-γ had a drastic reduction in viability after stimulation with LPS (Fig. 6.9). In addition, the decrease in viability occurred regardless of the amount of *rh*IFN-γ used. Hence, these results suggest that IFN-γ alone is insufficient to generate monocyte-derived cells that remain viable after stimulation with LPS. This also suggests that the supernatants from co-cultures contain an important survival factor that allows cells to remain viable after LPS stimulation.
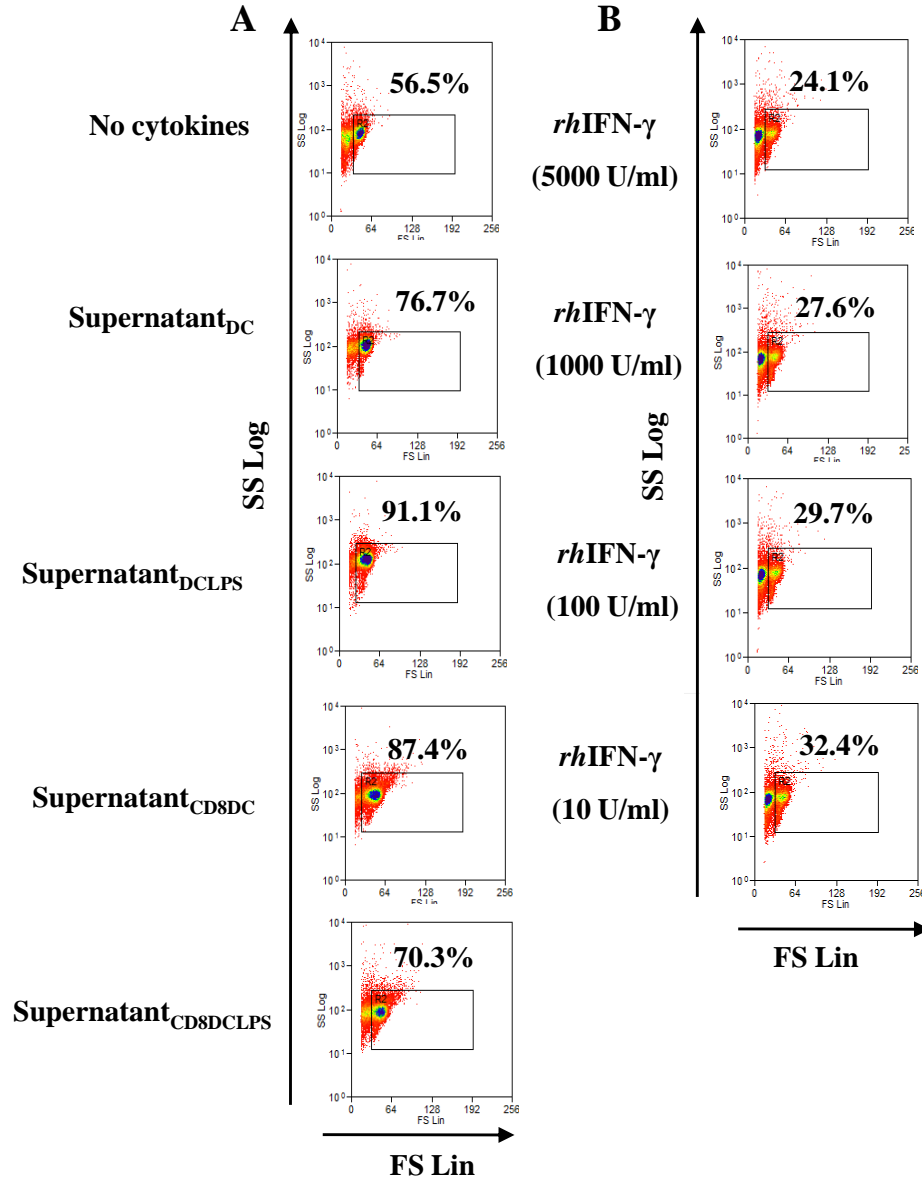
Fig. 6.8   Differentiation of monocytes with IFN-γ alone reduces their viability compared to monocytes differentiated with cultured supernatants.

Monocytes were differentiated with the (A) indicated supernatants or (B) increasing doses of *rh*IFN-γ for 48 h and stimulated with LPS for another 6 h. Cells were analyzed by flow cytometry and live cells were gated using forward and side scatter plots. Numbers represent percentages of live cells. Results are representative of three independent experiments.
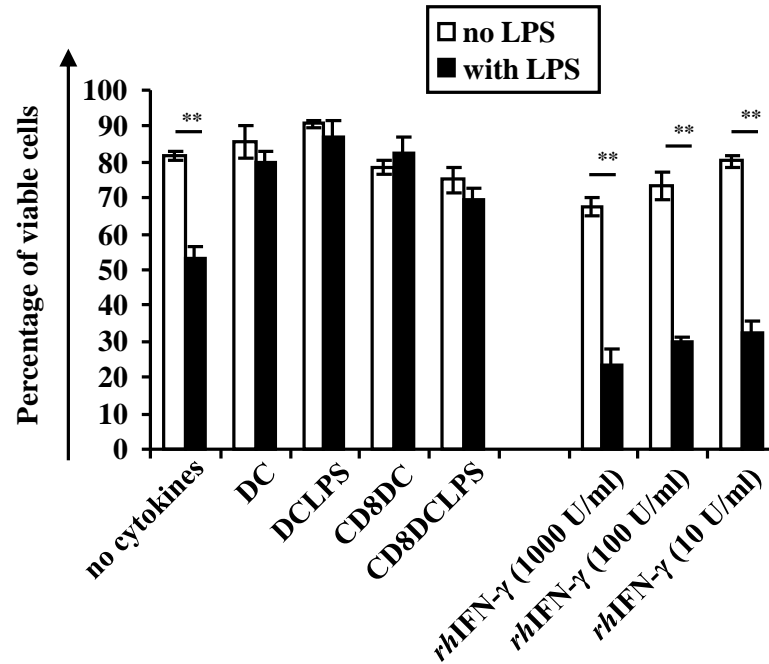
Fig. 6.9 Differentiation of monocytes with IFN-γ reduces their viability compared to monocytes differentiated with cultured supernatants.

Viability of differentiated monocytes from 3 donors (mean ± SD) with (black bars) or without (white bars) 6h LPS stimulation assessed by cells negative for the LIVE/DEAD® fixable violet dead cell stain through flow cytometry. **p<0.005 using a two-tailed unpaired student's t-test.

6.3   Discussion

We found previously that supernatants of CD8 T-cell-DC cytokine milieus contained increased amounts of TNF-α and IFN-γ compared to DC-only derived cytokine milieus. This led us to speculate that these two factors could possibly be the key mediators in the differentiation of monocytes into Tip-DCs. By using blocking antibodies against these cytokines, we found that in contrast to TNF-α which did not play a significant role in the differentiation of monocytes into Tip-DCs, blocking IFN-γ in CD8 T-cell-DC cytokine milieus resulted in a dose dependent decrease in the expression of CD40, HLA-DR, CD83 and TNF-α on Tip-DCs, as well as their ability to induce allogeneic T-cell proliferation in a MLR. However, we also found that IFN-γ alone was insufficient to generate Tip-DCs as monocytes differentiated with saturating levels of recombinant IFN-γ were still insufficient to generate viable DCs that can prime and express as high levels of TNF-α and iNOS as Tip-DCs. This suggests that while IFN-γ may be a key factor in the CD8 T-cell-DC cytokine milieus for differentiation of monocytes into Tip-DCs, it is ultimately not the only factor involved in their differentiation.

We observed that blocking TNF-α in our culture supernatants did not result in a significant decrease in the phenotype of Tip-DCs. This was surprising, as previous studies by Chomarat *et al.* (2003) and Iwamoto *et al.* (2007) have both shown that TNF-α drives the differentiation of monocytes into DCs. However, these discrepancies could be attributed to the differences in experimental setups. While studies from Chomarat's and

Iwamoto's groups studied the role of TNF-α on monocyte differentiation by adding recombinant TNF-α and GM-CSF to monocytes, our studies employed blocking antibodies against TNF-α receptor on monocytes before culturing them with CD8 T-cell-DC derived cytokine milieus. Since the CD8 T-cell-DC cytokine milieus have been shown to contain other cytokines, it is possible that these cytokines may compensate for the lack of TNF-α during the process of monocyte differentiation.

In contrast to the unchanged effect seen when blocking TNF-α, blocking IFN-γ in our culture supernatants resulted in a decrease in the expression levels of CD40, HLA-DR and CD83 on Tip-DCs, as well as their ability to express TNF-α and prime naive CD4 T-cells for proliferation. This is in line with a very recent study that showed the dependence of IFN-γ on Tip-DC differentiation performed in mice (Bosschaerts et al., 2010). In this study, they showed that IFN-γ^{-/-} mice that had been infected with *T.brucei* had significantly lower percentages of TNF-α and iNOS producing cells within the CD11b^{+}Ly6C^{+}CD11c^{+} inflammatory DCs in the liver. In addition, the expression levels of co-stimulatory molecules CD80/CD86 and MHC class II on these inflammatory DCs were also reduced in IFN-γ^{-/-} mice compared to their WT counterparts. These findings suggest that not only is IFN-γ important for the differentiation of Tip-DCs in mice, but it is also a significant factor for human monocytes to acquire the phenotypic and functional characteristics of Tip-DCs. Interestingly, the inability of monocytes to express more TNF-α and iNOS after differentiation with a certain level of IFN-γ suggests that a saturating effect occurs, thereby explaining why monocytes cultured with

Supernatant$_{CD8DC}$ or Supernatant$_{CD8DCLPS}$ could both differentiate into Tip-DCs despite the latter having higher amounts of IFN-γ and other cytokines.

While our current findings accord with Bosschaerts *et al.* 's study (2010), we found that previous groups have demonstrated that IFN-γ skews monocytes towards macrophages (Delneste et al., 2003) or tolerogenic DCs (Eljaafari et al., 2009) instead. Nevertheless, their results also revealed that IFN-γ increases the expression of CD80, CD86 and CD40 as well as down-regulation of phagocytosis which are consistent with characteristics of Tip-DCs observed in our model. These discrepancies could be attributed to differences in experimental models as both studies employed the use of recombinant IFN-γ, GM-CSF and IL-4 to differentiate monocytes in contrast to our usage of a cytokine milieu generated from cultured CD8 T-cells and DCs. Moreover, IFN-γ is not the only factor involved in Tip-DC differentiation, as monocytes differentiated with saturating levels of IFN-γ were still insufficient to generate viable DCs that can prime and express as high levels of TNF-α and iNOS as Tip-DCs. Notably, the decreased viability of monocytes differentiated with IFN-γ was not surprising, as previous studies have shown that IFN-γ induces the apoptosis of myeloid cells upon stimulation with LPS especially in the absence of other deactivating cytokines such as IL-4, IL-10 and TGF-ß (Bingisser et al., 1996; Chawla-Sarkar et al., 2003). Hence, we feel that other factors in the CD8-DC supernatants may act in concert with IFN-γ to induce the differentiation of Tip-DCs. In particular, IL-12p40 homodimer has been shown to increase the expression of TNF-α and iNOS in myeloid cells (Jana et al., 2003; Pahan et al., 2001) while GM-CSF may be an

important factor for the maintenance of viability in monocyte derived cells (Kinoshita et al., 1995; Witmer-Pack et al., 1987; Woltman et al., 2003).

IFN-γ, released by natural killer (NK) cells and activated T cells, is crucial for innate immune responses (Boehm et al., 1997). Despite the ability of both NK cells and activated T cells to secrete IFN-γ, CD4 Th1 cells were shown to skew monocytes into tolerogenic DCs (Mariotti et al., 2008) while the principal factor for NK induced DC differentiation was CD40L (Zhang et al., 2007). In contrast, we show that IFN-γ in our CD8 T-cell mediated cytokine milieu was an important factor for monocytes to differentiate into cells with Tip-DC characteristics. The role of CD8 T-cells observed in our findings can also be extrapolated to human psoriasis studies, where reduced infiltration of activated memory CD8 T-cells using alefacept showed decreased DC content, inflammation and tissue expression of IFN-γ and iNOS (Chamian et al., 2005). Collectively, our results highlight an important role for CD8 T-cells in mediating the differentiation of monocytes mainly through the production of IFN-γ which in turns supports Th1 responses through the differentiation of Tip-DCs.

CHAPTER 7: Differentiation of Tip-DCs in an *in vivo* mouse model of contact hypersensitivity

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7.1 Introduction

In the past few chapters, we found that human CD8 T-cells could prime DCs for IL-12p70 production *in vitro* and could further support Th1 responses by creating a cytokine milieu that allows the differentiation of monocytes into Th1-inducing Tip-DCs. We also found that IFN-γ was an important factor for the differentiation of Tip-DCs. However, it remains unknown if CD8 T-cells and IFN-γ are important for the differentiation of such Tip-DCs *in vivo*. To address this, we used a mouse model of contact hypersensitivity (CHS) to study the role of CD8 T-cells in the induction of Tip-DC differentiation. CD8 T-cells have been extensively shown to be the principal mediators of CHS responses (Akiba et al., 2002; Bouloc et al., 1998; Bour et al., 1995; Gocinski and Tigelaar, 1990; Xu et al., 1996). Studies have shown that their role in CHS involves the induction of keratinocyte apoptosis and the production of IFN-γ and IL-17 which contributes to further exacerbation of the inflammatory response (He et al., 2006; He et al., 2009). In addition, the presence of granulocytes such as neutrophils and monocytes has also been documented to play a significant role in the manifestation of clinical symptoms during the elicitation phase (Tuckermann et al., 2007). To date, Tip-DCs have been documented to play a pathogenic role in skin diseases such as psoriasis (Lowes et al., 2005; Wang et

al., 2006a). However, it remains unknown if Tip-DCs contribute to CHS responses and whether CD8 T-cells are the major contributors to their differentiation.

To further address the role of CD8 T-cells and Tip-DCs with CHS Th1 responses, we compared the CHS response using a contact allergen 2,4-dinitrochlorobenzene (DNCB) that induces the Th1-type allergic contact dermatitis, with another allergen, trimellitic anhydride (TMA), which preferentially provokes Th2-inducing diseases such as respiratory and skin hypersensitivity (Bernstein et al., 1982). Previous work has documented the triggering of selective immune responses in response to these two allergens (Ban et al., 2006; Dearman and Kimber, 1991). Dearman and colleagues (Dearman et al., 1995) have observed that draining lymph node cells obtained from mice that had repeated topical exposure to DNCB produced high levels of Th1 cytokine IFN-γ and low levels of Th2 cytokines IL-4 and IL-10, while the opposite was true for TMA. These distinct cytokine profiles thus reflect the allergies elicited upon exposure to each chemical and allow us to study the role of Tip-DCs in these two different settings.

In this chapter, we investigated the role of CD8 T-cells and Tip-DCs *in vivo* by first identifying the presence of Tip-DCs in the elicitation phase of the CHS response in mice that have been sensitized and challenged with either the Th1 inducing allergen, DNCB, or the Th2 inducing allergen, TMA. We next analyzed whether CD8 T-cells were involved in the differentiation of Tip-DCs during CHS by selective depletion of CD8 T-cells using monoclonal antibodies *in vivo* as well as performing CHS experiments in mice that have defective MHC-class I presentation through deficiency of beta-2-microglobulin ($\beta2M^{-/-}$).

Finally, we elucidated the role of IFN-γ for the differentiation of Tip-DCs using IFN-γ-/- mice as well as confirmed the origin of Tip-DCs to be derived from classical monocytes with the usage of CCR2$^{-/-}$ mice.

7.2 Results

7.2.1 DNCB induced significant inflammation in ears of mice compared to TMA

To investigate the differential ability of DNCB versus TMA to induce inflammatory responses, mice were subjected to CHS by first sensitizing them to chemical allergens, DNCB (1% w/v) or TMA (10% w/v), or the vehicle (1:4 olive oil: acetone) by topical application on shaved dorsal skin. Five days later, they were challenged with the same chemical on both the dorsal and ventral sides of the ears and observed for the inflammatory response after 18 hours. Severe hyperemia and reddening (erythema) was observed in ears of DNCB-treated mice (Fig. 7.1A). In contrast to DNCB-treated mice, TMA-treated mice developed only slight erythema while vehicle-treated mice did not show any significant signs of inflammation. Measurement of ear thickness using an engineer's micrometer revealed that DNCB-treated mice had a significant increase (**$p<0.005$) in ear swelling compared to vehicle-treated mice (Fig. 7.1B). In contrast, the increase in ear thickness of TMA-treated mice was not significantly different from vehicle-treated mice. Furthermore, the increase in ear thickness of DNCB-treated mice was also significantly larger compared to TMA-treated mice (**$p<0.005$). Finally, H&E staining of ear sections from treated mice indicated that DNCB-treated mice developed strong inflammatory reactions at the challenged sites which were characterized by marked spongiosis (edema) and extensive cell infiltration in the swollen dermis (Fig. 7.1C). In contrast, such reactions were substantially attenuated in the ears of TMA-treated mice.

A

vehicle   TMA   DNCB

B

C

vehicle

TMA
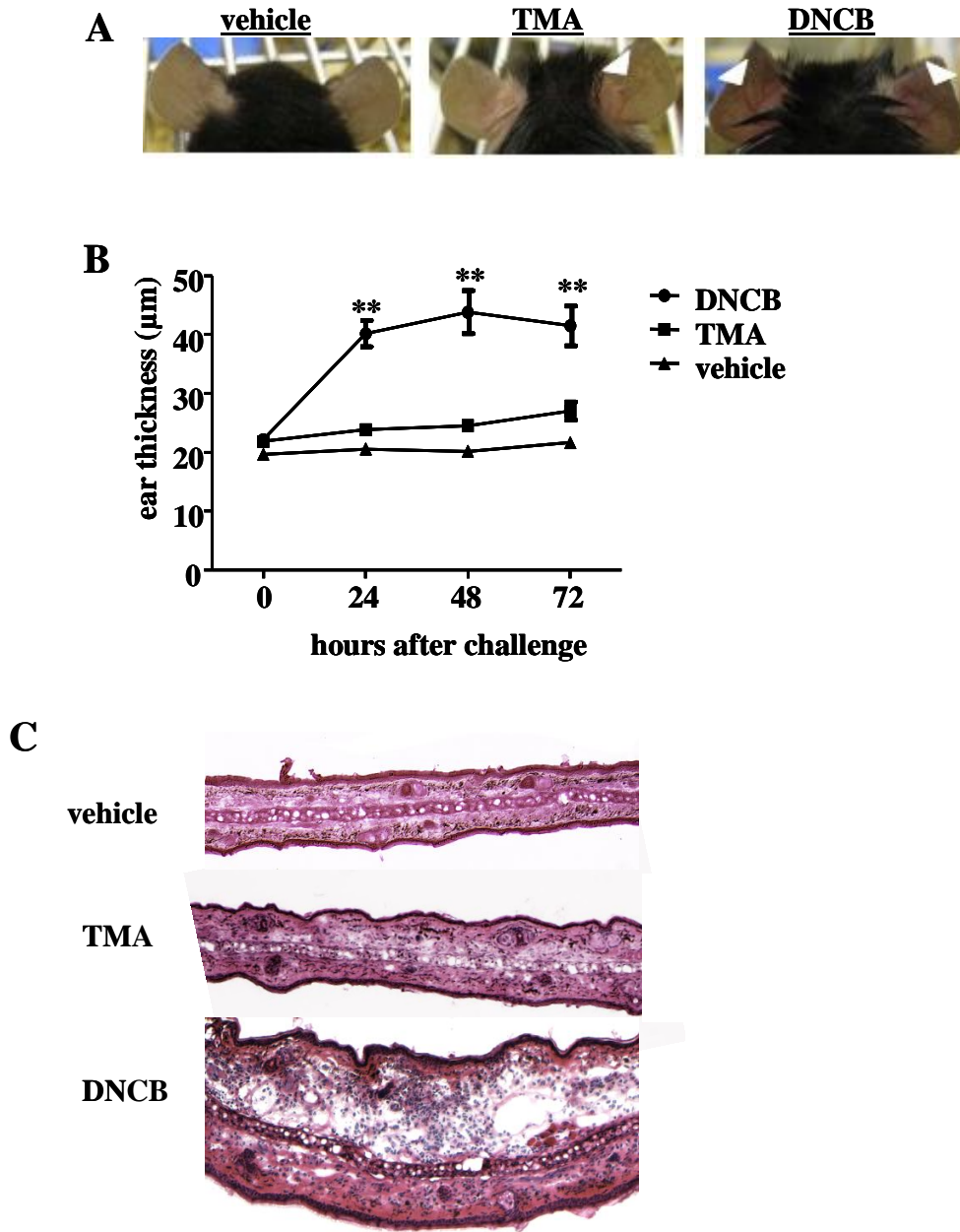
DNCB

Fig 7.1 DNCB elicitation, but not TMA, resulted in significant CHS responses.

WT mice were sensitized and challenged as described with DNCB, TMA or vehicle. (A) Photographs of ears of mice that have been treated with indicated chemical or vehicle. White arrows indicate the presence of dilated blood vessels and erythema (B) Ear thickness was measured after challenge at indicated time points using an engineer's micrometer (n=6 mice). **p<0.005 using one-way ANOVA (C) Ear skin tissues were harvested after challenge and stained with H&E. Results are representative of three experiments conducted

To ensure that the attenuated CHS response in TMA-treated, compared to DNCB-treated, mice was not due to the absence of immune priming or unresponsiveness to the chemical, the draining lymph nodes were examined. Auricular draining lymph nodes were first identified by an intradermal injection of a staining dye, Evan's blue, into the ears. Five minutes later, the cervical section was exposed and the draining lymph node was identified as the lymph node stained with Evan's blue, namely at the superficial cervical lymph node (Fig 7.2A). To study the CHS response, these auricular draining lymph nodes were excised 24, 48 and 72 hours after challenge with the chemical and cell counting was performed. DNCB-treated and TMA-treated mice showed no significant differences in cell numbers in the lymph nodes at all time points (Fig. 7.2B), suggesting that the attenuated inflammatory response seen in the ears of TMA-treated mice was not due to the absence of immune priming.
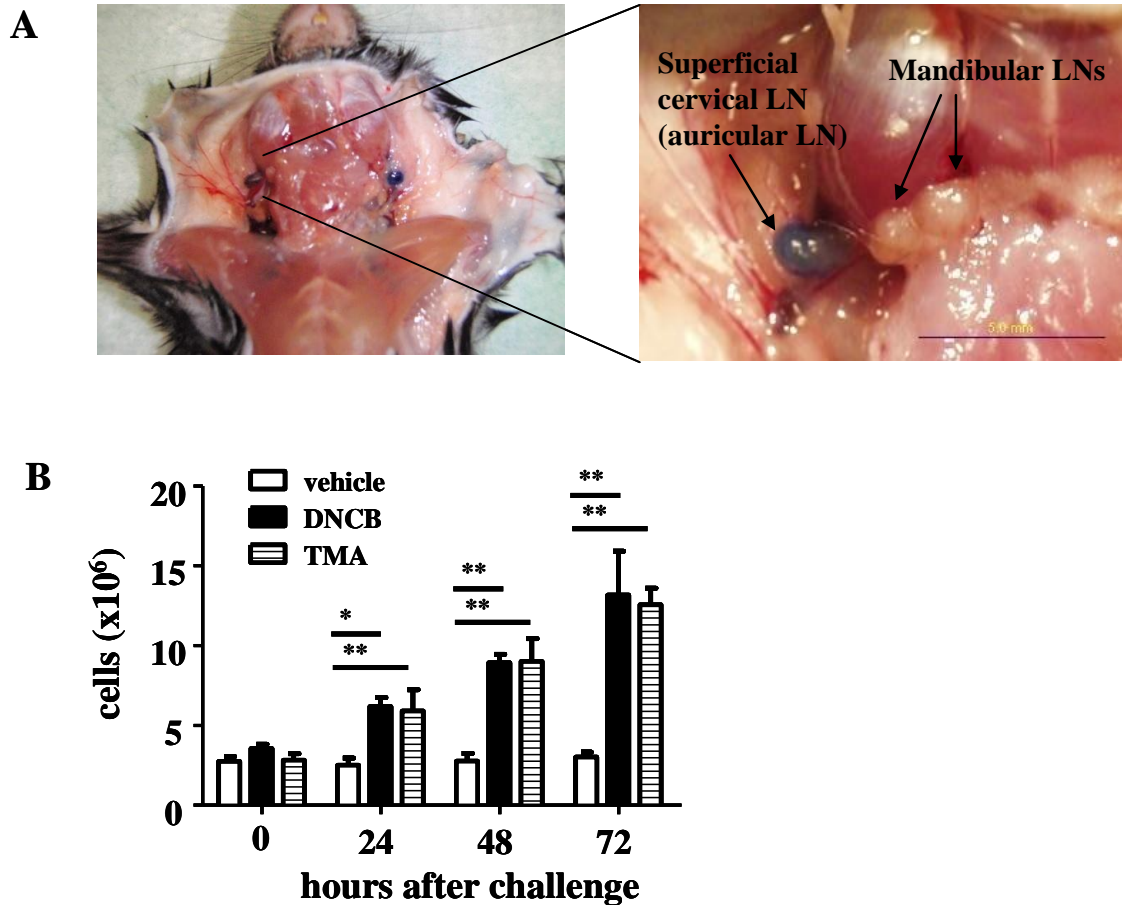
Fig 7.2 DNCB and TMA elicitation showed no difference in the activation of lymph node cells.

(A) To identify the auricular draining lymph node, Evan's blue was injected intradermally into each ear of the mice, euthanized after 5 min and the thoracic cavity exposed. The auricular draining lymph node is identified as the lymph node that have stained blue as shown in the photographs. (B) Draining auricular lymph nodes were harvested at indicated time points after challenge and cell counting was performed. Values are shown as means ± SEM of six mice per group. *p< 0.05, **p<0.001 analyzed using one-way ANOVA.

7.2.2 DNCB induced less Th2-type cytokines compared to TMA in sensitized mice

To further understand the increased inflammatory response seen in DNCB-treated compared with TMA-treated mice, cytokines in the ears and auricular lymph nodes were analyzed. Treated mice were subjected to CHS and ear tissue was harvested 24, 48 and 72 hours after challenge. The ear tissue was then homogenized and supernatants collected for cytokine analysis by ELISA. We found that DNCB-treated mice showed a significant increase (**$p<0.005$) in the presence of GM-CSF 48 and 72 hours after challenge compared to TMA-treated mice (Fig. 7.3A). However, we were unable to detect any significant changes in the levels of IFN-γ between DNCB-treated and TMA-treated mice (Fig. 7.3B). This suggests that the increased inflammatory response seen in DNCB-treated, but not TMA-treated mice, was not solely due to IFN-γ. We next looked for the presence of Th2 cytokines which may potentially antagonize the functions of IFN-γ. We found a significant increase (*$p<0.05$) in the levels of IL-5 in ears that have been excised 72 hours after challenge (Fig. 7.3C). Notably, we found a significant increase (*$p<0.05$) in the levels of IL-10 in ears 24 and 72 hours after challenge (Fig. 7.3D). In addition, the increase in IL-13 levels in TMA-treated mice as compared to DNCB-treated mice was statistically significant at all time points (**$p<0.005$) (Fig. 7.3E). These results demonstrate that the elicitation of CHS using TMA was associated with increased Th2 cytokines such as IL-10 and IL-13 which may have potential attenuation in the inflammatory response
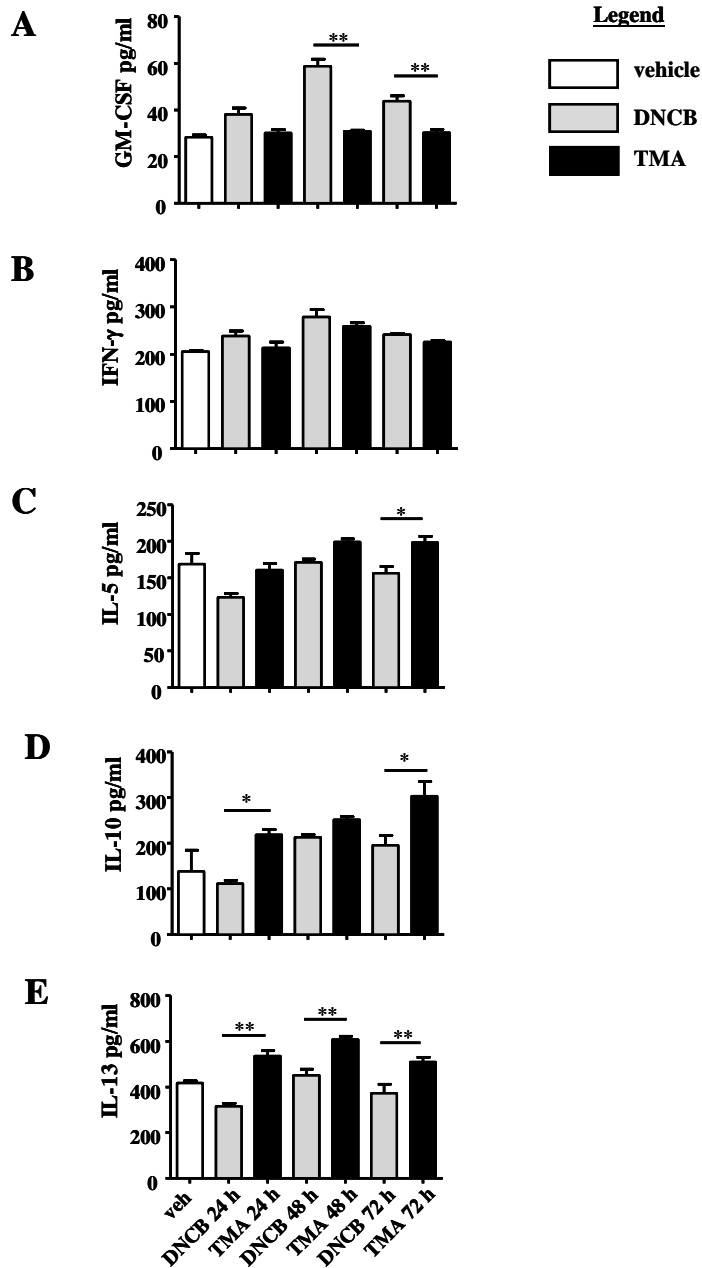
Fig 7.3 TMA-treated mice showed an increase presence of Th2 cytokines in the challenge site.

WT mice were treated with DNCB, TMA or vehicle as previously described. Ears were excised from mice at indicated time points after challenge, homogenized and supernatants collected. Cytokines (A) GM-CSF (B) IFN-γ (C) IL-5 (D) IL-10 and (E) IL-13 were analyzed via ELISA. Values are shown as means ± SEM of five mice per group. *p< 0.05, **p<0.001 analyzed using one-way ANOVA.

We further confirmed the Th2 cytokine profile of TMA-treated mice by analyzing the cells from the auricular lymph nodes. Lymph nodes were excised 72 hours after challenge and were re-stimulated with anti-CD3 and anti-CD28 *in vitro*. After an overnight culture, supernatants were harvested and cytokines were analyzed using ELISA. Consistent with the trend of IFN-γ detected in ear tissues, DNCB-treated and TMA-treated mice showed no significant differences in the production of IFN-γ (Fig. 7.4A). However, we discovered a significant increase (**p<0.005) in the quantities of IL-17 in the lymph nodes of DNCB-treated mice compared to TMA-treated mice (Fig. 7.4B). In particular, we detected substantial levels of IL-4, IL-5, IL-10 and IL-13 (**p<0.005) (Fig. 7.4C-F) in lymph nodes of TMA-treated mice which in contrast, were minimally or not detected in DNCB-treated mice. Hence, these results suggest that DNCB induces a Th1/Th17 response while TMA induces a Th1/Th2 response. In addition, the differential inflammatory responses could be due to the upregulation of Th2-inducing cytokines in TMA-treated, but not DNCB-treated, mice.
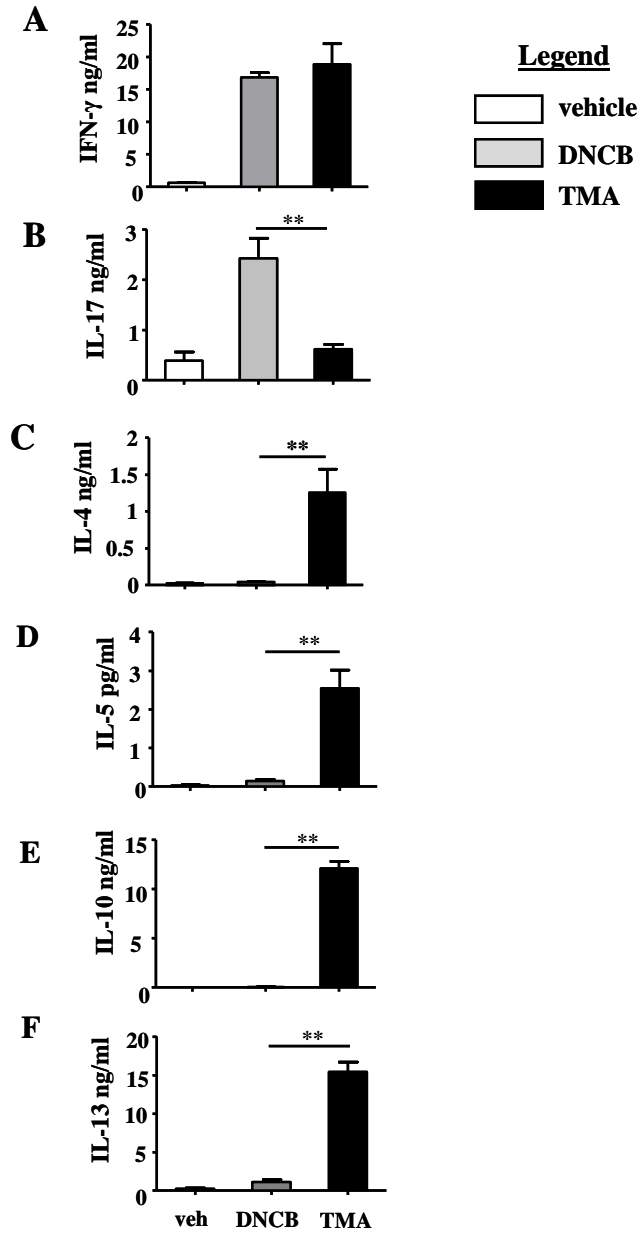
Fig 7.4 TMA-treated mice showed an increase presence of Th2 cytokines in auricular draining lymph nodes.

WT mice were treated with DNCB, TMA or vehicle as previously described. Draining lymph nodes were collected 72 h after challenge and cells were re-stimulated with 1 µg/ml of anti-CD3/anti-CD28 overnight in 48 well plates at a concentration of 2×10^6 cells/ml. Supernatants were harvested and cytokines (A) IFN-γ (B) IL-17 (C) IL-4 (D) IL-5 (E) IL-10 and (F) IL-13 were analyzed via ELISA. Values are shown as means ± SEM of six mice per group. *p< 0.05, **p<0.001 analyzed using one-way ANOVA.

7.2.3 Elicitation of CHS with DNCB resulted in a greater increase in CD45$^+$ immune cells

After determining the cytokine profile of skin tissues that have been exposed to DNCB or TMA, we next investigated the population of cells that have infiltrated into the skin tissue after CHS. Mice were topically sensitized, sacrificed and ears excised 24 hours after challenge. The populations of immune cells were analyzed by flow cytometry according to the materials and methods described in chapter 2.3.3. Briefly, the epidermis was separated from the dermis by incubating the ear tissue in dispase solution. Subsequently, the dermis was digested with Collagenase Type IV and filtered through a piece of 61μm gauze before staining with relevant antibodies for flow cytometry analysis.

Live cells were first gated using the forward and side scatter profile before excluding doublets using the side scatter linear and area profile. Next, viable cells were selected by gating on cells that stained negative for the aqua dead cell stain. This gating strategy was used for all subsequent flow cytometry analysis and is described in Fig. 7.5. As the majority of cells present in the dermal skin suspension are non-immune cells such as fibroblasts and endothelial cells, we stained the skin suspension with CD45, a marker for hematopoietic cells, to determine the percentage of immune cells in the skin. Flow cytometry analysis revealed that TMA-treated mice showed a small significant increase (*$p<0.05$) in the amount of CD45$^+$ cells in the dermis (Fig. 7.6A-B). In contrast to TMA-treated mice, DNCB-treated mice showed a greater significant increase (**$p<0.005$) in the percentage of CD45$^+$ immune cells in the dermis (Fig. 7.6A-B). Staining for infiltrating T-cells into the dermis using the anti-TCR$_{\alpha\beta}$ antibody revealed a significant

increase (*p<0.05) in T-cell numbers only in DNCB-treated mice (Fig. 7.6C). However, we were unable to differentiate the population of T-cells into CD4 or CD8 subsets as staining for these markers was very weak by flow cytometry (data not shown). DNCB-treated mice also displayed a significant increase (**p<0.005) in neutrophil numbers, identified by cells as $CD11b^+Ly6C^{mid}Ly6G^+$, compared to vehicle and TMA-treated mice (Fig. 7.6C). Lastly, monocytes, identified by $CD11b^+ Ly6C^+Ly6G^-$ cells, were increased significantly (**p<0.005) in both TMA-treated and DNCB-treated mice although DNCB-treated mice registered a much higher monocyte cell number compared to mice treated with TMA (Fig. 7.6C). Hence, these results demonstrate that DNCB elicitation on mice ears is accompanied by a greater infiltration of $CD45^+$ immune cells with a significant increase in T-cells, neutrophils and monocyte cell numbers.
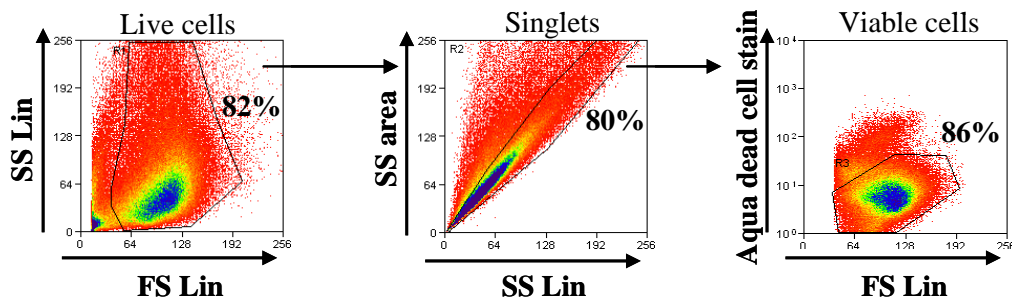
Fig 7.5 Gating strategy employed for analysis of dermal cells by flow cytometry.

WT mice were treated with DNCB, TMA or vehicle as previously described. Ears were excised from mice and digested into single cell suspensions before flow cytometry analysis. Live cells were gated using the forward and side scatter profile and were selected for singlets by gating on cells using the side scatter linear versus side scatter area plot. Finally, viable cells were selected by gating on cells that stain negative for the aqua dead cell stain.
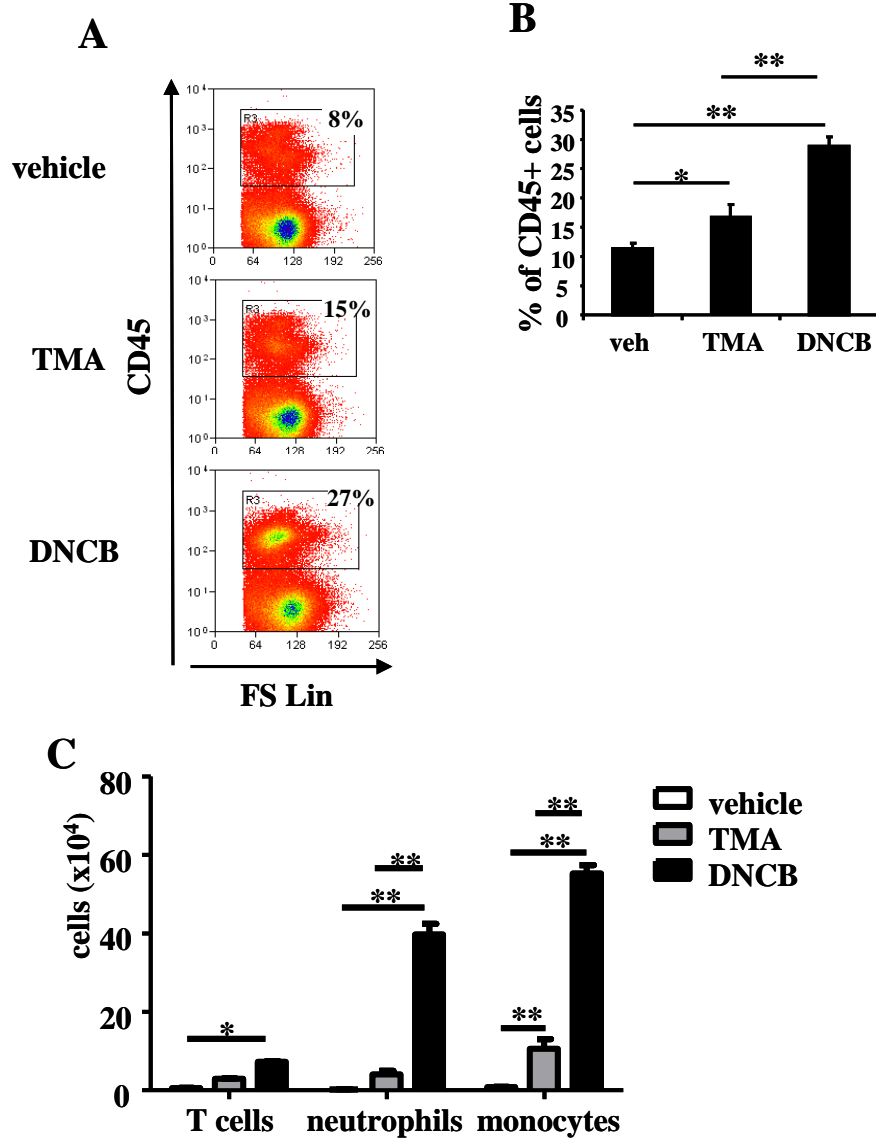
Fig 7.6   DNCB elicitation resulted in a larger significant increase in immune cell infiltration into the challenge site.

WT mice were sensitized and challenged as described. Ear skin tissues were harvested 24 h after challenge and digested according to the materials and methods. (A) Cells were stained with anti-CD45 antibody and analyzed using flow cytometry. Numbers represent percentages of positive cells (B) Statistical analysis of samples. (C) Cells were stained with anti-TCR$_{\alpha\beta}$ to measure T-cells, anti-Ly6G to measure neutrophils and anti-Ly6C excluding Ly6G$^+$ cells to measure monocytes. Graph represents the number of cells in challenge site. Values are shown as means ± SEM of six mice per group. *p< 0.05, **p<0.005 analyzed using one-way ANOVA.

7.2.4 DNCB elicitation resulted in the increased presence of Tip-DCs in the skin dermis

We next analyzed the dermis from treated mice for Tip-DCs by first examining for $CD45^+MHC$ class II^+ ($MHCII^+$) cells that express TNF-α and iNOS by flow cytometry. In contrast to TMA-treated mice which did not display any significant differences in $CD45^+MHCII^+$ cells, we found a significant increase (*p<0.005) in the percentage of $CD45^+MHCII^+$ cells in the dermis of DNCB-treated mice as compared to mice treated with vehicle alone (Fig. 7.7A-B). In addition, this increase in $CD45^+MHCII^+$ cells in DNCB-treated mice was also statistically significant when compared to TMA-treated mice (Fig. 7.7B). When gated on these $CD45^+MHCII^+$ cells, we found a slight significant increase (*p<0.05) in iNOS-expressing cells in TMA-treated compared to vehicle-treated mice (Fig. 7.7C-D). Notably, we found an approximate 4-fold increase in iNOS-expressing cells in DNCB-treated compared to vehicle-treated mice (Fig. 7.7B) and this increase was statistically significant (**p<0.005) compared to both vehicle and TMA-treated mice (Fig. 7.7D). We also determined the expression of TNF-α among the MHC II expressing cells and only found a significant increase (**p<0.005) in TNF-α-expressing cells among DNCB-treated mice compared to vehicle-treated mice (Fig. 7.7E-F). In addition, this increase in DNCB-treated mice was also statistically significant (*p<0.05) when compared to TMA-treated mice (Fig. 7.7F). Notably, the TNF-α and iNOS expressing cells among the $CD45^+MHCII^+$ population all expressed CD11b (Fig. 7.7C, E), suggesting that they were of myeloid origin. Hence, these results demonstrate that only CHS elicitation with DNCB could result in the increase in $CD45^+MHCII^+$ cells

and that the increased expression of both TNF-α and iNOS among these cells was also only associated with DNCB treatment.
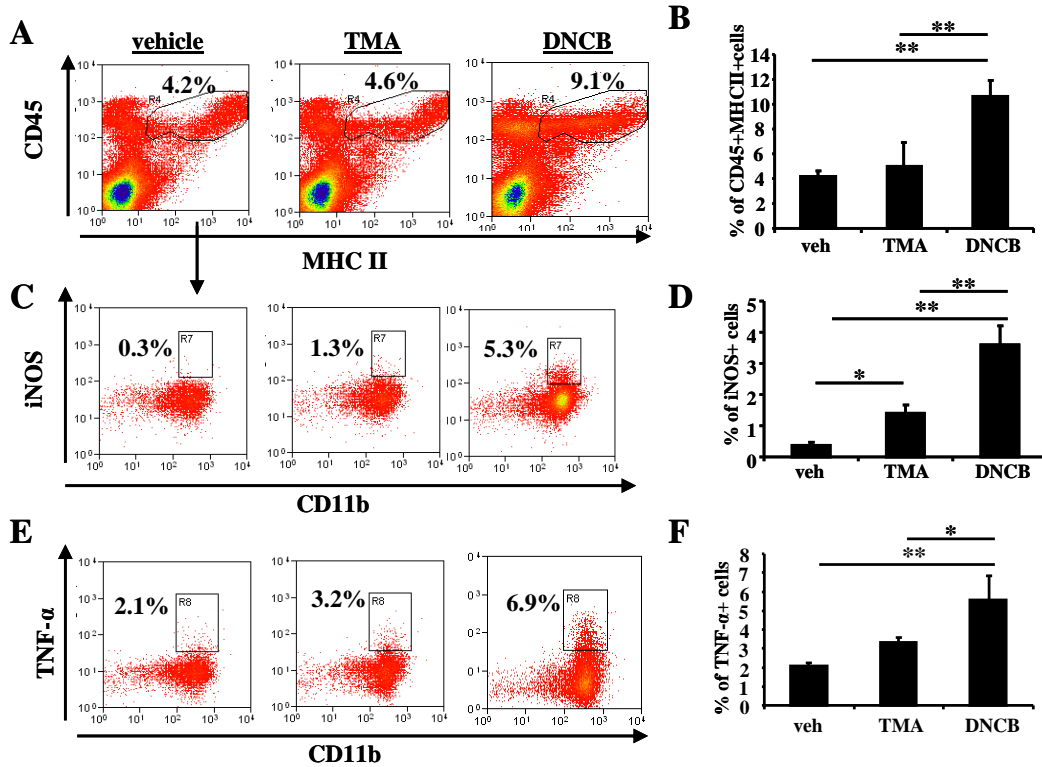
Fig 7.7 DNCB elicitation resulted in significant infiltration of TNF/iNOS-producing DCs.

WT mice were sensitized and challenged as described. Ear skin tissues were harvested 24 h after challenge and digested according to the materials and methods. (A) Cells were stained with anti-CD45 and anti-MHCII. Numbers represent percentages of positive cells (B) Average percentage of CD45$^+$MHCII$^+$ cells in treated samples (C) Cells were fixed and permeabilized before staining with anti-iNOS. Numbers represent percentages of iNOS$^+$ cells among CD45$^+$MHCII$^+$ cells (D) Average percentage of iNOS expressing cells among CD45+MHCII+ cells. (E) Cells were stained for intracellular TNF-α. Numbers represent percentages of TNF-α expressing cells among CD45$^+$MHCII$^+$ cells. (F) Average percentage of TNF-α expressing cells among CD45$^+$MHCII$^+$ cells. Values are shown as means ± SEM of three mice per group. *p< 0.05, **p<0.005 analyzed using one-way ANOVA. Results are representative of three experiments.

We next determined whether these CD45$^+$MHCII$^+$ cells were more representative of Tip-DCs than macrophages and if they expressed higher levels of TNF-α and iNOS compared to the CD45$^+$, non-MHCII expressing counterparts. We found that CD45$^+$MHCII$^+$ cells present in DNCB-treated mice displayed a higher MFI expression of CD11c, a marker commonly associated with DCs, compared to CD45$^+$MHCII$^-$ cells (Fig. 7.8A). In addition, these CD45$^+$MHCII$^+$ cells expressed lower levels of CD68, a marker commonly expressed on macrophages, as shown by the lower MFI expression compared to CD45$^+$MHCII$^-$ cells (Fig. 7.8A). Notably, these CD45$^+$MHCII$^+$ cells also had a higher MFI expression of TNF-α and iNOS compared to CD45$^+$MHCII$^-$ cells (Fig. 7.8A). Moreover, the differences in the MFI expressions of CD11c, CD68, iNOS and TNF-α between these two populations of cells were also statistically significant (Fig. 7.8B). Hence, these results suggest that CD45$^+$MHCII$^+$ cells were representative of Tip-DCs and that the expression of MHC II on CD45$^+$ cells is also associated with a lower expression of CD68 but higher expression of CD11c, iNOS and TNF-α.
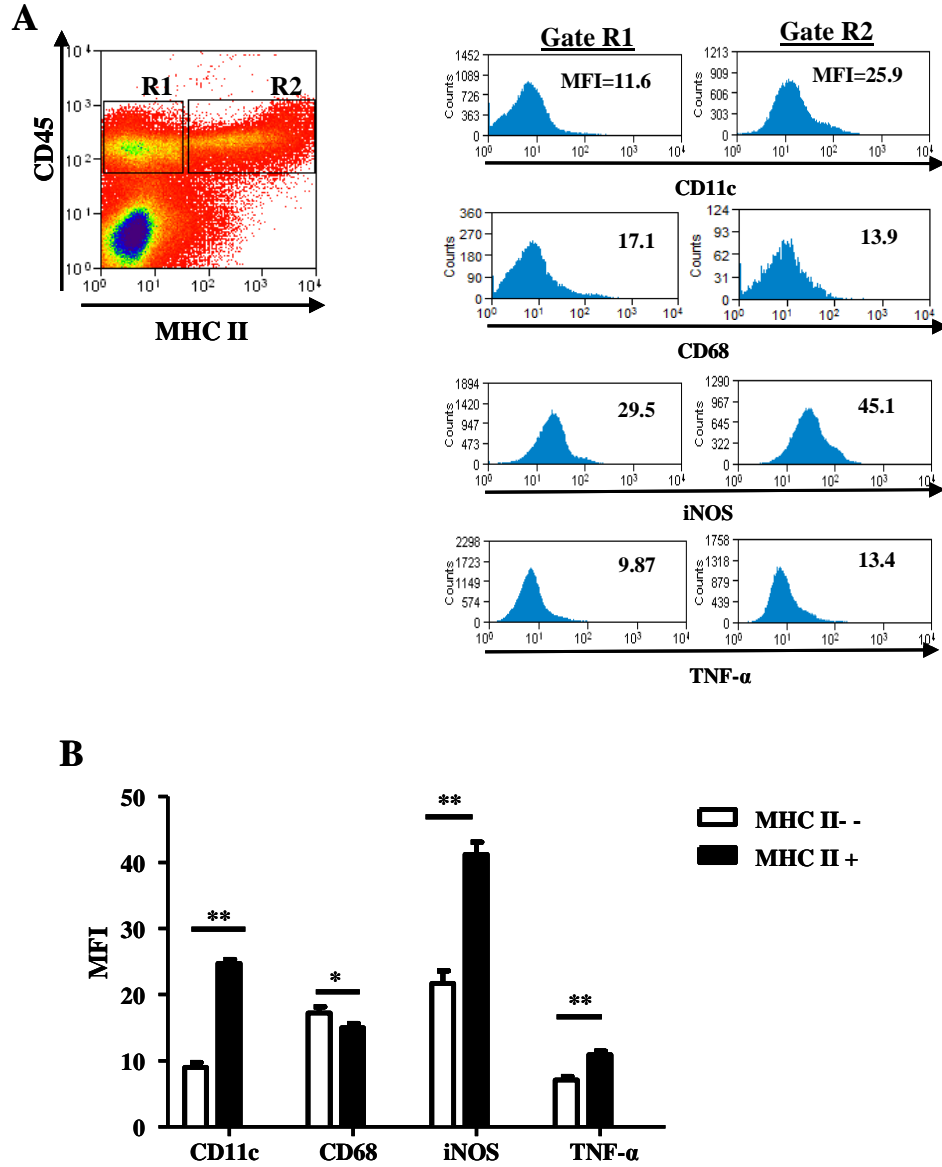
Fig.7.8 Further characterization of Tip-DCs.

WT mice were sensitized and challenged with DNCB as described. Ear skin tissues were harvested after challenge, digested and analyzed using flow cytometry. (A) Cells were gated into CD45$^+$MHCII$^-$ (R1 gate) or CD45$^+$MHCII$^+$ (R2 gate) and analyzed for CD11c, CD68, iNOS and TNF-α expression. Numbers represent mean fluorescence index (MFI). (B) Statistical analysis of MFI expression of indicated markers. Values are shown as means ± SEM of nine mice per group. *p<0.05, **p<0.005 analyzed using one-way ANOVA.

Since Ly6C$^+$ monocytes have been shown to infiltrate into the periphery during inflammation (Shi and Pamer, 2011), we determined if the increase in CD45$^+$MHCII$^+$ cells (Tip-DCs) seen in DNCB-treated mice could be due to an increase in differentiated Ly6C$^+$ monocytes. We found that vehicle-treated mice had no Ly6C$^+$ expressing cells in the dermis (Fig. 7.9A). When mice were treated with TMA, we found a 20% increase in total Ly6C$^+$ cells with half of them expressing MHCII (Fig. 7.9A). In comparison, mice treated with DNCB demonstrated a 19% increase in Ly6C$^+$MHCII$^-$ cells and 38% increase in Ly6C$^+$MHCII$^+$ cells, resulting in a increase of 57% of total Ly6C$^+$ cells (Fig. 7.9A). Gating on either Ly6C$^-$MHCII$^+$ or Ly6C$^+$MHCII$^+$ cells revealed that both populations expressed TNF-α and iNOS, although the latter was shown to have a higher percentage of TNF-α/iNOS expressing cells (Fig. 7.9B). Notably, the increase in Ly6C$^+$ cell populations in DNCB-treated mice were also significantly higher than TMA-treated or vehicle-treated mice (Fig. 7.9C). Hence, these results suggest that the increase in Tip-DCs or CD45$^+$MHCII$^+$ cells seen in DNCB-treated mice could be due to the increased presence of differentiated Ly6C$^+$ monocytes.
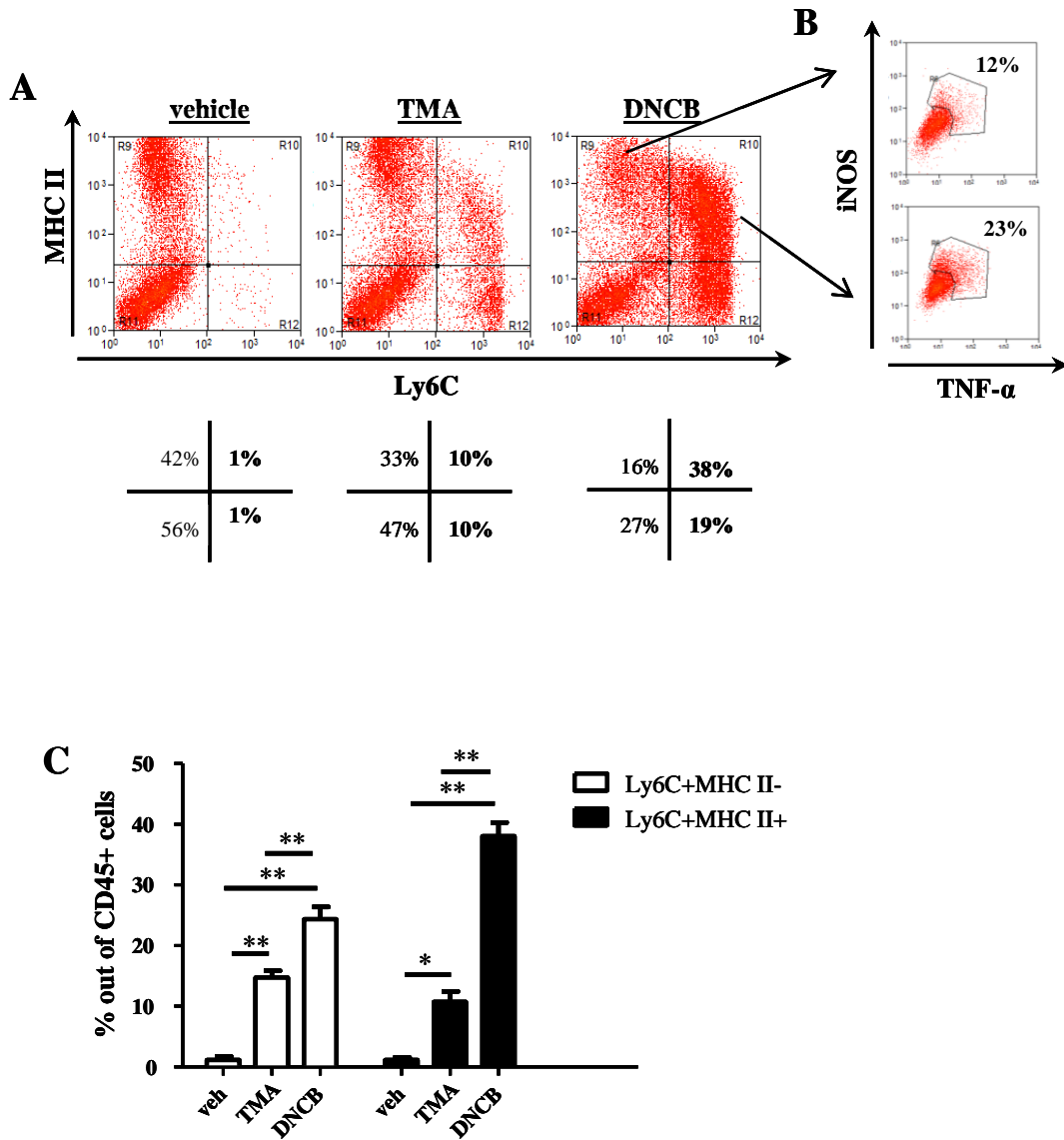
Fig. 7.9 TMA-treated samples showed reduced presence of Ly6C⁺MHCII⁺ cells.

WT mice were sensitized and challenged with TMA, DNCB or vehicle as described. Ear skin tissues were harvested after challenge, digested and analyzed using flow cytometry. (A) Cells were gated for $CD45^+Ly6G^-$ cells to exclude neutrophils before analyzing for $Ly6C^+MHCII^+$ cells. Numbers represent percentages of cells in each quadrant. (B) Cells were further gated on each indicated quadrant and analyzed for intracellular TNF-α and iNOS. Numbers represent percentages of positive cells. (C) Average percentage of $Ly6C^+MHCII^-$ and $Ly6C^+MHCII^+$ cells out of $CD45^+$ cells. Values are shown as means ± SEM of six mice per group. *$p < 0.05$ **$p < 0.005$ analyzed using one-way ANOVA.

7.2.5 Deficiency in CD8 T-cells, but not CD4 T-cells, resulted in reduction of Tip-DC accumulation and a decrease in CHS responses.

In the earlier chapters, we showed that CD8 T-cells played a role in the differentiation of human monocytes into Tip-DCs *in vitro*. Thus, we investigated if the Tip-DCs seen during CHS elicitation with DNCB could also be mediated by CD8 T-cells *in vivo*. We first used mice that were deficient in ß2-microglobulin (ß2m$^{-/-}$), an important component of the MHC class I molecule structure. These ß2m$^{-/-}$ mice have defective MHC class I presentation and hence do not have any CD8 T-cells (Koller et al., 1990). We confirmed the phenotype of these mice by analyzing the blood and lymph nodes and confirmed that CD8 T-cells were indeed absent in these ß2m$^{-/-}$ mice (Fig. 7.10A-B). While CD8 T-cells were consistently lacking in ß2m$^{-/-}$ mice, the percentage of CD4 T-cells in the lymph nodes were sometimes elevated (Fig. 7.10B) and, although this was not consistently seen in all experiments, has been reported previously by others (Giese and Davidson, 1995; Vikingsson et al., 1996). Hence, we also used depleting antibodies to remove CD8 T-cells and CD4 T-cells for comparative purposes. CD8 T-cells or CD4 T-cells were depleted by injecting 250 μg of anti-CD8α (clone: 53-6.7) or anti-CD4 (clone: GK1.5) respectively two days before the last challenge. Analysis of the blood (Fig. 7.10A) and lymph nodes (Fig. 7.10B) demonstrate more than 98% depletion of these cells each time.
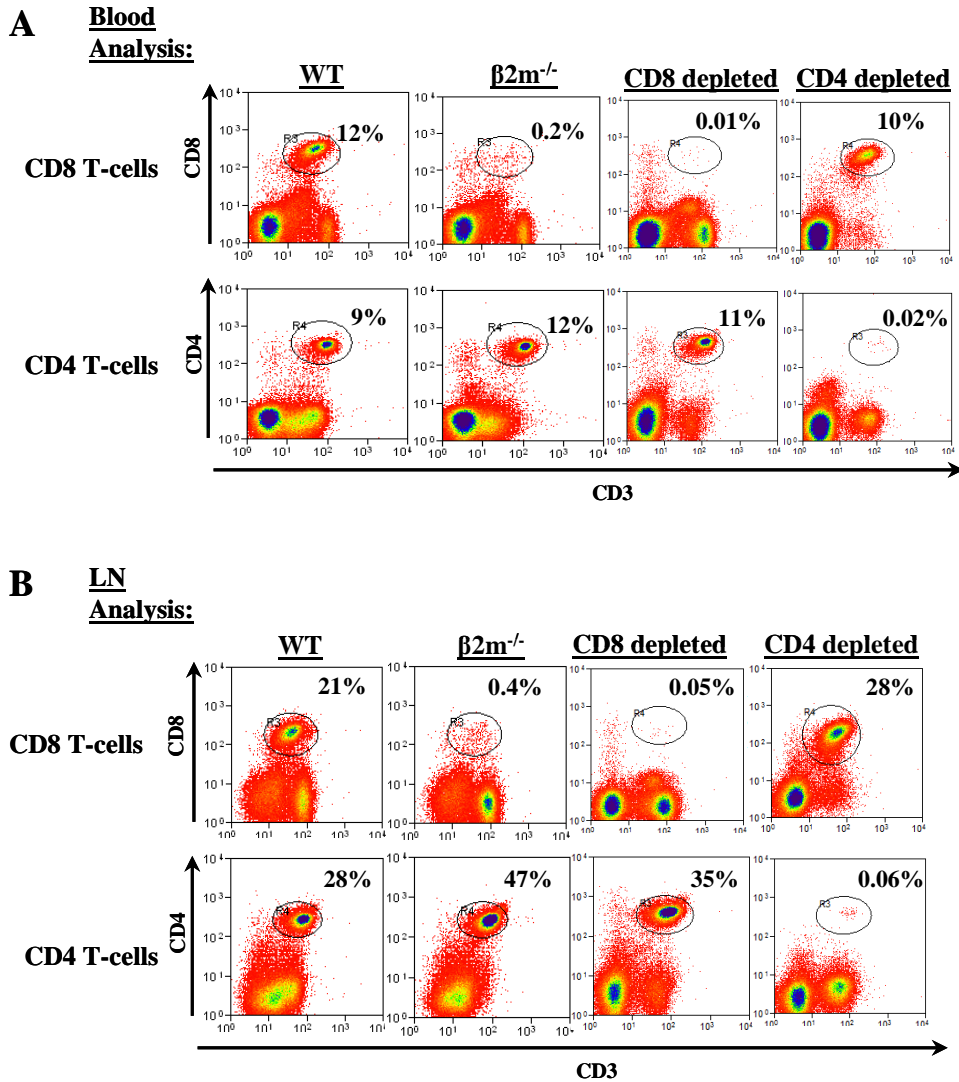
Fig. 7.10 Blood and lymph node analysis of β2m$^{-/-}$ mice and mice that have been treated with anti-CD8 or anti-CD4 depleting antibodies.

WT or β2m$^{-/-}$ mice were sensitized and challenged with DNCB as described. 3 days after sensitization, 250 μg of anti-CD8 or anti-CD4 antibodies was injected i.p. into WT mice. 24 hours after challenge, CD8 T-cells and CD4 T-cells were analyzed via flow cytometry in (A) blood harvested through cardiac puncture and in (B) draining auricular lymph nodes to ensure the absence of indicated cells. Numbers represent percentages of positive cells. Results are representative of three experiments conducted.

To determine if the ablation of CD8 T-cells or CD4 T-cells may have a profound effect on the CHS response, histology was performed on ear sections through H&E staining. We found that ß2m$^{-/-}$ mice and CD8dep mice had a reduction in edema and fluid deposits compared to WT mice when subjected to CHS with DNCB, although this was not seen in CD4dep mice (Fig. 7.11A). In addition, this decrease in edema was associated with a significant decrease in ear thickness for both the ß2m$^{-/-}$ mice (*p<0.05) and CD8dep mice (**p<0.005) (Fig. 7.11B). However, there was no significant change in ear thickness in CD4dep mice (Fig. 7.11B). Therefore, we conclude that CD8 T-cells, and not CD4 T-cells, are the dominant modulators of CHS responses.

A

WT

β2m⁻/⁻

CD8 depleted

CD4 depleted

B
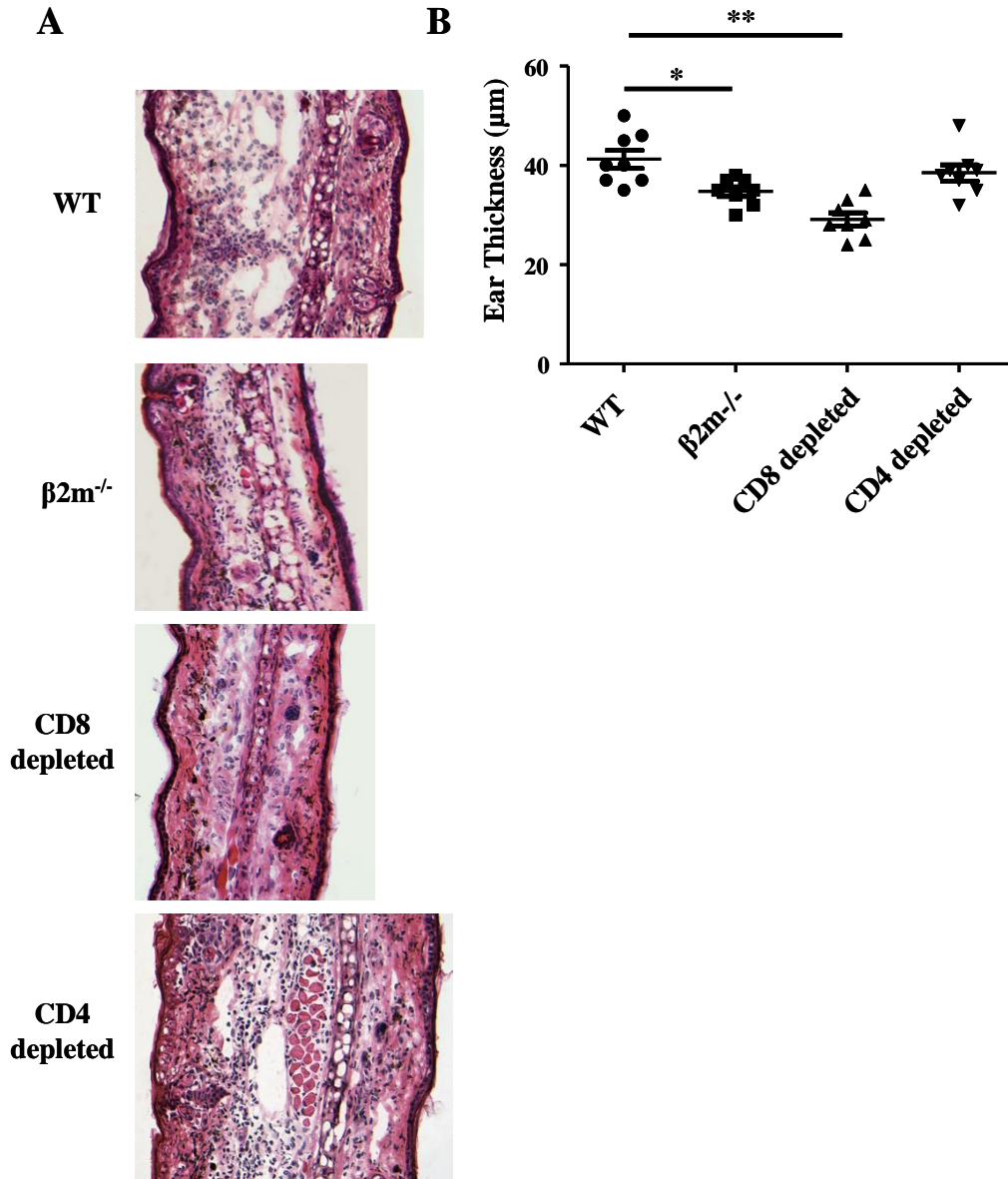
Fig 7.11   CD8 T-cells, but not CD4 T-cells, are important for CHS responses.

WT or β2m⁻/⁻ mice or WT mice that have been depleted of CD8 T-cells or CD4 T-cells were sensitized and challenged as described with DNCB. (A) Ear skin tissues were harvested after challenge and stained with H&E. (B) Ear thickness was measured 18 hours after challenge using a micrometer. Values are shown as means ± SEM of eight mice per group. *p<0.05 **p<0.005 analyzed using one-way ANOVA. Results are representative of three experiments conducted.

When CHS was performed using DNCB on ß2m$^{-/-}$ mice, we found a significant decrease

(**p<0.005) in CD45$^+$MHCII$^+$ cells compared to WT mice in the dermis (Fig. 7.12A-B).

In comparison, mice treated with CD8 depleting (CD8dep) antibodies also showed a

significant reduction (**p<0.005) in CD45$^+$MHCII$^+$ cells although this reduction was not

seen in mice treated with CD4 depleting (CD4dep) antibodies (Fig. 7.12A-B). Upon

analyzing the CD45$^+$MHCII$^+$ cells for iNOS expression, we found that ß2m$^{-/-}$ mice and

CD8dep mice both exhibited a drastic reduction in iNOS expression (Fig. 7.12C) and this

reduction was statistically significant (**p<0.005) (Fig. 7.12D). In contrast, we did not

detect any significant differences in iNOS expression in CD4dep mice compared to mice

which did not receive any depleting antibodies (Fig. 7.12C-D). We also noted that TNF-α

expression among CD45$^+$MHCII$^+$ cells was slightly reduced in ß2m$^{-/-}$ mice and CD8dep

mice (Fig. 7.12E), although this reduction was only statistically significant (*p<0.05) in

CD8dep mice (Fig. 7.12F). In addition, we found no significant differences in TNF-α

expression in CD4dep mice (Fig. 7.12E-F). Hence, these results suggest that CD8 T-cells,

but not CD4 T-cells, are able to modulate the presence of Tip-DCs during CHS using

DNCB by affecting their ability to express TNF-α and iNOS.
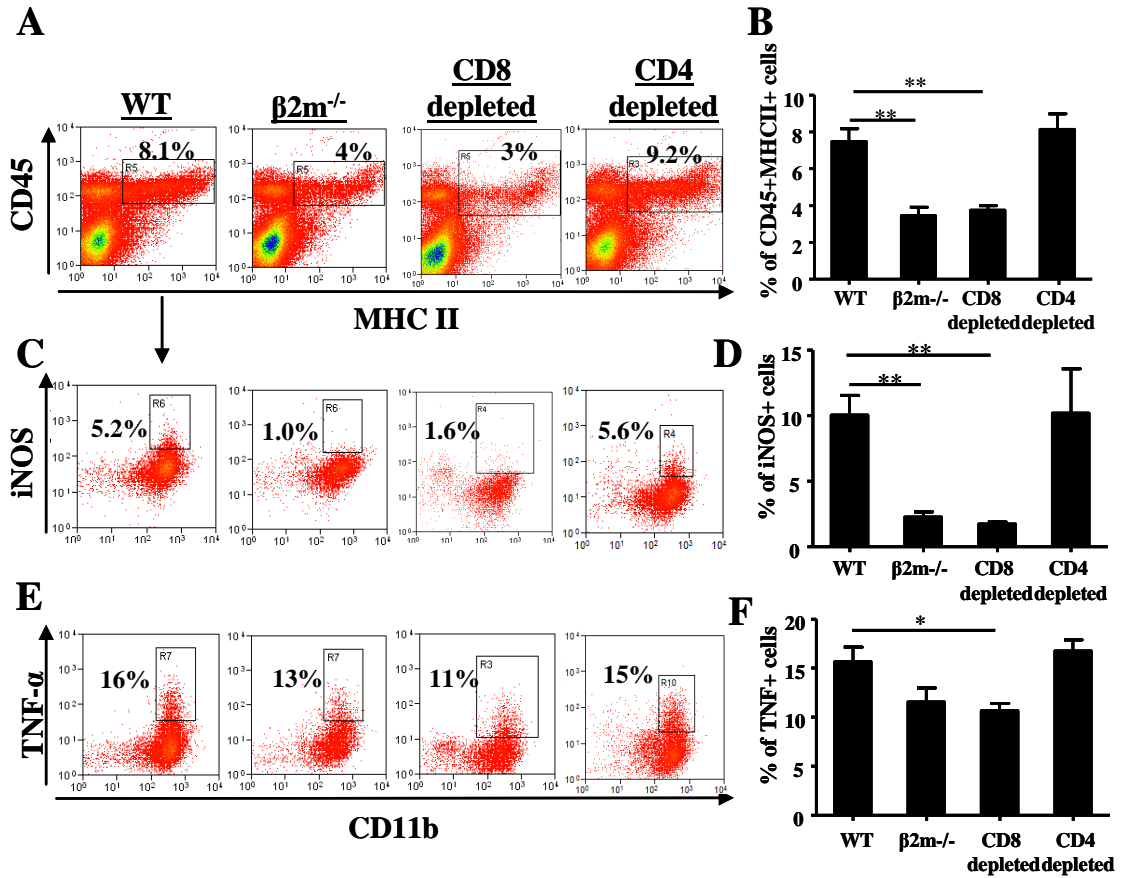
Fig 7.12 TNF/iNOS-producing DCs were reduced in β2m$^{-/-}$ and CD8 depleted mice.

WT or β2m$^{-/-}$ mice or WT mice that have been depleted of CD8 T-cells or CD4 T-cells were sensitized and challenged as described with DNCB. Ear skin tissues were harvested 24 h after challenge and digested according to the materials and methods. (A) Cells were stained with anti-CD45 and anti-MHCII. Numbers represent percentages of positive cells (B) Average percentage of CD45$^+$MHCII$^+$ cells in treated samples (C) Cells were fixed and permeabilized before staining with anti-iNOS. Numbers represent percentages of iNOS$^+$ cells among CD45$^+$MHCII$^+$ cells (D) Average percentage of iNOS expressing cells among CD45$^+$MHCII$^+$ cells. (E) Cells were stained for intracellular TNF-α. Numbers represent percentages of TNF-α expressing cells among CD45$^+$MHCII$^+$ cells. (F) Average percentage of TNF-α expressing cells among CD45$^+$MHCII$^+$ cells. Values are shown as means ± SEM of three mice per group. *p< 0.05, **p<0.005 analyzed using one-way ANOVA. Results are representative of three experiments.

We next investigated if the reduction in Tip-DCs seen in ß2m$^{-/-}$ mice and CD8dep mice was due to a decrease in the presence and differentiation of Ly6C$^+$ monocytes. We found a slight increase in percentage of Ly6C$^+$MHCII$^-$ cells among the CD45$^+$ immune cells in ß2m$^{-/-}$ mice and CD8dep mice (Fig. 7.13A), although this was not statistically significant (Fig. 7.13B). In contrast, we found a significant decrease (**p<0.005) in percentage of Ly6C$^+$MHCII$^+$ cells in ß2m$^{-/-}$ mice and CD8dep mice although this decrease was not detected in CD4dep mice (Fig. 7.13A-B). Thus, these results demonstrate that the decrease in Tip-DCs in ß2m$^{-/-}$ mice and CD8dep mice could also be due to a decrease in Ly6C$^+$ cell differentiation.
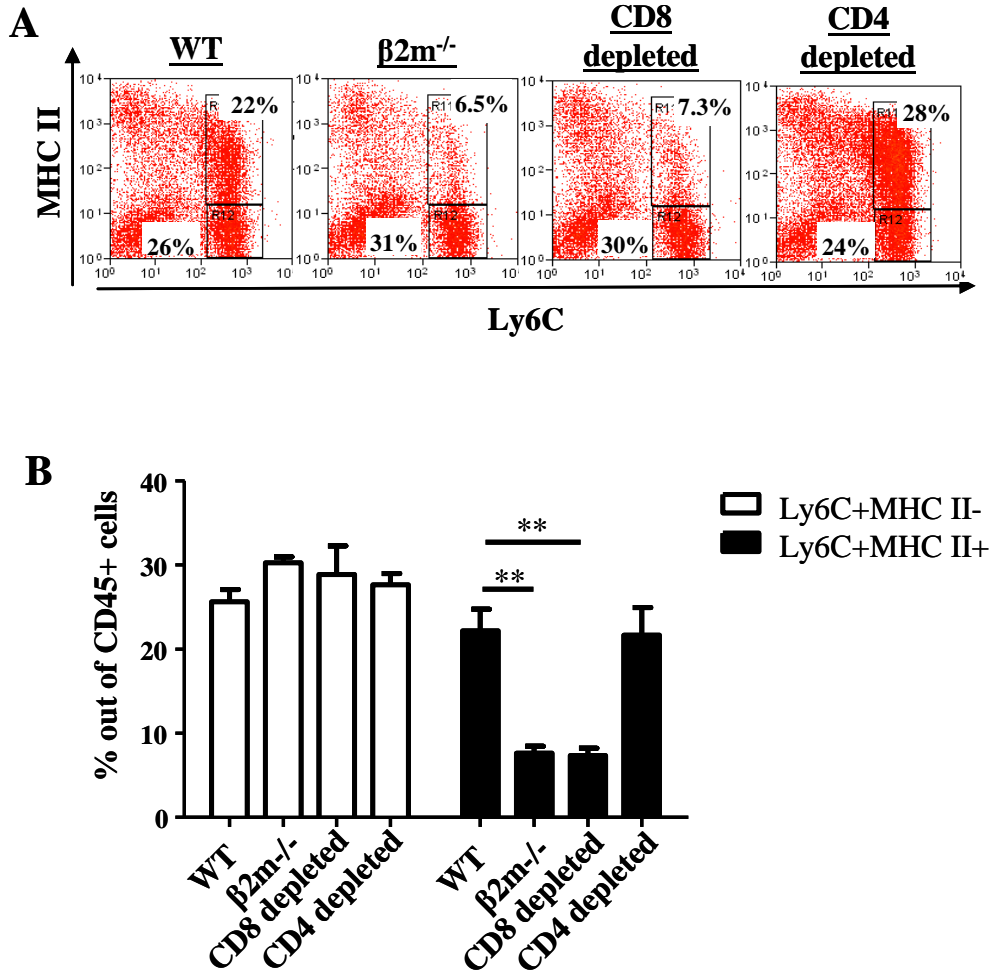
Fig.7.13 β2m⁻/⁻ and CD8 depleted mice had reduced presence of Ly6C+MHCII+ cells.

WT or $\beta 2m^{-/-}$ mice or WT mice that have been depleted of CD8 T-cells or CD4 T-cells were sensitized and challenged as described with DNCB. Ear skin tissues were harvested after challenge, digested and analyzed using flow cytometry. (A) Cells were gated for $CD45^+Ly6G^-$ cells to exclude neutrophils before analyzing for $Ly6C^+MHCII^+$ cells. Numbers represent percentages of cells in each quadrant. (B) Average percentage of $Ly6C^+MHCII^-$ and $Ly6C^+MHCII^+$ cells out of $CD45^+$ cells. Values are shown as means ± SEM of six mice per group. *p<0.05 **p<0.005 analyzed using one-way ANOVA.

7.2.6 Tip-DCs are reduced in IFN-γ$^{-/-}$ and CCR2$^{-/-}$ transgenic mice during CHS responses.

Previous reports have shown that Ly6C^{+} monocytes require CCR2 to infiltrate into inflammatory sites (Serbina et al., 2003). In addition, we showed previously *in vitro* that the differentiation of Tip-DCs was dependent on IFN-γ. Thus, we determined if Tip-DCs in our CHS model is dependent on the presence of IFN-γ and CCR2 signalling. When CHS was performed on IFN-γ$^{-/-}$ mice, we found a 3-fold decrease in the presence of CD45^{+}MHCII^{+} cells compared to WT mice (Fig. 7.14A). In addition, CCR2$^{-/-}$ mice had a severe decrease in the presence of CD45^{+}MHCII^{+} cells with only 1.3% CD45^{+}MHCII^{+} cells compared to 9.1% of these cells in WT mice (Fig. 7.14A). Notably, the decrease in CD45^{+}MHCII^{+} cells in IFN-γ$^{-/-}$ and CCR2$^{-/-}$ mice compared to WT mice was also statistically significant (**p<0.005) (Fig. 7.14B). We next examined the CD45^{+}MHCII^{+} cell population for iNOS and TNF-α expression. We found a significant decrease (**p<0.005) in the percentage of iNOS-expressing cells in both IFN-γ$^{-/-}$ and CCR2$^{-/-}$ mice compared to WT mice (Fig. 7.14C-D). In contrast, CCR2$^{-/-}$ mice showed a significant increase (**p<0.005) in percentage of TNF-α-expressing cells among the CD45^{+}MHCII^{+} cell population compared to WT mice (Fig. 7.14E-F). However, because the population of CD45^{+}MHCII^{+} cells in IFN-γ$^{-/-}$ and CCR2$^{-/-}$ mice are much lesser than WT mice, we found that IFN-γ$^{-/-}$ and CCR2$^{-/-}$ mice still had an overall decrease in percentage of CD45^{+}MHCII^{+}TNF-α^{+} cells among total cells and this decrease was statistically significant (Fig. 7.14G). Hence these results suggest that both IFN-γ and CCR2 signalling were important for modulating the presence of Tip-DCs and expression of TNF-α and iNOS during CHS.
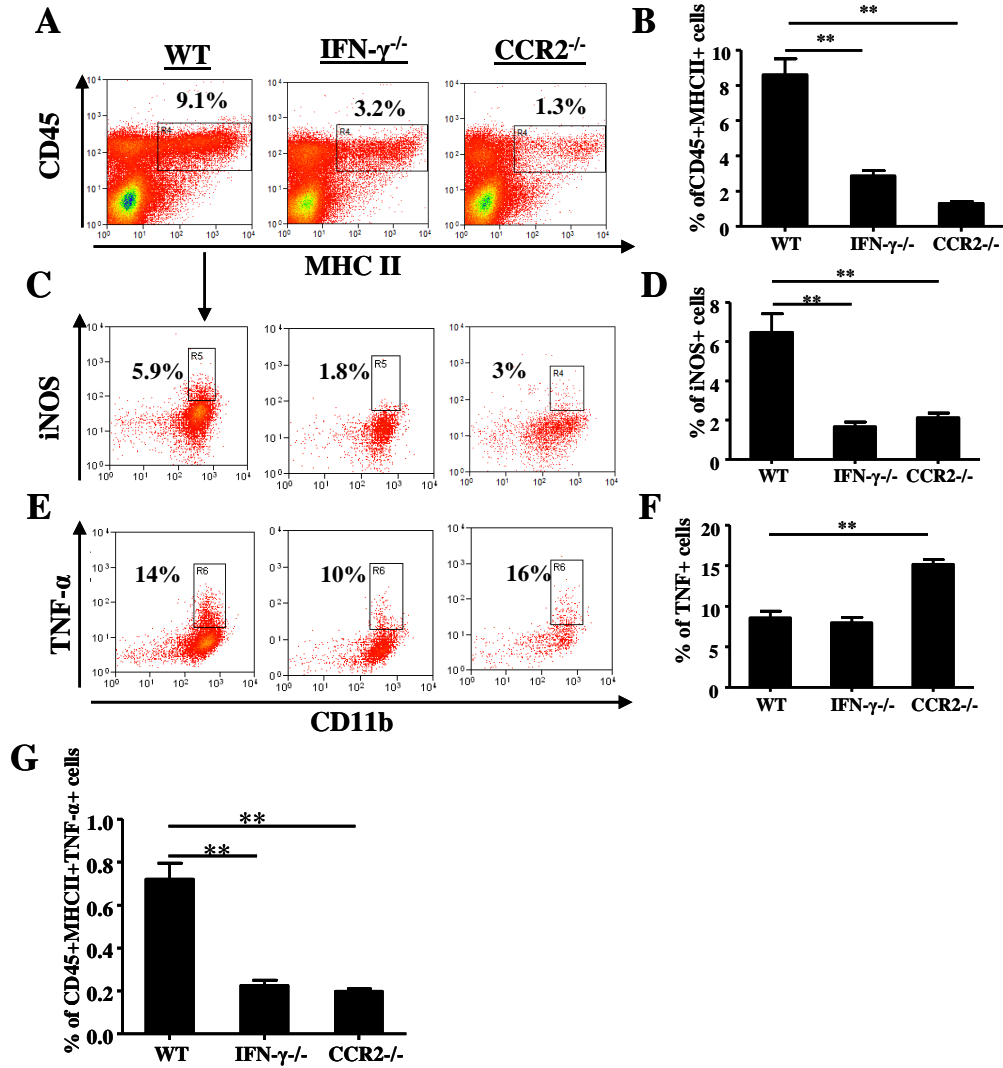
Fig 7.14 TNF/iNOS-producing DCs were reduced in IFN-γ-/- and CCR2-/- mice.

WT, IFN-γ-/- and CCR2-/- mice were sensitized and challenged as described with DNCB. Ear skin tissues were harvested 24 h after challenge and digested according to the materials and methods. (A) Cells were stained with anti-CD45 and anti-MHCII. Numbers represent percentages of positive cells (B) Average percentage of $CD45^+MHCII^+$ cells among total cells in treated samples (C) Cells were fixed and permeabilized before staining with anti-iNOS. Numbers represent percentages of $iNOS^+$ cells among $CD45^+MHCII^+$ cells (D) Average percentage of iNOS expressing cells among $CD45^+MHCII^+$ cells. (E) Cells were stained for intracellular TNF-α. Numbers represent percentages of TNF-α expressing cells among $CD45^+MHCII^+$ cells. (F) Average percentage of TNF-α expressing cells among $CD45^+MHCII^+$ cells. (G) Average percentage of $CD45^+MHCII^+TNF-α^+$ cells among total cells. Values are shown as mean ± SEM of three mice per group. *$p< 0.05$, **$p<0.005$ analyzed using one-way ANOVA. Results are representative of three experiments.

We also investigated if the reduction in Tip-DCs in IFN-$\gamma^{-/-}$ and CCR2$^{-/-}$ mice was due to a decrease in the presence and differentiation of Ly6C$^+$ monocytes. We found that IFN-$\gamma^{-/-}$ mice had a comparable percentage of total Ly6C$^+$ monocytes (61%) compared to WT (64%) (Fig. 7.15A). However, IFN-$\gamma^{-/-}$ mice had a significant increase (**p<0.005) in percentage of undifferentiated Ly6C$^+$ cells (Ly6C$^+$MHCII$^-$) compared to WT mice after CHS elicitation (Fig. 7.15A-B). At the same time, this was accompanied by a significant decrease (**p<0.005) in percentage of differentiated Ly6C$^+$ cells (Ly6C$^+$MHCII$^+$) compared to WT mice (Fig. 7.15A-B). We also found that CCR2$^{-/-}$ mice had a severe deficiency in total Ly6C$^+$ cells (6%) in the dermis compared to WT mice (64%) after CHS elicitation that was also statistically significant (**p<0.005) (Fig. 7.15A-B). Hence, these results suggest that the reduction of Tip-DCs seen in IFN-$\gamma^{-/-}$ mice could also be due to a defect in Ly6C$^+$ cell differentiation while the minimal percentage of Tip-DCs in CCR2$^{-/-}$ mice was due to a drastic reduction in the presence of Ly6C$^+$ cells.

A

B
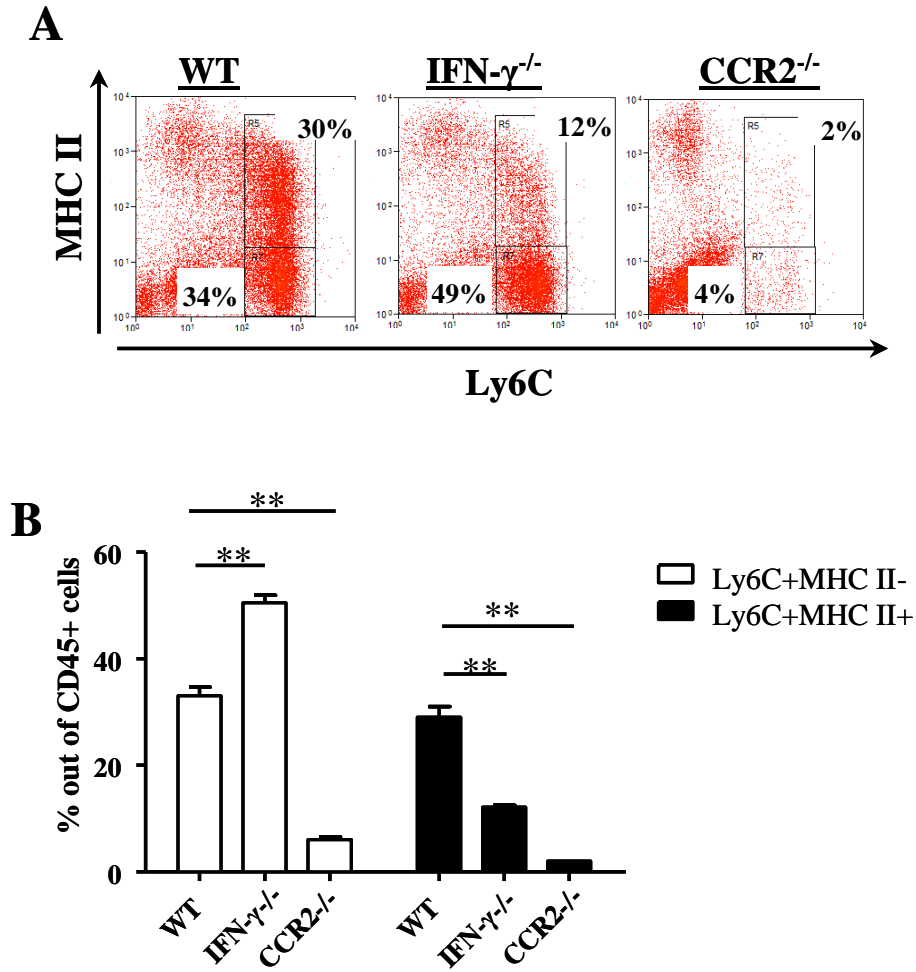
Fig.7.15 IFN-γ$^{-/-}$ and CCR2$^{-/-}$ mice had reduced presence of Ly6C+MHCII+ cells.

WT, IFN-γ$^{-/-}$ and CCR2$^{-/-}$ mice were sensitized and challenged as described with DNCB. Ear skin tissues were harvested 24 h after challenge and digested according to the materials and methods.(A) Cells were gated for CD45^{+}Ly6G^{-} cells to exclude neutrophils before analyzing for Ly6C^{+}MHCII^{+} cells. Numbers represent percentages of cells in each quadrant. (B) Average percentage of Ly6C^{+}MHCII^{-} and Ly6C^{+}MHCII^{+} cells out of CD45^{+} cells. Values are shown as means ± SEM of six mice per group. *p<0.05 **p<0.005 analyzed using one-way ANOVA.

Finally, we determined if the absence of IFN-γ or CCR2 signalling affects the CHS response elicited by DNCB. Histological analysis of challenged sites showed that CHS in WT mice was associated with vascular enlargement, dermal edema and infiltration of mononuclear cells (Fig. 7.16A). However, these observations were present at a much lesser extent in IFN-γ$^{-/-}$ and CCR2$^{-/-}$ mice (Fig. 7.16A). In addition, measurement of mouse ears with CHS demonstrated a significant decrease in ear swelling in IFN-γ$^{-/-}$ (*p<0.05) and CCR2$^{-/-}$ (**p<0.005) mice compared to their WT counterparts (Fig. 7.16B). These results suggest that IFN-γ and CCR2 are not only important for the presence of Tip-DCs during CHS responses, but are also crucial for the pathological changes during CHS.

A

WT

IFN-γ⁻/⁻

CCR2⁻/⁻

B
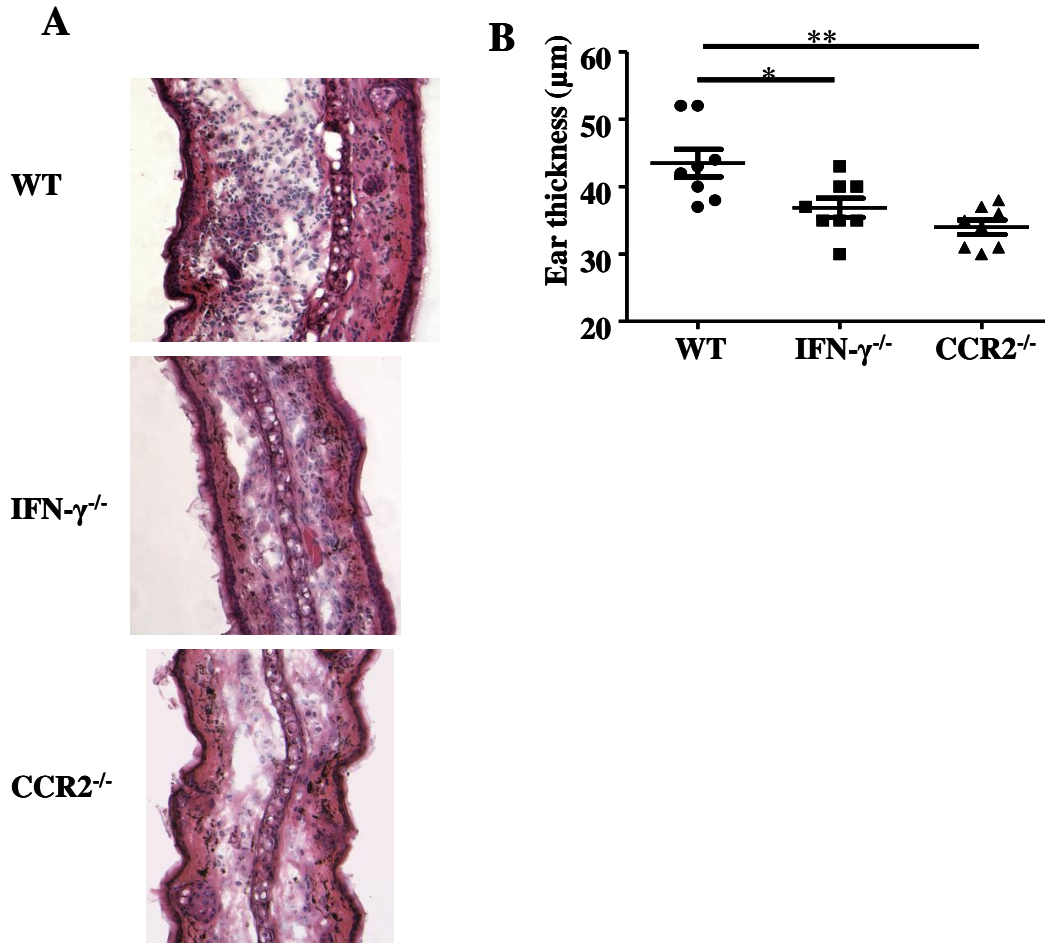
Fig 7.16 IFN-γ⁻/⁻ and CCR2⁻/⁻ mice had reduced CHS responses.

WT, IFN-γ⁻/⁻ and CCR2⁻/⁻ mice were sensitized and challenged as described with DNCB. (A) Ear skin tissues were harvested after challenge and stained with H&E. (B) Ear thickness was measured 18 hours after challenge using an engineer's micrometer. Values are shown as means ± SEM of eight mice per group. *p<0.05 **p<0.005 analyzed using one-way ANOVA. Results are representative of three experiments conducted.

7.2.7 Tip-DCs are located proximately to endothelial structures and T-cells during DNCB elicitation

We further examined the presence of Tip-DCs in the dermis of DNCB-treated mice by performing immunostaining on frozen ear tissue cryosections. We noticed that vehicle-treated mice had a small number of MHCII$^+$ cells and were distributed both in the epidermis and dermis where they represent Langerhans cells and dermal DCs respectively (Fig.7.17A) In addition, staining for CD31, a marker which detects endothelial structures such as lymphatic and blood vessel endothelium, revealed that these structures are relatively constricted at the steady state. In addition, we were unable to detect any iNOS in the dermis of ear sections in vehicle-treated mice (Fig. 7.17A). When ear sections from DNCB-treated mice were stained with MHCII, we noticed a increase in MHCII$^+$ staining in the dermis and this was accompanied by dilation of lymphatic and blood vessels as observed by the opened circular structures when stained with CD31 (Fig. 7.17B). Staining DNCB-treated ear sections with iNOS revealed a brightly stained epidermis which is consistent with literature findings of iNOS-expressing keratinocytes during inflammation (Bécherel et al., 1997; Robertson et al., 1996; Sirsjö et al., 1996). Closer examination of iNOS production in the dermis revealed iNOS-expressing cells near endothelial structures (Fig 7.17C) and these iNOS-expressing cells were also co-localized with MHCII$^+$ cells (Fig. 7.17D). We also analyzed ear sections for CD3$^+$ cells. Vehicle-treated ears showed minimal CD3 staining in the dermis but displayed substantial staining in the epidermis (Fig. 7.18A) where they exhibit dendritic-like structures and have been shown to be γδ T-cells (or dendritic epidermal T-cells) essential for homeostasis and wound healing (Havran and Jameson, 2010; Hayday, 2009;

Jameson and Havran, 2007). In contrast, staining for CD3 in DNCB-treated skin samples revealed an increased amount of CD3$^+$ cells in the dermis (Fig. 7.18B) and they were found to cluster with iNOS-expressing MHCII+ cells (Fig. 7.18C-E). These immunostaining results suggest that the proximity of Tip-DCs near endothelial structures or T-cells during elicitation of CHS may have profound effects during inflammation which remains to be explored.
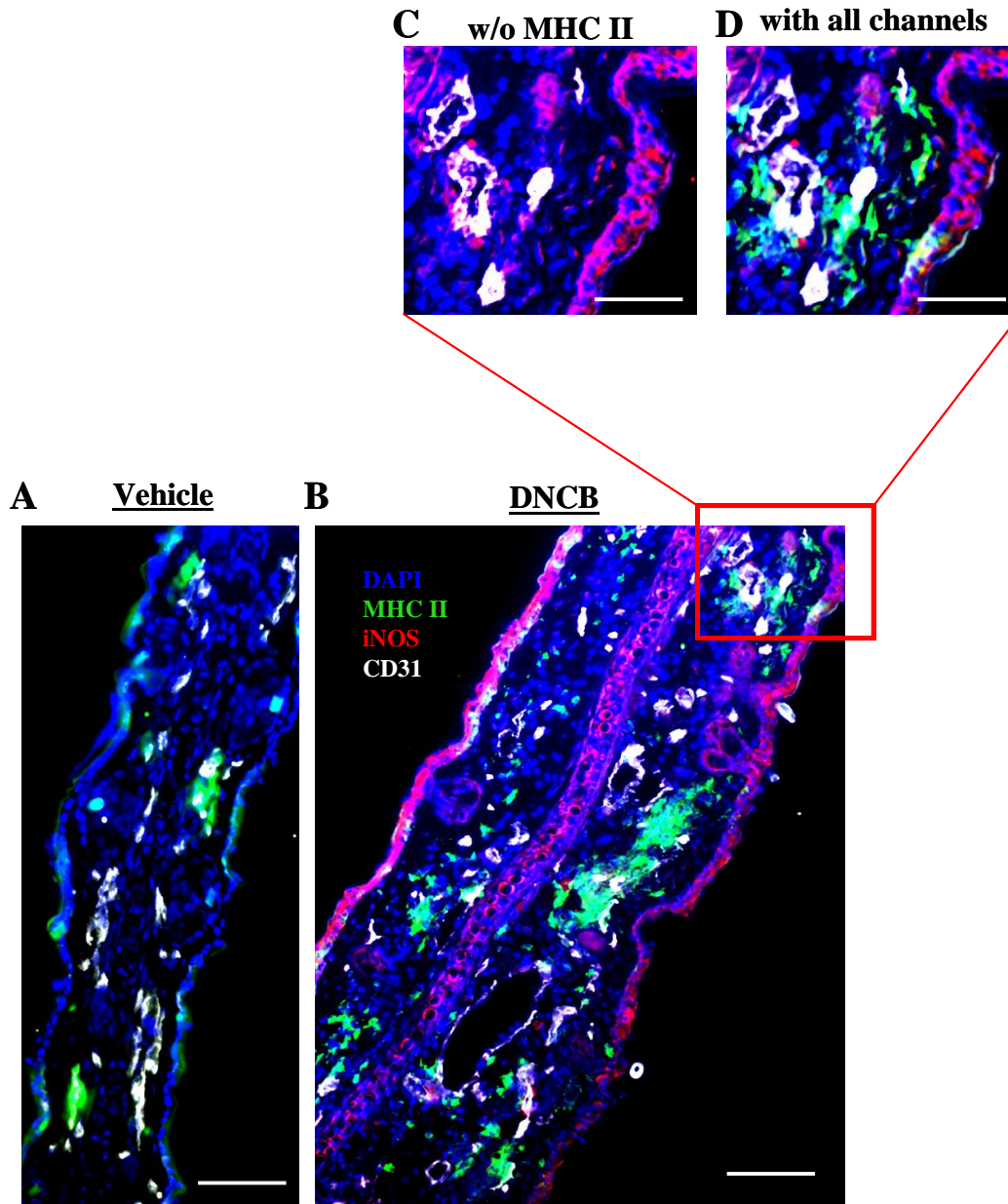
Fig 7.17   Tip-DCs are proximately located to endothelial vessels.

WT mice were sensitized and challenged as described with (A) vehicle or (B) DNCB. Immunostaining was performed on 7μm of ear cryosections and DAPI (blue), MHCII (green), iNOS (red) and CD31 (white) was detected. Scale bar represents 100μm. (C-D) Magnified analysis of MHCII$^+$iNOS$^+$ cells near CD31$^+$ endothelial cells. Scale bar represents 200μm. Results are representative of three experiments.
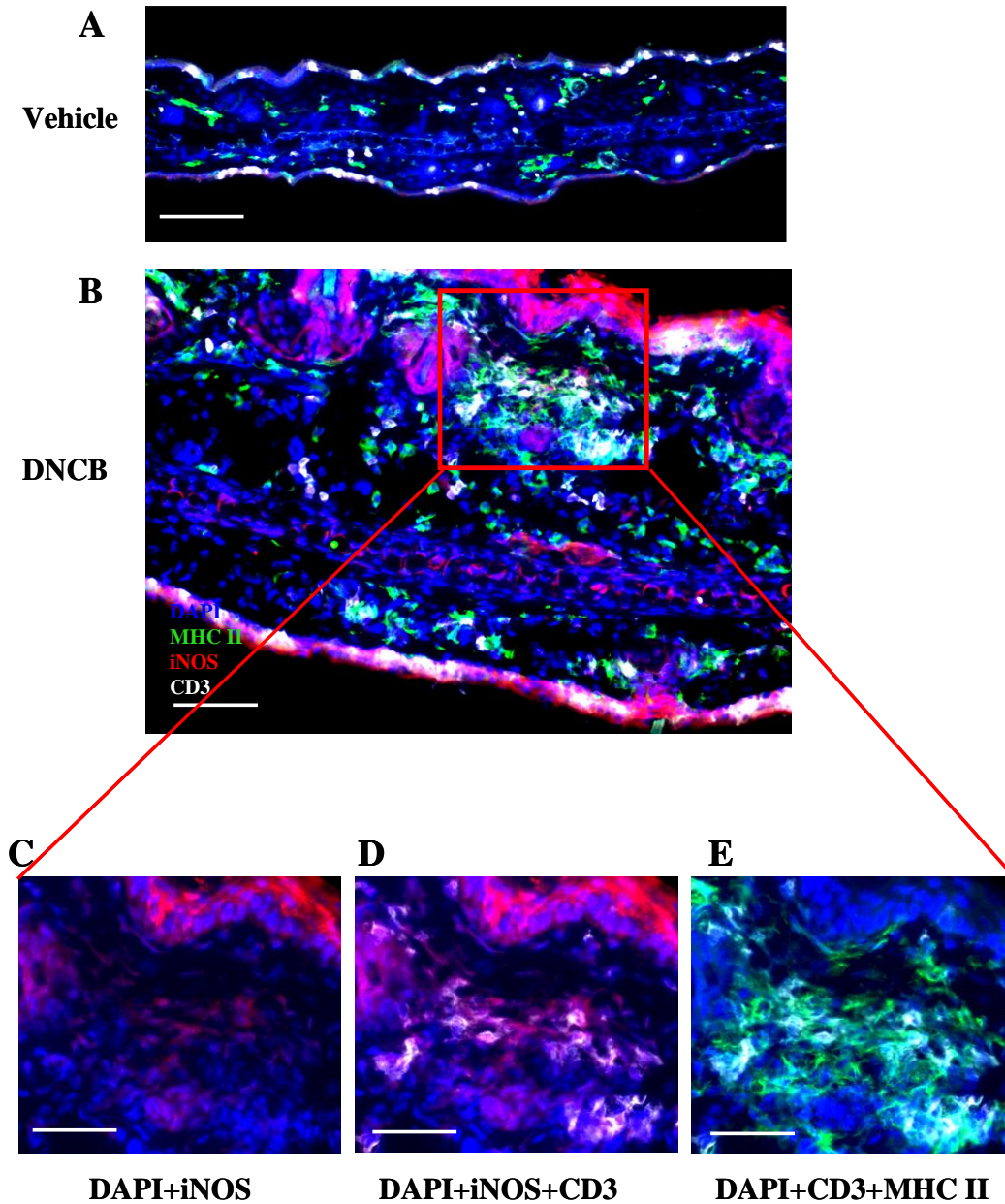
Fig 7.18   Tip-DCs are proximately located to T-cells.

WT mice were sensitized and challenged as described with (A) vehicle or (B) DNCB. Ears were harvested 72 h after challenge. Immunostaining was performed on 7μm of ear cryosections and DAPI (blue), MHCII (green), iNOS (red) and CD3 (white) was detected. Scale bar represents 100μm. (C-E) Magnified analysis of MHCII$^+$iNOS$^+$ cells with CD3$^+$ T-cells. Scale bar represents 200μm. Results are representative of three experiments.

7.3   Discussion

In the previous chapters, we showed that human CD8 T-cells can support Th1 responses by differentiating monocytes into Tip-DCs *in vitro*. In this chapter, we demonstrate that Tip-DCs can also be detected *in vivo* in a model of contact hypersensitivity in the dermis of mice treated with a chemical hapten. We showed that in contrast to TMA, a chemical that favours immune polarization towards Th2 responses, the presence of Tip-DCs was significantly increased in mice that have been treated with DNCB, a chemical that polarizes Th1 responses. We also found that while TMA-treated mice showed minimal ear swelling when challenged with the chemical, DNCB-treated mice showed pathological changes in the ears associated with CHS such as vascular enlargement, dermal edema and increased infiltration of mononuclear cells. In addition, we demonstrated that CD8 T-cells, but not CD4 T-cells, were the major modulators of Tip-DCs during DNCB-elicitated CHS responses through the use of blocking antibodies *in vivo*. Finally, we also showed that the differentiation and recruitment of Tip-DCs was largely dependent on IFN-γ and CCR2 signalling.

Chemical allergens that cause allergic contact dermatitis or occupational asthma, namely DNCB or TMA, have been shown to induce divergent immune responses characteristic of Th1 and Th2-type cell activation respectively (Bernstein et al., 1982; Botham et al., 1989; Dearman and Kimber, 1991). In line with these findings, we also observed that sensitization of mice to DNCB or TMA resulted in the production of different cytokine patterns. In particular, we found that auricular draining lymph nodes excised from mice

after challenge with TMA showed the presence of Th2 cytokines, IL-4, IL-5, IL-10 and IL-13, which were not detected in DNCB-treated samples. Notably, while there were significant differences in Th2 cytokines produced in TMA versus DNCB-treated mice, we did not detect any significant difference in IFN-γ production between these two samples. While this observation was in contrast to earlier studies which demonstrated lower IFN-γ production in TMA samples (Dearman et al., 1996; Moussavi et al., 1998), we found that a recent finding had supported our observation by demonstrating that the deviating immune responses induced in mice by different classes of chemical allergens are independent of IFN-γ-producing cells, but are associated with differential frequencies of IL-4-expressing cells (Dearman et al., 2005). In addition, we also found a significant increase in IL-17-producing cells in lymph nodes of DNCB-treated compared to TMA-treated mice. This finding has not been reported in literature thus far and may also help to explain the divergent pathological changes seen in DNCB versus TMA-elicited responses, since IL-17 has been associated with the infiltration of granulocytes and regulation of inflammatory chemokines (Aggarwal and Gurney, 2002; He et al., 2009; Kish et al., 2009; Kolls and Lindén, 2004). This differing cytokine patterns seen in lymph nodes of DNCB versus TMA-treated mice was also noticed in the challenged site, as we are the first to show that while there were no significant differences in IFN-γ, we found a significant increase in IL-10 and IL-13 in TMA-treated compared to DNCB-treated ear tissues. Moreover, we also found a significant increase in GM-CSF in DNCB-treated compared to TMA-treated ear tissues. Since GM-CSF has been associated with the development of inflammatory DCs (Auffray et al., 2009; Miller et al., 2002; Xu et al.,

2007), the increase in GM-CSF seen in DNCB-treated samples may also be associated with the increased presence of Tip-DCs which were not detected in TMA-treated mice.

We noticed that mice that have been sensitized with DNCB developed typical symptoms associated with CHS such as vascular enlargement, dermal edema and erythema. In contrast, these observations were not detected in mice that have been sensitized and challenged with TMA. While these observations may suggest that TMA may not elicitate any significant dermatological pathogenesis, studies have shown that chronic topical exposure to TMA results in atopic dermatitis characterized by significant edema and Th2 cytokine profile, accompanied by infiltration of T-cells, MHCII$^+$ cells, eosinophils and mast cells, as well as a strong increase of serum IgE levels (Schneider et al., 2009). Since we have adopted an acute protocol of CHS with only one sensitization and one challenge procedure compared to the chronic protocol with two sensitization and three challenge procedures, this may help to explain the attenuated response seen in our model with TMA as opposed to that seen by Schneider and colleagues (2009). This attenuated response in comparison to DNCB-treated mice may also be accounted by the increased presence of IL-10 detected in TMA but not DNCB-treated mice, since IL-10 has been shown to down-regulate immune responses in contact dermatitis (Grimbaldeston et al., 2007; Pestka et al., 2004).

We also found an increase in immune cell infiltration into DNCB-elicitated ear tissues detected by flow cytometry. In particular, we are the first to show an increase in TNF-α and iNOS expressing-CD45$^+$MHCII$^+$CD11b$^+$ cells in DNCB as compared to TMA-

elicitated ear tissues. Notably, these cells also expressed higher levels of CD11c and lower levels of CD68 than MHCII$^-$ cells, suggesting that these cells closely resemble Tip-DCs as described in other models (Aldridge et al., 2009; Bosschaerts et al., 2010; Serbina et al., 2003). In particular, we found that CD8 T-cells were important for the presence of such Tip-DCs during CHS responses to DNCB, as depletion of CD8 T-cells, but not CD4 T-cells, resulted in a significant decrease in these cells. Although the expression of TNF-α by Tip-DCs was not totally abrogated, the loss of CD8 T-cells resulted in the inability of these cells to express iNOS, thereby demonstrating the importance of CD8 T-cells in the activation of Tip-DCs. Moreover, we found that the reduced presence of MHCII$^+$ Tip-DCs in mice depleted of CD8 T-cells was accompanied by a reduction in differentiated monocytes (Ly6C$^+$MHCII$^+$ cells) after elicitation of CHS. This phenomenon was also observed in IFN-γ$^{-/-}$ mice whereby the absence of IFN-γ also resulted in a decrease in differentiated monocytes. To further confirm that Tip-DCs derive mainly from monocytes, we found that the absence of CCR2 signalling, important for the recruitment of Ly6C$^+$ monocytes (Jia et al., 2008; Serbina et al., 2003; Tsou et al., 2007), caused a drastic reduction in the presence of Tip-DCs. These findings suggest that the infiltration of Tip-DCs is dependent on the recruitment of classical Ly6C$^+$ monocytes via CCR2 and that their differentiation and activation is dependent on the presence of CD8 T-cells and IFN-γ. Since CD8 T-cells have been shown to be the predominant effectors in CHS, compared to CD4 T-cells which infiltrate at a much later time point and produce Th2 cytokines instead (Akiba et al., 2002; Desvignes et al., 1996; Xu et al., 1996), it is tempting to speculate that CD8 T-cells may rely on IFN-γ to differentiate infiltrating

Ly6C$^+$ monocytes into MHCII$^+$ cells resembling Tip-DCs as well as for them to acquire iNOS and a increase in TNF-α expression.

While studies in IFN-γ$^{-/-}$ mice have shown that the differentiation and activation of Tip-DCs during CHS is dependent on IFN-γ, it was surprising that TMA-treated mice had a significant reduction in the presence of Tip-DCs compared to DNCB-treated mice even though both treatments showed similar levels of IFN-γ in the draining lymph nodes and challenged sites. These results suggest that the differentiation of Tip-DCs can be down modulated by other cytokines. Notably, IL-10 has been shown to reduce the presence and pathogenicity of Tip-DCs (Bosschaerts (Bosschaerts et al., 2010; Guilliams et al., 2009) and since increased IL-10 concentrations were detected in TMA-treated compared to DNCB-treated samples, this could possibly account for the reduced presence of Tip-DCs seen in TMA-treated samples. It is also interesting to note that Tip-DCs were detected in substantial numbers only in DNCB-treated mice, which was associated with a Th1 phenotype, as opposed to TMA-treated mice which was associated with a Th2 cytokine profile. This suggests that the presence of such Tip-DCs may represent a major class of Th1 regulated effector cell population which may serve to further support Th1 responses. Similarly, its minimal presence in a Th2 dominant environment, such as in TMA-treated mice, may imply that these Tip-DCs do not serve to exacerbate Th2 responses. In support of this observation, CCR2$^{-/-}$ mice have been shown to have enhanced Th2 but reduced Th1 differentiation (Rivera et al., 2011; Traynor et al., 2002) and that Tip-DCs are important for the clearance of pathogens that require Th1 regulated responses (Aldridge et al., 2009; De Trez et al., 2009; Serbina et al., 2003). While the expression of

TNF-α and iNOS by Tip-DCs may be important for the clearance of invasive pathogens, TNF-α is also a mediator of septic shock (Tracey and Cerami, 1994) and NO exerts significant tissue damage through oxidative stress (Aldridge et al., 2009; Ishiyama et al., 2009). Therefore, in the presence of sterile inflammation such as in CHS responses, the differentiation of Tip-DCs by CD8 T-cells may serve to exacerbate the pathology of CHS which is supported by our findings demonstrating significant reduction in CHS responses when Tip-DCs were absent in CCR2$^{-/-}$ mice. Collectively, our results hence provide further insight to the immunomodulatory functions of CD8 T-cells by demonstrating the importance of these cells in the differentiation of Tip-DCs as part of a Th1 regulated response in an *in vivo* model of allergic contact dermatitis.

CHAPTER 8: Final Discussion

8.1 Summary of Main Findings

In this thesis, we first demonstrated the ability of human CD8 T-cells to support Th1 responses by priming DCs for IL-12p70 and inducing the development of monocytes into Tip-DCs (Chong et al., 2011). We show that activated human CD8 T-cells could induce DCs to produce IL-12p70 *in vitro* in the presence of LPS through IFN-γ and that this mechanism was independent of CD40L signaling. This CD8 T-cell-DC interaction also resulted in the production of a cytokine milieu containing GM-CSF, IL-1, IL-2, IL-6, IFN-γ, and TNF-α that promoted the differentiation of monocytes into Tip-DCs. These Tip-DCs expressed high levels of TNF-α and iNOS, MHC class II and upregulated co-stimulatory molecules CD40, CD80 and CD86. They also had increased expression of toll-like receptors TLR2, TLR3, TLR4 and chemokine receptors CCR1 and CX_3CR1. Importantly, Tip-DCs expressed the classical DC maturation marker CD83, exhibited T-cell priming ability and were capable of further driving Th1 responses, through their expression of TNF-α and iNOS, by priming naive CD4 T-cells for IFN-γ production. Differentiation of monocytes into Tip-DCs was partially dependent on IFN-γ as blocking the IFN-γ receptor on monocytes resulted in a significant decrease in CD40, CD83 expression and TNF-α production. Finally, we showed that the ability of CD8 T-cells to differentiate monocytes into Tip-DCs can also be demonstrated in an *in vivo* mouse model of allergic contact hypersensitivity (CHS). This differentiation and activation of Tip-DCs during CHS responses was compromised in ß2m$^{-/-}$, IFN-γ$^{-/-}$ and CCR2$^{-/-}$ mice

and mice that were depleted of CD8, but not CD4, T-cells. In particular, the presence of Tip-DCs was significantly increased in mice that have been treated with a Th1-inducing topical sensitizer, DNCB, but not in mice that have been treated with a Th2-inducing sensitizer, TMA. Collectively, our results identify a role for CD8 T-cells in orchestrating Th1-mediating signals, not only through the rapid initiation of DC IL-12p70, but also through the differentiation of monocytes into Tip-DCs. A schematic illustration of this mechanism is represented in Fig 8.1.
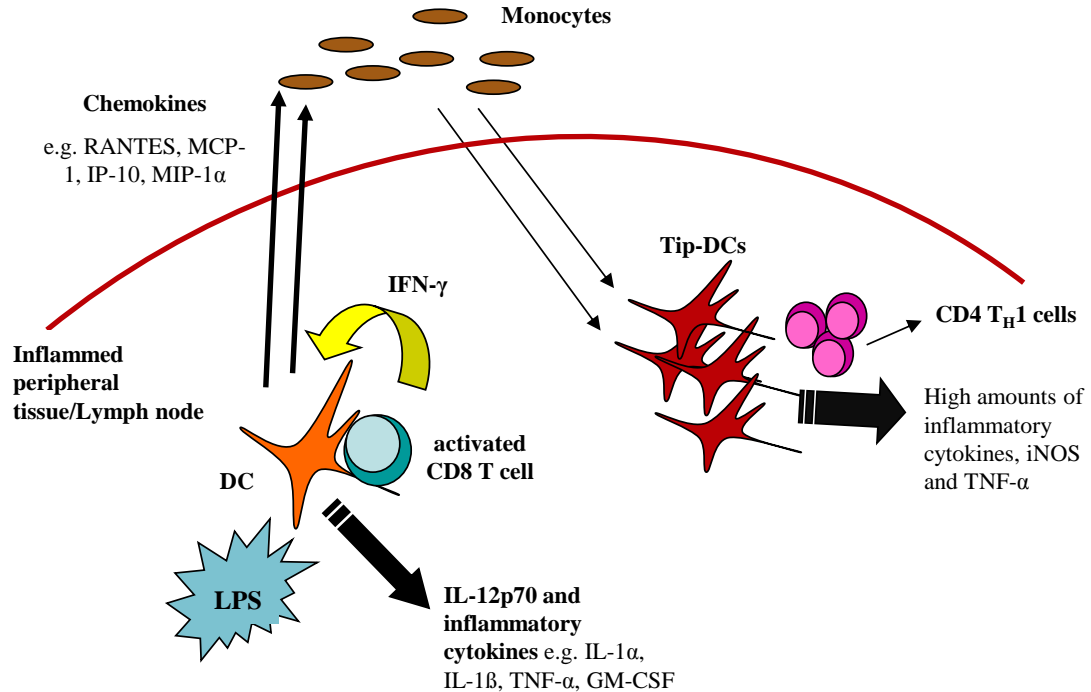
Fig.8.1 Model for the role of CD8 T cells in orchestrating a Th1 response.

8.2 Limitations of study

One of the main limitations of this study is the physiological relevance of the model that was proposed of how human CD8 T-cells could interact with DCs to produce IL-12p70 and support Th1 responses through the differentiation of Tip-DCs. As this model was demonstrated *in vitro*, it remains unknown where and how this could occur *in vivo*. In addition, since we have only adopted supernatants from co-cultures of CD8 T-cells and DCs to differentiate monocytes, it is unclear if other soluble factors may play a role in an *in vivo* setting. To try and understand how this may take place, we have investigated CD8 T-cells and Tip-DCs in an *in vivo* murine model of contact hypersensitivity and demonstrated that CD8 T-cells are indeed important for the differentiation of Tip-DCs. In addition, several studies in mice have shown that CD8 T-cells are essential for the promotion of Th1 responses *in vivo* which may occur through promoting IL-12p70 from DCs (Gurunathan et al., 2000; MacAry et al., 1998; Peterson et al., 2002; Thomas et al., 2002; Uzonna et al., 2004). However, while these *in vivo* studies in mice have supported our model of human CD8 T-cell's role in Th1 responses *in vitro*, the translational implications from mice to humans still remains unknown.

Human psoriasis studies, which allow investigations through skin biopsies obtained from normal skin and psoriatic lesions, have provided several indications that CD8 T-cells may play an important role in the pathology of this disease. CD8 T-cells have been shown to be the key player in the maintenance of chronic human psoriatic lesions, due to the association of MHC class I with psoriasis (Henseler and Christophers, 1985), the

persistence of Vß3 and Vß13.1-bearing CD8 T-cells in lesions (Chang et al., 1994) as well as the rapid and selective clearing of CD8 T-cells with successful UVB therapy (Krueger et al., 1995) in association with the clinical resolution of the lesion. CD8 T-cells have also been shown to interact with DCs in psoriatic lesions where monocytes were also found to accumulate (Ghoreschi et al., 2007). Moreover, increased expression of IL-12p70 by DC subsets has been detected in these lesions (Rosmarin and Strober, 2005; Yawalkar et al., 2009). Notably, a clinical study conducted by Chamian and colleagues (2004) has shown that administration of alefacept, an antibody that reduces the infiltration of activated memory CD8 T-cells into the skin, reduced DC content, inflammation and tissue expression of iNOS and IFN-γ in psoriasis patients. While this study did not mention the ability of CD8 T-cells to differentiate monocytes into Tip-DCs, the presence of Tip-DCs have also been recently described in human psoriasis studies (Lowes et al., 2005; Zaba et al., 2008). Taken together, these studies strongly suggest that human CD8 T-cells could mediate Th1 responses through DC IL-12p70 and differentiation of Tip-DCs.

8.3 CD8 T-cells and the importance of their Th1-inducing responses

While the ability of CD8 T-cells to eliminate pathogens and transformed cells through CTL killing mechanisms is well established, their helper role in maintaining Th1 responses has only recently been appreciated. Our results demonstrate that activated IFN-γ producing CD8 T-cells can rapidly prime both allogeneic and peptide-pulsed DCs for IL-12p70 production. IL-12p70 is a hallmark inflammatory cytokine essential for

inducing potent immune responses (Brunda et al., 1993; Trinchieri, 2003; Weiss et al., 2007). Notably, its production is limited to innate immune cells such as DCs, macrophages, and neutrophils, thereby providing a critical bridge between innate and adaptive immunity (Trinchieri, 2003).

The potential therapeutic uses of IL-12 in correcting Th2 dominant diseases have been described. In particular, many murine studies have shown that this cytokine can potently inhibit allergic airway inflammation in the lung (Gavett et al., 1995; Hogan et al., 1998; Stämpfli et al., 1999). In addition, adoptive transfer of CD8 Tc1 cells into asthmatic mice suppressed airway hyperresponsiveness and allergic airway inflammation (Ishimitsu et al., 2001; Renz, 1994; Takeda et al., 2009; Wells et al., 2007). Unfortunately, administration of IL-12 to patients with asthma has been limited due to toxicity and adverse side effects (Bryan et al., 2000). Nevertheless, the use of IL-12 linked to polyethylene glycol (PEG) moieties serves as another treatment option that has the potential of inducing a Th1 cytokine response (Leonard and Sur, 2003). This mode of administration is likely to enhance cytokine delivery to the target organ, while decreasing its toxicity, thereby serving as a promising IL-12 therapy for asthma.

In cancer, the use of IL-12 in human clinical trials has shown promising results in some forms of cancer (Anwer et al., 2010; Atkins et al., 1997; Younes et al., 2004). Importantly, studies have shown that transfer of type-1 polarized DCs that are capable of producing high amounts of IL-12 have proven effective in treating patients with recurrent maglinant glioma (Okada et al., 2011). In particular, Kalinski and colleagues (Nakamura

et al., 2007) have shown that heterologous IFN-γ-producing CD8 T-cells can support the ability of DCs to produce IL-12p70 *in vivo* which strongly enhances the IL-12p70-dependent immunogenic and therapeutic effects of vaccination in the animals bearing established tumors. Recently, Kerka and colleagues (Kerkar et al., 2011) also demonstrated that engineering tumor-specific CD8 T-cells to secrete IL-12 resulted in improved therapeutic efficacy in a B16 mouse model of established melanoma. Interestingly, they report that these engineered CD8 T-cells interacted with myeloid derived suppressor cells, macrophages, and DCs within tumors via MHC class I and upon producing IL-12, triggered a programmatic change in these suppressive APCs which allow them to improve the ability of antigen-specific CD8+ T cells to eliminate large established tumors. These results suggest that the use of CD8 T-cells or type-1 polarized DCs to re-program the suppressive tumor environment into a Th1 inflammatory state is a promising therapy for the treatment of solid tumors.

8.4 Challenges of characterizing monocyte-derived cells into DCs or macrophages

The role and function of DCs and macrophages have been clearly distinctive until recent years with the discovery of increasing subsets of DCs and macrophages in different anatomical sites that appear similar in function and phenotype to each other. This is particularly true during inflammation when monocytes can differentiate into DCs or macrophages and is compounded by the lack of reliable surface markers that truly differentiates DCs from macrophages. For example, while CD11c has been shown to be a classical DC marker for mouse studies, alveolar macrophages in the lung have also been

found to express CD11c (Gonzalez-Juarrero et al., 2003; Jakubzick et al., 2008); while CD11b and F4/80, markers universally thought to identify macrophages have been shown to be expressed very poorly on splenic and pulmonary macrophages (Gonzalez-Juarrero et al., 2003; Idoyaga et al., 2009; Jakubzick et al., 2008). The functional paradigm of DCs and macrophages has also been challenged when recent studies show that LCs are not important for T-cell priming (Kissenpfennig et al., 2005) and while some macrophages such as Kupffer cells are actively phagocytic, other macrophages like microglial cells are not (Geissmann et al., 2010). Nevertheless, the general consensus is that DCs are the most potent antigen-presenting cells (Geissmann et al., 2010)

The characterization of differentiated monocytes into Tip-DCs has also been controversial. Some have proposed that these Tip-DCs, first described during infection with *Listeria monocytogenes* (Serbina et al., 2003), resemble activated M1 macrophages (Geissmann et al., 2010) as M1 macrophages have also been shown to express high levels of co-stimulatory molecules, oxidative metabolites and inflammatory cytokines (Gordon and Taylor, 2005). However, although Tip-DCs and M1 macrophages share several functions and express common genes, we have shown in our studies that human monocytes differentiated with CD8 T-cell-DC supernatants develop functional and phenotypic characteristics of Tip-DCs and differed slightly from M1 macrophages. We found that these Tip-DCs had lower phagocytic activity, higher MHC II expression and were more efficient in priming naive T-cells compared to M1 macrophages. Moreover, a study performed by Delneste and colleagues (2003) has shown that human monocytes differentiated with IFN-γ and GM-CSF, two important factors for Tip-DC differentiation,

exhibited more DC-like properties compared to monocytes cultured with IFN-γ and M-CSF. Therefore, it would be interesting to determine if the levels of GM-CSF versus M-CSF during inflammation may also alter the functional outcome of differentiated monocytes into DCs or macrophages respectively *in vivo*. Moreover, a clearer distinction between Tip-DCs and M1 macrophages can only be made when both Tip-DCs and M1 macrophages can be found and distinguished in the same anatomical inflammatory setting *in vivo*. Therefore, it would be of interest to further characterize the Tip-DCs from other macrophage-like cells seen in inflamed skin of CHS mice to establish these differences. In addition, it is hoped that microarray and proteomic analysis will yield new markers to detect proteins that will better distinguish macrophages from DCs.

8.5 Potential role of Tip-DCs in allergic contact dermatitis

We found that a decrease in Tip-DCs was associated with a reduction in CHS responses in our *in vivo* model of contact hypersensitivity. In addition, our preliminary data showing the clustering of Tip-DCs with T-cells in the inflamed skin, as well as with endothelial structures, suggest that Tip-DCs may potentially play a role in the induction of T-cell responses and vascular changes. Tip-DCs have been shown to interact with T-cells in many peripheral inflammatory settings. In skin infection with *L. major*, Tip-DCs have been implicated in the induction of CD4 Th1 responses (León et al., 2007). Infection of *Cryptococcus neoformans,* an environmental fungus that causes pulmonary infections following inhalation, also caused a skewing of Th2 from Th1 CD4 T-cell responses when Tip-DCs were absent in CCR2$^{-/-}$ mice, thereby resulting in prolonged and

more severe pulmonary infection and pathology (Traynor et al., 2002). In addition, Tip-DCs were also found to be important in supporting virus-specific CD8 T-cells in influenza-infected lungs (Aldridge et al., 2009). Lastly, Tip-DCs were also essential for supporting both Th1 and Th17 responses in skin lesions of psoriatic patients (Zaba et al., 2007). Since Tip-DCs were demonstrated to prime Th1 responses in our human *in vitro* data, it would be of interest to determine if Tip-DCs present in CHS-elicitated skin are also capable of mediating such Th1 responses.

In particular, monocyte-derived cells have been shown to be involved in modulation of vascular structures as they express high levels of TNF-α, ROS as well as vascular endothelial growth factors (VEGFs) (Eubank et al., 2003; Gordon and Taylor, 2005; Gröne et al., 1995; Hojo et al., 2000). In particular, TNF-α is a major mediator of vascular permeability (Clauss et al., 2001; Ferrero et al., 2001; Hofmann et al., 2002) while the production of NO from iNOS-expressing cells can induce vasodilation and vascular leakage, resulting in edema at peripheral sites (Hughes et al., 1990; Ialenti et al., 1992). Moreover, NO can interact with the superoxide anion to produce a reactive peroxynitrite radical that causes cell damage and is cytotoxic to endothelial cells leading to further vascular damage (Beckman et al., 1990; Parks and Granger, 1983). VEGF production not only leads to an increase in angiogenesis and lymphangiogenesis, but also an increase in vascular permeability when present in excessive amounts (Murohara et al., 1998; Sirois and Edelman, 1997; Weis and Cheresh, 2005). Since Tip-DCs also express high levels of TNF-α and iNOS and are found to locate proximately to endothelial structures, it would be interesting to determine if they also express VEGFs and if they

contribute to vascular permeability leading to exacerbation of edema seen in CHS responses.

8.6 Monocytes and immunotherapy

The rapid infiltration of classical monocytes during inflammation is important for the clearance of pathogens (Geissmann et al., 2010). However, their excessive and prolonged recruitment hinders the resolution of inflammation and is a hallmark of numerous diseases. Studies of allergic contact dermatitis have shown that treatment with corticosteroids, which resolves symptoms associated with inflammation, is accompanied by a reduction in monocytes (Tuckermann et al., 2007). Since we also showed that the decreased presence of monocyte-derived Tip-DCs was accompanied with a reduction in CHS responses, targeting monocytes could be of therapeutic value to reduce the pathology of diseases.

In particular, CCR2$^{-/-}$ mice have attenuated levels of inflammation in many disease models (Shi and Pamer, 2011). Indeed, a recent study (Leuschner et al., 2011) showed that monocytes can be targeted via CCR2 by developing a lipid nanoparticle that encapsulated a short interfering RNA (siRNA) that selects for *Ccr2* mRNA (siCCR2). By labelling the siRNA with a near-infrared fluorochrome, siCCR2 could be tracked in mice using fluorescence molecular tomography. siCCR2 was injected intravenously and was shown to accumulate in sites of monocyte reservoirs, such as the spleen and bone marrow, with the highest uptake of siCCR2 by Ly6C^{hi} monocytes. Importantly, the

authors showed that knocking down CCR2 with siRNA yielded therapeutic benefits in a couple of disease models. Pre-treatment of mice for 3 days before or 1 hour following induction of ischaemia–reperfusion injury with siCCR2 resulted in a decrease in the infarct size by 34%. Similarly, treatment of atherosclerotic mice with siCCR2 decreased the number of Ly6C^{hi} monocytes in the atherosclerotic plaques and reduced the lesion size in the aortic root and siCCR2 treatment of mice with diabetes prior to pancreatic islet transplantation significantly extended the normoglycaemic period. Finally, siCCR2 treatment of mice that had palpable tumors resulted in a reduction in tumor growth and tumor-associated macrophage numbers, as well as decreased vascular endothelial growth factor levels and number of microvessels in the tumor.

8.7 Future directions

Determining if murine monocytes differentiate towards Tip-DCs *in vitro*

Although we have shown that human monocytes are able to differentiate into Tip-DCs *in vitro* with CD8 T-cell-DC cytokine milieus, whether this may occur using murine monocytes *in vitro* remains unknown. Therefore a few key experiments demonstrating this phenomenon would be useful to complete the mechanism that has been shown in the mouse model of contact hypersensitivity *in vivo*.

Determining other factors important for the differentiation of Tip-DCs

We showed that IFN-γ was an important factor for the differentiation of human monocytes into Tip-DCs. However, differentiation of monocytes with IFN-γ alone was

insufficient to generate viable cells that could prime T-cells as well as Tip-DCs. In particular, GM-CSF has been shown to be a promising factor in mouse studies for the differentiation and support of inflammatory DCs (Campbell et al., 2011; Shortman and Naik, 2007; Xu et al., 2007). It would hence be of interest to determine if GM-CSF is the missing factor in our human *in vitro* studies in the differentiation of viable Tip-DCs. In addition, the IL-12p40 homodimer has recently been implicated in the stimulation of myeloid cells to produce TNF-α and NO (Jana et al., 2003; Pahan et al., 2001). Therefore a greater understanding of the factors that are necessary for the differentiation of Tip-DCs, this would help with the manipulation of cytokine therapies beneficial for improving diseases.

Exploring the dichotomy of CD8 T-cells in DNCB vs TMA elicitation of CHS

In support of previous findings, we showed that TMA-treated mice had a significant increase in Th2 cytokines such as IL-4, IL-5, IL-10 and IL-13 as compared to DNCB-treated mice. While studies have shown that such Th2 cytokines are mainly produced by CD4 T-cells during CHS (Dearman et al., 1996; Gorbachev et al., 2001b; Xu et al., 1996), CD8 T-cells expressing Th2 cytokines, known as Tc2 cells, have also been reported in other disease models (Ito et al., 2005a; Salgame et al., 1991; Ying et al., 1997). Given that CD8 T-cells infiltrate as early as 3 hours after challenge into the elicitation site (Akiba et al., 2002; Kish et al., 2011), it would be of interest to determine if CD8 Tc2 cells are present in TMA-treated mice and if they play a role in attenuating the inflammatory response.

Understanding the physiological and immunological role of Tip-DCs in skin inflammation

We noted that CCR2$^{-/-}$ mice had a dramatic decrease in the infiltration of Tip-DCs during CHS elicitation. This was accompanied by a reduction in the amount of edema seen in the ears. Neutrophils and mast cells have been shown to be the main mediators of vascular permeability (Di Gennaro et al., 2009; Dvorak, 2005; Edens and Parkos, 2003; Oschatz et al., 2011). However, it is unknown if Tip-DCs can exacerbate vascular permeability to aggravate the inflammatory response. It would therefore be of interest to determine, for the first time, if Tip-DCs are responsible for the exacerbation of vascular permeability by conducting *in vitro* assays with Tip-DCs and dermal blood endothelial cells. In addition, Tip-DCs were shown to interact with T-cells in the inflamed skin of WT mice. The repertoire of T-cells in the dermal skin is heterogeneous, consisting of both αß$^+$ and γδ$^+$ T-cells. While the role of αß$^+$ T-cells in CHS is well established, γδ$^+$ T-cells have shown both inflammatory and down-regulatory functions (Guan et al., 2002; Sumaria et al., 2011) and further studies on how Tip-DCs may modulate γδ$^+$ versus αß$^+$ T-cells can be conducted to further understand their role in the exacerbation or resolution of inflammatory responses.

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