

CHARACTERIZATION OF HUMAN IL-15-INDUCED DENDRITIC CELLS AND
MOLECULAR SIGNALING MECHANISMS INVOLVED IN DENDRITIC CELL
MATURATION AND IMMUNE FUNCTIONS

By

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This is dedicated to my grandmother who has been my motivation for this journey.

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LIST OF ABBREVIATIONS

Ag	Antigen
APC	Antigen presenting cell
APC	Allophycocyanin
BFA	Brefeldin A
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid (DNA)
CMV	Human cytomegalovirus
CyC	Cy-chrome
CSFE	Carboxyfluorescein diacetate, succinimidyl ester
DC	Dendritic cell
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule -3 grabbing non-integrin
DMSO	Dimethylsulfoxide
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated kinase
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
γ c	Cytokine receptor common subunit gamma (interleukin-2 receptor subunit gamma)
GM-CSF	Granulocyte macrophage-colony stimulating factor
GM-CSFR α	Granulocyte macrophage-colony stimulating factor receptor subunit alpha
HCV	Human hepatitis C virus
HLA	Histocompatibility leukocyte antigen

JAK	Janus kinase
IFN- γ	Interferon gamma
IL-1 α	Interleukin-1 alpha
IL-1 β	Interleukin-1 beta
IL-2R β	Interleukin-2 receptor subunit beta
IL-4	Interleukin-4
IL-4R α	Interleukin-4 receptor subunit alpha
IL-6	Interleukin-6
IL-15	Interleukin-15
IL-15R α	Interleukin-15 receptor subunit alpha
imDC	Immature dendritic cell
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinases
mDC	Mature dendritic cell
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
NK cell	Natural killer cell
OVA	Ovalbumin
PBL	Peripheral blood lymphocyte
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PD-L1	Programmed cell death ligand 1
PE	Phycoerythrin
PMA	Phorbol-12-myristate-13-acetate

qRT-PCR	Quantitative real time-polymerase chain reaction
RT-PCR	Reverse transcriptase-polymerase chain reaction
SEM	Standard error of the mean
STAT3	Signal transducer and activator of transcription 3
STAT5	Signal transducer and activator of transcription 5
STAT6	Signal transducer and activator of transcription 6
TBS	Tris-buffered saline
TCR	T cell receptor
TLR4	Toll-like receptor 4
TNF α	Tumor necrosis factor alpha

Abstract of Dissertation Presented to the Graduate School
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Dendritic cells (DCs) have an essential role in inducing and modulating innate and adaptive immunity. The discovery of how to generate large numbers of human immunostimulatory DCs *in vitro* using monocytes, GM-CSF, and IL-4 lead to numerous studies attempting to exploit the immunogenic nature of DCs for immune therapies. Currently most DC-immune studies and DC-based clinical trials utilize these IL-4 DCs. IL-4 DC-based immunotherapy shows promising results but low efficacy. Thus alternative cytokine combinations to generate DCs with stronger immunostimulatory abilities have been evaluated. DCs generated with GM-CSF and IL-15 (IL-15 DCs) were the first alternative DC subset to the IL-4 DCs that are capable of priming cytolytic CD8⁺ T cell responses. IL-15 DCs stimulate stronger T cells responses than IL-4 DCs, but the mechanism(s) how this occurs is unknown.

In our work, IL-15 DCs elicited significantly greater antigen-specific cytolytic CD8⁺ and CD4⁺ T cell responses compared to IL-4 DCs. We found that IL-15 DCs were phenotypically less differentiated and mature than IL-4 DCs; however, many surface molecules involved in priming T cells were similarly expressed on both DC subsets.

Compared to IL-4 DCs, IL-15 DCs secreted more pro-inflammatory cytokines including IL-6, IFN- γ , and TNF α which may facilitate polarization of cytolytic T cell responses.

Our assessment of IL-4- and IL-15-mediated molecular signaling pathways indicated that each of the DC subsets have their own distinctive signaling profile which consequentially modulates their particular immune functions. The STAT5, STAT6, and ERK1/2 pathways were significantly more activated in IL-4 DCs compared to IL-15 DCs. IL-15 does not directly activate the STAT5 or STAT3 pathways in DCs although it strongly up-regulates pSTAT5 levels in lymphocytes. Although IL-15 does not directly activate the conventional STAT pathways, activation of the p38 and STAT3 pathways are increased during IL-15 DC maturation but not in IL-4 DCs. Furthermore, the STAT3 pathway appears to regulate IFN- γ production in the DCs. These results suggest that in comparison to IL-4 DCs, greater activation of STAT3 in mature IL-15 DCs helps to increase IFN- γ production by the IL-15 DCs, and the subsequent polarization of more antigen-specific cytolytic T cell responses.

CHAPTER 1 OVERVIEW

Human Dendritic Cells

Dendritic cells (DCs) are bone marrow-derived professional antigen presenting cells (APCs) which play an essential role in the induction and modulation of adaptive and innate immune responses. DCs are a heterogeneous population of cells that are identified primarily by their abilities to up-take antigens (Ags) in the peripheral blood and tissues, migrate to lymph nodes, and stimulate lymphocytes responses specifically against the particular Ag. DCs were first identified and characterized in mice by Steinman and Cohn in a series of publications from 1973 to 1974 (1-3). Since then, two major types of human DCs have been recognized which can be differentiated from each other based on a distinctive expression pattern of surface markers and unique immune behaviors. These DC types are called myeloid DCs, which are the more conventional and longer studied DC type, and more newly discovered plasmacytoid DCs (pDCs) which are also referred to as interferon-producing cells (IPCs). Human myeloid DCs are CD11c⁺ TLR4⁺ TLR7⁻ DC-SIGN⁺ whereas pDCs are CD11c^{low} TLR4⁻ TLR7⁺ IL-3R⁺, dependent on IL-3 rather than GM-CSF for differentiation, and secrete very high levels of type 1 interferons (IFN- $\alpha\beta$) (4, 5). In this report, we will focus on the more highly studied myeloid DCs.

Myeloid DCs have two distinct functional states, the immature and mature. After differentiation into immature DCs (imDCs), the imDCs enter the peripheral blood into non-lymphoid tissues. CD11c⁺ immature DCs (imDCs) typically express a few other DC-identifying surface markers such as CD1a and DC-specific intercellular adhesion molecule -3 (ICAM-3) grabbing non-integrin (DC-SIGN). CD1a is involved in the

presentation of primarily self- or microbial-lipids or glycolipid Ags to T cells and its expression may be sensitive to the availability of serum (6, 7). DC-SIGN is considered to be a DC-specific surface marker. It is highly expressed on DCs and its expression is positively regulated by IL-4 (8). DC-SIGN stabilizes physical interactions between ICAM3-expressing naïve T cells and consequently promotes primary T cell responses (8-11). It is also involved in capturing human immunodeficiency virus (HIV) and the subsequent transmission of HIV to permissive T cells.

imDCs are functionally characterized by their high capability for endocytosing Ags or pathogens allowing the Ags to be processed and eventual presented to neighboring T cells (11, 12). Ag peptides are presented to T cells by the major histocompatibility complex (MHC) class I or class II molecules in order to elicit CD8⁺ or CD4⁺ T cell immune responses respectively. imDCs characteristically exhibit the phenotype of CD14^{low}, CD11c⁺, MHC class I⁺, MHC class II^{low}, DC-SIGN⁺, co-stimulatory molecules (CD80, CD86, CD83)^{low}, CCR7^{low}, PD-L1^{low} (10, 13, 14). Due to the low expression levels of the classical co-stimulatory molecules (CD80, CD86, CD83), stimulation of T cells by imDCs usually induces the T cells to become anergic (14-16). Also their low expression of MHC class II molecules impairs the physical interactions between imDCs and CD4⁺ T cells. Detection of microbial components via toll-like receptors (TLRs) or other similar receptors and/or stimulation by pro-inflammatory cytokines induce imDCs to undergo maturation.

DC maturation is typically marked by reduction in Ag up-take capacity as well as the up-regulation of MHC class II molecules like HLA-DR, co-stimulatory molecules such as CD80, CD83, and CD86, as well as the CCR7 receptor on the surface of the

cell. The significant increase in the molecule expression levels correlate with the abilities of mature DCs (mDCs) to stimulate both CD8⁺ and CD4⁺ T cell immune responses, provide a high level of co-stimulatory signaling, and effectively mobilize to the lymphoid organs (13, 17). mDCs also up-regulate the surface expression of other co-stimulatory molecules such as CD40. Increased expression of CD40 on the activated DCs leads to enhanced production of IL-12p70 when stimulated by CD40 ligand (CD40L) which is expressed on T cells and B cells. Secretion of IL-12 is an important stimulatory factor for T cell activities which will be described in more depth later. Finally another surface marker up-regulated upon DC maturation is the programmed cell death ligand 1 (PD-L1) (18). PD-L1 on mDCs binds to the programmed cell death protein 1 (PD-1) expressed on the surface of activated T cells and consequentially negatively regulates the effector functions of the particular T cell.

The ability to generate large numbers of functional DCs *ex vivo* has fueled the interest in developing DC-based immune therapeutic applications that can exploit the immunogenic nature of DCs. Up to now most of the DC vaccine clinical trials use monocyte-derived IL-4 DCs that are loaded with a tumor Ag or tumor-associated Ag to stimulate the patient's immune responses against the particular cancer or virus. These DCs would then present the tumor/tumor-associated Ag to the patient's T cells, and consequently induce robust cytolytic T cell responses specifically targeting the cancer cells expressing the particular tumor/tumor-associated Ag. Many studies have evaluated various DC preparation methods and Ag loading techniques at initiating tumor Ag- or viral Ag- specific immune responses *in vitro* and *ex vivo* including imDCs, mDCs, DCs loaded *ex vivo* with Ags, DCs that are genetically modified to express specific Ag(s),

and DCs loading with defined Ag peptides or solid tumor lysates (19-25). Nearly all of these DC vaccine strategies, except imDCs, are capable of inducing strong Ag-specific T cell responses. Additionally, many of the cancer patients treated with IL-4 DC vaccines demonstrated Ag-specific immune responses (19, 22-24, 26-28). However, the major problem that prevents IL-4 DC vaccine strategies from becoming a standard cancer immune treatment is that only a few DC vaccine clinical trials report substantial objective clinical responses.

***In Vitro* Monocyte-derived Dendritic Cells**

In vitro derived human DCs are routinely generated using purified peripheral blood monocytes. The first method to generate human immune stimulatory DCs *in vitro* was described in 1994 by Sallusto et al (29). Since then, differentiation of monocyte-derived DCs *in vitro* using GM-CSF and IL-4 has been the most used combination of cytokines and consequently the most comprehensively characterized human DC subset. As other groups evaluated other factors involved in the process of monocyte-to-DC differentiation and maturation, it became clear that DC differentiation was a fairly permissive event which is influenced by its surround environmental conditions (30, 31). Monocyte-derived DC differentiation can be affected by the manner of monocyte purification (magnetic activated cell sorting (MACS) or by plastic adherence), the cell media, and presence of serum (7, 32). Investigations of the effect of different cytokines on DC differentiation determined that GM-CSF appeared to be necessary for survival of the monocytes and promote monocyte-to-DC differentiation over monocyte-to-macrophage differentiation. The presence of GM-CSF is necessary for generating monocyte-derived DCs that are capable of eliciting cytolytic IFN- γ -producing immune responses. Studies investigated monocyte-derived DCs differentiated with only IL-4 or IL-3 and IL-4 compared to the

traditional IL-4 DCs showed that the DCs prepared without GM-CSF secreted less IFN- γ and IL-12p70 and less IFN- γ T cell responses than IL-4 DCs (33, 34).

IL-4 is used in conjunction with GM-CSF since it can also inhibit macrophage differentiation (35). In the absence of IL-4, monocyte-derived DCs that were generated with GM-CSF alone appeared less mature due to their lower expression of the classical co-stimulatory molecules in comparison to the traditional IL-4 DCs (36-38). They also secreted more IL-10 and only low amounts of IL-12, thus indicating that this DC subset would likely stimulate less cytolytic T cell responses than conventional IL-4 DCs. Other substitutions for IL-4 was used to generate monocyte-derived DCs included thymic stromal lymphopoietin (TSLP), IFN- α , or IL-15 and then their immunostimulatory capabilities were compared to IL-4 DCs. DCs generated with GM-CSF and TSLP induced strong proliferation of CD4⁺ T cells but did not induce activation of the CD8⁺ T cell population (39, 40). DCs prepared from monocytes using GM-CSF and IFN- α could not express the typical surface markers associated with DC maturation and demonstrated only limited abilities to stimulate T cell responses compared to the more traditional IL-4 DCs (41).

DCs generated using GM-CSF paired with IL-4 which are called IL-15-induced DCs or simply IL-15 DCs (42-46). In 2001 Mohamadzadeh et al. and Saikh et al. were the first to independently demonstrate that the presence of IL-15 alone or GM-CSF and IL-15 can differentiate monocytes into immune regulatory DCs (42, 44). IL-15 DCs can even be generated by culturing monocytes with conditioned media from alveolar or bronchial epithelial tumor cells that secrete high levels of IL-15 (47). These IL-15 DCs are the first alternative DC subset to the conventional IL-4 DCs that are capable of

eliciting Ag-specific cytolytic CD8⁺ T cell activity. IL-15 DCs were described to express the following DC surface markers CD14^{low} HLA-DR⁺ HLA class I⁺ CD11c⁺ CD86⁺ at similar expression levels to the classical IL-4 DCs. IL-15 DCs can stimulate melanoma-specific autologous and allogeneic CD8⁺ cytolytic T cell responses more efficiently than the conventional IL-4 DCs (43, 45). They were also shown to be able to initiate more cytolytic CD8⁺ T cells activity against viral Ags as well (46). And in contrast with the conventional imIL-4 DCs, stimulation by imIL-15 DCs does not induce immune tolerance but stimulates CD8⁺ T cell proliferation. However, how IL-15 DCs stimulate more CD8⁺ effector T cell responses against Ags compared to the more commonly studied IL-4 DCs remains unclear (43, 45). Also how the molecular signaling mechanisms regulated by either IL-15 or IL-4 affect monocyte-derived DC differentiation and/or maturation as well as subsequent immunostimulatory functions are unknown.

T Cells

T cells can be identified by their expression of the CD3 complex which is an essential component of the overall T cell receptor (TCR) complex. The TCR can recognize fragments of the Ag peptides being presented to them by DCs. T cells can be divided into two major classes that are identified by the expression of one of the two TCR co-receptors, CD8 or CD4 (48). CD8⁺ T cells interact with MHC class I molecules whereas CD4⁺ T cells associate with MHC class II molecules. Upon activation, CD8⁺ T cells display Ag-specific cytotoxic responses including directed cell lysis, degranulation of cytolytic granules containing perforin and granzyme, and production of IFN- γ (49). In contrast, CD4⁺ T cells are traditionally called helper T cells due to their involvement in facilitating the activation of other immune cells. Upon activation, CD4⁺ T cells differentiate into specialized CD4⁺ populations (50, 51). The two most studied types of

CD4⁺ T cells subsets are the type 1 helper (Th1) and type 2 helper (Th2) cells. Th1 cells characteristically produce IFN- γ , IL-2, and TNF α which help activate APCs and protect against intracellular microbial and viral infections, whereas Th2 typically secrete IL-4, IL-5, and IL-13 to help activate B cells and address extracellular parasites.

In addition CD8⁺ and CD4⁺ T cell can be further subdivided into three functional subsets known as central memory (T_{cm}), effector memory (T_{em}), and effector T cell subsets (T_{eff}) (52, 53). These subsets can be identified by the expression of certain surface markers such as markers to indicated differentiation or activated status (CD45RA, CD27, and CD28) and ability to mobilize to lymphoid tissues (CD62L and CCR7). T_{cm} cells (CD45RA⁻ CD27⁺ CD28⁺ CD62L⁺ CCR7⁺) are normally found in secondary lymphoid tissues. They exhibit marginal levels of cytolytic activity and are considered to have only a limited migratory potential. T_{em} cells (CD45RA⁻ CD27⁺ CD28⁺ CD62L⁻ CCR7⁻) circulate in non-lymphoid tissues and exhibit some cytolytic activity. T_{eff} cells (CD45RA⁻ CD27⁻ CD28⁻ CD62L⁻ CCR7⁻) produce the highest amounts of IFN- γ , perforin, and granzyme.

As a heterogeneous group of immune cells, DCs are defined by their capacity to elicit Ag-specific responses from T cells as measured by consequential T cell proliferation, cytolytic activity, and/or production of immunoregulatory cytokines. DCs prime Ag-specific T cell immune activities by providing three major signals (13, 54). Signal 1 is the presentation of Ags which is mediated by MHC class I and II molecules and other molecules such as CD1a (6, 11, 54). Signal 2 is the sufficiently expression of co-stimulatory molecules such as CD80, CD83, CD86, and CD40 which are up-regulated after DC maturation. The availability of signal 3 determines whether CD4⁺ T

cells will differentiate into Th1 or Th2 cells or for CD8⁺ T cells it helps to promote the activation of the cytolytic CD8⁺ T cell responses. Signal 3 is classically the production of IL-12 by DCs but signal 3 can also include other pro-inflammatory cytokines that induce IFN- γ production (50, 55-57).

Natural Killer Cells

In addition to priming T cell immune response, DCs can also activate natural killer (NK) cells. NK cells are specialized innate immune cells that respond to detected viral- or pathogen-infected and tumor cells by either lysing the target cells or secreting immunoregulatory cytokines. Human NK cells are identified as CD3⁻ CD56⁺ lymphocytes (58-60). They, like T cells, are functionally heterogeneous and can be subdivided into two major subsets based on their distinctive immune activities. These NK cell subsets are often referred to as the cytolytic or immunoregulatory NK cells and can be identified by the differential expression of CD56 and CD16. CD56 is a neural cell adhesion molecule with unknown functionality on NK cells and CD16, which also called Fc γ receptor III (Fc γ RIII), binds to antibody-coated target cells and then facilitates antibody-dependent cellular cytotoxicity (ADCC). Most of the circulating NK cells are CD56^{dim} CD16⁺ and considered to be the cytotoxic subset on NK cells. Upon activation, CD56^{dim} CD16⁺ NK cells are readily able to directly kill target cells by either exocytosis of cytolytic granules containing perforin and granzyme and/or the death receptor pathway. Additionally CD56^{dim} CD16⁺ NK cells can exhibit high levels of ADCC which is mediated by CD16. The second major subset of NK cells are CD56^{bright} CD16⁻ and commonly referred to as immunoregulatory NK cells. CD56^{dim} CD16⁺ NK cells can kill target cells without engagement of CD16 or prior stimulation. Activated CD56^{bright} CD16⁻

NK cells are generally not efficient at killing their target cells; however, they can produce large amounts of immunoregulatory cytokines such as IFN- γ , TNF α , and IL-10 (61).

Unlike T cells and their TCRs, NK cells do not rearrange genes that encode Ag-specific receptors for activation. Instead they express a variety of surface receptors called the NK cell receptors (NKR) that can either activate or inhibit the NK cell immune functions. NK cell activation is mediated by the balance of activating and inhibitory signals from the NKR as well as the presence of inflammatory cytokines.

Along with their cytolytic functions, NK cells can regulate immune functions of other cells including T cells and DCs. CD56^{bright} NK cells secrete high amounts of IFN- γ which can facilitate the polarization of IFN- γ responses by T cells and restrict tumor angiogenesis (58, 62, 63). The presence of activated NK cells synergistically enhances the anti-tumor cytolytic T cell responses primed by mDCs (64). Production of IFN- γ by CD56^{bright} NK cells is generally based on two signals. The first signal usually being the availability of IL-12 and the second one includes the presence of IL-2, IL-15, IL-18 or stimulation of an NK activating receptor like CD16. In contrast, NK cells stimulated with IL-12 paired with IL-15 can secrete large amounts of the anti-inflammatory cytokine IL-10 which can inhibit the T cell IFN- γ responses. Additionally, highly cytolytic NK cells can provide particles of the infected or tumor cells for DCs to up-take, process, and present for priming T cell responses.

Crosstalk between DCs and NK cells is important for both cell types. Cell-to-cell interaction between DCs and NK cells enhances NK cell cytokine production, proliferation, and cytolytic activity (65-67). mDCs are the main producers of IL-12 which is essential for inducing IFN- γ production in the NK cells (61). mDCs also secrete IL-2,

IL-15, and IL-18 which can enhance the production of IFN- γ by the NK cells.

Furthermore secretion of IL-15 by mDCs helps to maintain the NK cell population and increase NK cells' cytotoxicity functions (68-70). Additionally mDCs present IL-15 bound to the IL-15 receptor alpha subunit (IL-15R α) *in trans* to neighboring NK cells which necessary for NK cell development and survival (71-74). IL-15 trans-presentation is also demonstrated to be essential for NK cell activation and the enhancement of NK cell cytolytic functions (72-75). In murine models it was shown that NK cells are not consistently poised on the brink of activation *in vivo* thus they require trans-presentation of IL-15 by mDCs to prime these resting NK cells for immune activation (76, 77). Soluble IL-15 can bind to IL-15R α expressed on the surface of mDCs but the optimal trans-presentation of IL-15 occurs in mDCs co-expressing both IL-15 and IL-15R α (73, 78, 79).

NK cells can directly lyse imDCs at high NK cell-to-DC ratios due to low expression levels of MHC class I molecules on the imDCs, a classic NK cell activation scenario (80, 81). Alternatively, at low NK cell-to-DC ratios physical interaction with NK cells and the availability of large amounts of NK cell-derived IFN- γ and TNF α can induce DC maturation and enhance the immune stimulatory functions of the resulting mDCs (67, 80, 82, 83). Additionally, NK cells also produce GM-CSF which supports DC maturation as well as monocyte-to-DC differentiation and survival (63, 81, 84). The decision for NK cells to lyse imDCs or induce maturation was hypothesized to be dependent on the NK cell-to-DC density but the results of later studies suggested that other factors including DC-NK cell co-culture duration or culture conditions for imDC differentiation may influence the outcome (77, 85).

IL-4

IL-4 is mainly produced by activated type 2 helper (Th2) CD4⁺ T cells, basophils, and mast cells (86, 87). IL-4 is a critical factor for inducing differentiation of naïve CD4⁺ T cells into the specialized Th2 CD4⁺ T cell subset. It inhibits IFN- γ transcription, which ultimately prevents the differentiation of the naïve CD4⁺ T cells into Th1 cells, the other specialized CD4⁺ T cell subset. IL-4 also inhibits the production of inflammatory cytokines such as IL-6, IL-15, and TNF α (88). In DC precursor cells, IL-4 plays a role in the *in vitro* differentiation of monocyte-derived myeloid DCs (29). It enhances LPS-induced maturation and IL-12 production in murine bone marrow-derived DCs as well as induces IL-4 production (89-91). Moreover, the presence of IL-4 inhibited the bone-marrow precursor cells from differentiating to macrophages, and consequently promoted DC differentiation (35, 92).

In hematopoietic cells, the IL-4 receptor (IL-4R) is composed of two subunits the IL-4 receptor alpha (IL-4R α) and common gamma chain (γ_c , CD132) which is a subunit shared among several cytokine receptors including IL-2 and IL-15 (87, 88, 93, 94) (Figure 1-1). IL-4 signaling is initiated with IL-4R α (CD124) first binding with high affinity to IL-4 ($K_d = 20 - 300^{-12}$ M) and then dimerizing with the γ_c chain. This leads to the activation of the Janus kinase 1 (Jak1) and Jak3 which are respectively associated with IL-4R α and γ_c which, subsequently results in the activation of the signal transducer and activator of transcription 6 (STAT6) (86).

IL-15

IL-15 is pleiotropic cytokine which is mainly secreted by monocytes and DCs. IL-15 is essential for NK cell development and activation as well as proliferation and maintenance of CD8⁺ memory T cells (68, 72, 75, 95-97). IL-15 is highly regulated

through multiple mechanisms at the transcriptional, translational, and the post-translational levels (98-100). IL-15 mRNA is expressed in a wide variety of cells and tissues including monocytes, DCs, epithelial cells, and fibroblasts but it is absent in T cells (98-101). The IL-15 transcription is regulated by the 10 transcriptional initiation sites (AUG sites) in the 5' untranslated region (UTR) and alternative splicing (98, 102). Alternative splicing generates two IL-15 isoform precursor proteins which can be distinguished from each other by the length of their signal peptide sequences (102, 103). One of the IL-15 precursor proteins has a 48 amino acid (aa) long signal peptide (IL-15LSP) and the other has a 21 aa short signal peptide (IL-15SSP). Notably, the longer IL-15 isoform is secreted while the shorter isoform is not secreted.

The IL-15LSP precursor protein is translocated to the Golgi apparatus for further protein modification while the IL-15SSP remains in the cytoplasm. While in the Golgi apparatus, the maturing IL-15 protein binds to the IL-15 receptor alpha (IL-15R α) chain that also localizes in the Golgi apparatus (75, 104). The IL-15R α acts as a chaperone protein for the associated IL-15 protein where it directs mobilization from the Golgi apparatus to the surface membrane. IL-15 can also be stored intracellularly until the appropriate stimulation (LPS or GM-CSF) which then causes the sequestered IL-15 to translocate to the surface membrane (105). Once on the cell surface, the IL-15R α -IL-15 complex is released by proteolytic cleavage of matrix metalloproteinases.

The IL-15 receptor (IL-15R) is composed of IL-15R α , IL-2 receptor beta (IL-2R β), and γ c (Figure 1-1). Unlike IL-4, there are three distinct signaling mechanisms mediated by IL-15, which occur in different cell types and are commonly called IL-15R-mediated signaling, reverse signaling, and trans-presentation (Figure 1-2). In the classic IL-15R-

mediated mechanism, IL-15R α first binds to IL-15 and then complexes with the IL-2R β - γ c dimer (98-100). Jak1 is rapidly recruited to the IL-2R β chain to be activated while Jak3, which is associated with the γ c chain, is also activated. The phosphorylated Jak 1 and Jak3 proteins will then activate the STAT3 and STAT5 pathways respectively. An alternative IL-15R complex called IL-15RX has been discovered in mast cells (98, 100). The IL-15RX complex is not well studied but it does not associate with the traditional IL-15R α , IL-15R β , or γ c chains and activates the JAK2–STAT5 and tyrosine kinase 2 (TYK2)–STAT6 pathways.

The IL-15R α chain binds IL-15 with very high affinity ($K_d=10^{-11}$ M) but it does not transduce intracellular signaling by itself (101). It is needed for IL-15R-mediated signaling but seems more involved in anchoring IL-15 to the membrane of mainly DCs or monocytes. The presentation of IL-15 *in trans* to the IL-2R β - γ c dimer on a neighboring NK or T cell is another IL-15 signaling mechanism referred to as IL-15 trans-presentation (73-75). IL-15 trans-presentation is critical for NK cell development and activation as well as for the maintenance of CD8⁺ memory T cells (68, 75, 97). Addition of soluble IL-15 can be “loaded” onto the existent IL-15R α on the cell surface but IL-15 is mostly likely associated with IL-15R α in the cytoplasm of IL-15-producing cells such as monocytes and DCs (73, 75, 79, 106). IL-15 bound to IL-15R α on the cell membrane can undergo trans-endosomal recycling which prolongs the effect of IL-15 trans-presentation on responding NK and T cells (78). The engagement of IL-15 with the IL-2R β - γ c dimer ($K_d\sim 10^{-9}$ M) activates the Jak1-STAT3 and Jak3-STAT5 pathways in a similar manner as the IL-15R-mediated mechanism.

The third IL-15 signaling mechanism called IL-15 reverse signaling also involves biologically active membrane-bound IL-15 on monocytes and monocytic cell lines. However in this mechanism, IL-15 is not attached to the surface membrane using the IL-15R α chain but an unidentified anchor (107, 108). Cross-linking membrane-bound IL-15 using anti-IL-15 antibodies on monocytes activated Rac3, a Rho GTPase involved in actin rearrangement and consequently increased cellular adherence (109). In prostate cancer and renal cancer cells, activation of the membrane-bound IL-15 leads to phosphorylation and activation of focal adhesion kinase-1 (FAK) which mediates cell motility and adhesion (107, 110). Additionally, cross-linking of the IL-15 on the cell surface also activates two mitogen-activated protein kinase (MAPK) family members the extracellular signal-regulated kinase 1/2 (ERK1/2) and p38 (107, 109, 110). Activation of ERK1/2 and p38 in monocytes and monocytic cell lines increased production of the inflammatory IL-8, IL-6, and TNF α mRNA transcript and protein levels (107, 109).

JAK-STAT Signaling Pathway

The Jak-STAT signaling pathway is activated by many cytokines, growth factors, and hormones. The Jak kinase family consists of four protein kinases JAK1, JAK2, JAK3, and TYK2. Jak kinases are usually bound to the cytoplasmic domains of their receptors, where they function to phosphorylate tyrosine residues on the receptor chains and recruited STAT proteins. There are seven STAT proteins STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6. Before activation, the non-phosphorylated STAT proteins are mainly found in the cytoplasm. Activated STAT proteins have dual functions as molecular signaling molecules in the cytoplasm and transcription factors (TFs) in the nucleus. Each STAT family protein responds to a defined set of cytokines, and each also regulates, with other transcription factors and/or

cofactors, a group of specific genes. In this report, we will focus on STAT6, STAT5, and the STAT3 proteins since they are known to be directly activated by at least one of the three cytokines used in IL-4 DC or IL-15 DC differentiation.

The start of the JAK-STAT signaling cascade begins with receptor-cytokine association leading to the dimerization or trimerization of all of the activated receptor subunits. This allows the associated JAKs to become activated and cross-phosphorylate tyrosine residues on the receptor chains' cytoplasmic domains. The phospho-tyrosine residues consequently recruit certain STAT proteins to these docking sites. After binding to the phospho-tyrosine docking site with their Src homology 2 (SH2)-domain, the STAT proteins are phosphorylated by nearby JAKs, released from the receptor, form homo- or heterodimers, and then translocate into the nucleus to regulate target gene transcription. Depending on the particular STAT protein, STAT proteins either bind directly to the STAT-specific DNA binding sequence in the gene promoter called interferon (IFN) γ -activated sequences (GAS) or first associate with co-activator proteins such as p300 and creb binding protein (CBP) before binding to the gene promoter (111-113). The phosphorylated state of the STAT protein is transient due to its numerous negative regulatory mechanisms. Phosphorylation is negatively regulated by wide array of proteins ranging from protein tyrosine dephosphorylases, protein inhibitors of activated STATs (PIASs) which directly bind to tyrosine phosphatases, suppressor of cytokine signaling (SOCS) which bind phospho-tyrosine sites on the receptor and/or the JAK catalytic sites (114-116).

STAT6

IL-4 is the primary activator for the STAT6 pathway (117-119). STAT6 activation occurs by the phosphorylation of tyrosine 641 (Tyr641) by the IL-4R α -associated JAK1

or γ -associated JAK3. Phosphorylation of STAT6 (pSTAT6) leads to their homodimerization before they are translocated to the nucleus. STAT6 is necessary for IL-4 responsiveness by T cells and differentiation into Th2 cells which characteristically secrete IL-4 and stimulate B cells immune responses. Activation of the STAT6 pathway induces transcription of genes involved with differentiation and functions of Th2 cells such as MHC class II, IL-4 and IL-4R α (112, 118, 120, 121). pSTAT6 also negatively regulates the transcription of genes associated with Th1 cell differentiation and functions like IFN- γ , in addition to anti-apoptosis genes such as GATA3, BCL-xL and BCL-2. Murine STAT6 knock out (KO) models demonstrated the necessity of the STAT6 pathway for IL-4-mediated signaling (117, 122). STAT6 KO mice were deficient for Th2 cells. Additionally, lymphocytes from STAT6 KO mice could not up-regulate the normally IL-4-responsive MHC class II and IL-4R α upon IL-4 stimulation.

STAT5

The term "STAT5" usually refers to a heterodimer consisting of STAT5a and STAT5b proteins but in some cell types it sometimes is used to describe the STAT5a and/or STAT5b homodimers. STAT5a and STAT5b are independently encoded by two genes and share about 96% homology with each other at the protein level. Both proteins are expressed in many types of cells and tissues, but some cells and tissues predominantly express much higher amounts of only one of the STAT5 proteins. For example, STAT5a is the preferred form in the mammary gland yet in the liver STAT5b is more favored STAT5 protein (123, 124). STAT5a and STAT5b are activated by a wide range of cytokines and growth factors, including IL-2, IL-7, IL-15, GM-CSF, growth hormone, and prolactin. Upon appropriate stimulation, Tyr694 is phosphorylated on STAT5a and Tyr699 is phosphorylated on STAT5b (125). Activated STAT5a and

STAT5b can heterodimerize or occasionally homodimerize which may be dependent on cell type.

Originally STAT5a was determined to be necessary for prolactin signaling and subsequently directed mammary gland development, whereas STAT5b is essential in growth factor-mediated signaling and involved in body mass growth in males (111, 112, 124, 126, 127). Only when both STAT proteins were knocked out were there more severe immunological deficiencies such as reduction in the number of circulating NK and T cells. Recently, pSTAT5 has also been discovered to be essential for the development and/or maintenance of pDCs, regulatory T cells (Tregs), and NK cells by regulating the transcription of TFs essential for those immune cell types such as forkhead box P3 (FoxP3) for Tregs and interferon regulatory factor 8 (IRF8 or interferon consensus sequence-binding protein (ICSBP)) which negatively regulates pDC development (126, 128, 129). In addition to the FoxP3 and IRF8 genes, STAT5 is a transcriptional activator of many other genes involved myeloid DC differentiation (Id2), survival (c-myc, Bcl-x, cyclinD1), and negative regulatory proteins for its own pathway (Socs-1, Socs-3, Cis-1) (111, 123, 126, 130). Constitutive activation of STAT5 is frequently observed in many myeloid-related leukemia types and solid tumors.

STAT3

STAT3 is activated by several ligands such as IL-6, IL-10, and epidermal growth factor (EGF) which results in phosphorylation of the STAT3 protein on Tyr705 by JAK1, JAK2, or TYK2 (131-133). STAT3 is also phosphorylated on a serine residue Ser727 by an unidentified kinase (120, 131, 134). Phosphorylation of Ser727 does not affect the phosphorylation of the two tyrosine residues nor does it enhance nuclear translocation

of the activated STAT3 dimer. Ser727 phosphorylation has been suggested to increase the DNA binding capacity of activated STAT3 but it has not been definitively proven yet.

Upon activation, STAT3 homodimerizes with another activated STAT3 or heterodimerizes with an activated STAT1 protein. Activation of STAT3 positively regulates transcription of anti-apoptosis genes such as c-myc, Bcl-2, BCL-xL, and cyclin D1 (120, 132, 133, 135). Activated STAT3 also up-regulates transcription of IL-6 and IL-10 and down-regulates transcription of IFN- γ , TNF α , and IL-12 as well as MHC class II molecules and co-stimulatory molecules (CD80, CD86) (135, 136). Due to its positive regulation of inflammatory cytokines and anti-apoptotic genes, constitutive activation of STAT3 is found in many cancer types such as leukemias, lymphomas, breast carcinoma, and multiple myeloma (120, 132, 133, 135-137).

STAT3 is essential for embryonic development since attempts to establish a murine STAT3 KO model has proven to be embryonic lethal. However conditional STAT3 KO in murine macrophages and neutrophils displayed enhanced production of inflammatory cytokines like TNF α , IL-1, and IFN- γ following LPS stimulation (136). The increased IFN- γ , TNF α , and IL-6 production by murine macrophages, NK cells, or tumor cells after disruption of the STAT3 signaling pathway resulted in enhanced anti-tumor T cell responses (120, 137). Recently, other murine conditional STAT3 KO and inhibition of the up-stream JAK (JAK2) in murine DCs indicated that activation of STAT3 is detrimental for DC immunostimulatory functions (138, 139).

MAPK Signaling Pathway

Mitogen-activated protein kinases (MAPKs) are ubiquitously expressed serine-threonine kinases that regulate a plethora of diverse cellular activity including gene expression, metabolism, motility/adhesion, survival/apoptosis, and differentiation (140-

145). There are three major groups of MAPKs characterized in mammals, the extracellular signal-regulated kinase (ERK) family, c-Jun amino-terminal kinase (JNK) family, and the p38 family. We will focus on the ERK 1 and 2 kinases (ERK1/2) and the p38 kinase family in this report since they are activated by at least one of the cytokines used for DC differentiation and maturation (86, 146-149). Each MAPK group is activated by a distinct kinase cascade which is initiated by activation of a MAPK group-specific MAPK kinase kinase (MAPKKK or MAP3K) or a MAPK/ERK kinase kinase (MEKK), which then phosphorylates a downstream MAPK kinase (MAPKK or MAP2K) or a MAPK/ERK kinase (MEK), that subsequently activates the MAPK protein. The MAPK protein is activated by dual phosphorylation of two threonine and tyrosine residues that are located within activation loops of the kinase. Phosphorylation of both of the threonine and tyrosine residues causes activation loops, which originally sterically hinder access to the kinase catalytic sites, to undergo conformational changes which, then, allow the kinase to associate with its substrate. MAPK protein activities are negatively regulated by three general groups of phosphatases that exhibit a preference for dephosphorylating phospho-tyrosine or phospho-serine/threonine. The last group of phosphatases readily dephosphorylate both tyrosine and serine/threonine residues and are called a dual-specificity phosphatases (DUSPs) (142).

MAPK proteins are activated by a variety of different stimuli, but in general, ERK1/2 are mainly activated by growth factors and phorbol esters whereas the p38 kinases are more responsive to cellular stress (oxidative stress, DNA damage) and cytokine stimulation. LPS-mediated activation of the ERK1/2 and/or p38 pathways

increase expression of the classical co-stimulatory molecules (CD80, CD86, CD83) and negatively regulate transcription of MHC class II molecules (150-152)

ERK

The ERK family contains two isoforms, ERK1 and ERK2, which are commonly referred to as ERK1/2 collectively due to their structural and functional similarities. ERK1 and ERK2 are also called p44 MAPK or p42 MAPK respectively (or p44/42 MAPK). ERK is activated by the phosphorylation of both Thr202 and Tyr204 residues on ERK1 and residues Thr183 and Tyr185 on ERK2. ERK1/2 is activated by many growth factors (epidermal growth factor (EGF) and GM-CSF), phorbol esters, LPS, and inflammatory cytokines (TNF α , IL-1 β) (153-156). ERK1/2 activation induces transcription of TFs such as Egr-1 which in turn enhances transcription of the TNF α gene (154). It can also negatively regulate transcription of TFs like class II transactivator gene (CIITA) which is involved in the transcription of MHC class II molecules (151, 152). Activation of ERK1/2 also activates TFs involved in tumor suppression (p53), anti-apoptosis (Bim, Bad, Bcl-2, BCL-xL), and cell cycle-modulation (c-myc, p21^{CIP1}, and cyclin D1). Activation of the ERK1/2 pathway also leads to the phosphorylation of other essential TFs such as c-jun, Ets-1, inhibitor kappa B kinase (IKK), retinoblastoma protein (Rb), CREB binding protein (CBP), CCAAT/enhancer-binding protein- β (C/EBP β) and Elk-1 (140, 155, 157). Activated Elk-1 initiates transcription of c-fos which can then dimerize with c-jun to form the TF activator protein 1 (AP-1) that is involved in facilitating IFN- γ and GM-CSF transcription.

p38

The p38 MAPK family consists of four isoforms p38 α , β , γ , and δ which are encoded by separate genes. The p38 isotypes are about 60% homologous with each

another and differentially expressed among various tissues and cell types. Of the four p38 kinase isotypes, p38 α the most abundant and expressed in most cell types while p38 β is also widely expressed but it is found at lower levels than p38 α (158, 159). Expression of the p38 γ and p38 δ are the most cell-restricted which is suggestive of having more specialized functions than p38 α and p38 β . In general, p38 becomes activated by the dual phosphorylation at Thr180 and Tyr182 residues in response to cellular stress (oxidative stress or DNA damage), growth factors (GM-CSF), LPS, inflammatory cytokines (TNF α , IL-6), and IL-4 (146, 147, 158, 160). p38 activation regulates transcription of TFs like c-fos and CIITA which promotes as well as induces phosphorylation of other TFs such as tumor suppressor p53, oncogene c-myc, myocyte enhance factor 2C (MEF2C), MEF2A, C/EBP β , c-jun, and activating transcription factor 2 (ATF2). Finally, activation of the p38 pathway up-regulates production of many pro-inflammatory cytokines like IFN- γ , IL-1, IL-6, IL-8, IL-12p40 by either inducing transcription or stabilizing the mRNA transcripts and consequently enhancing translation of the cytokine protein.

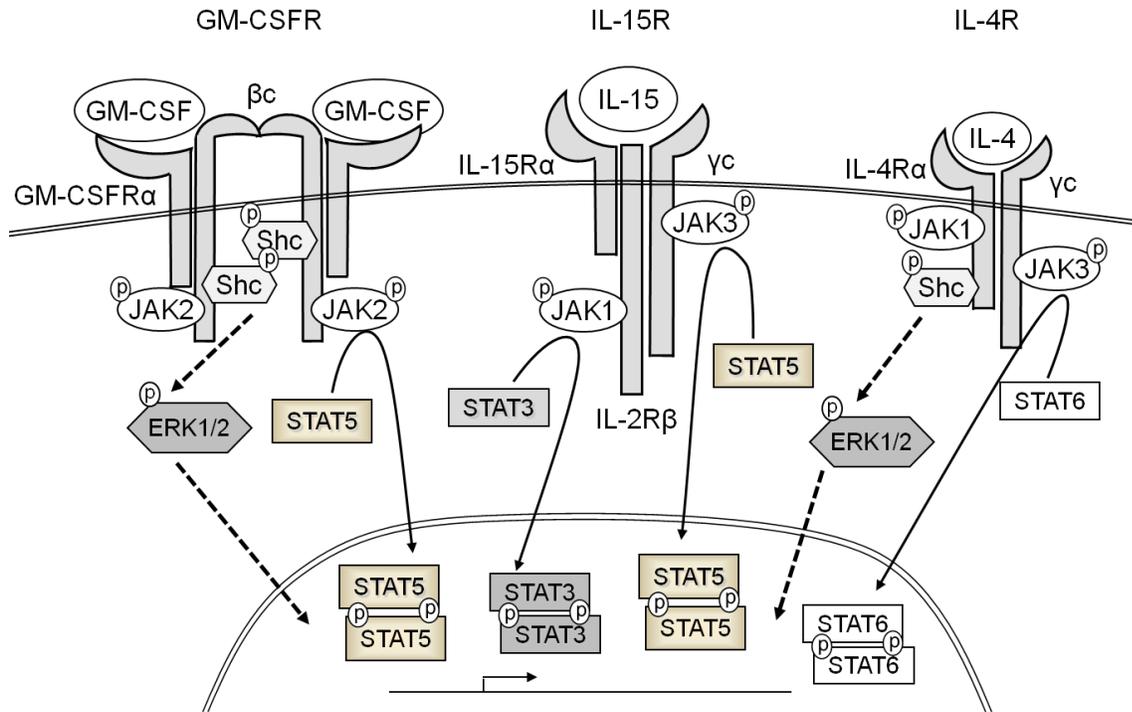


Figure 1-1. Diagram of the GM-CSFR, IL-15R, and IL-4R complexes along with the classically activated STAT or MAPK pathways. GM-CSFR consists of two GM-CSFR α chains and two common β chains (β c) that forms a hexameric complex when bound to two GM-CSF molecules. GM-CSF activates STAT5 and ERK1/2 pathways. IL-15R consists of IL-15R α , IL-2R β , and common γ chain (γ c). IL-15 primarily activates STAT5 but sometimes STAT3. IL-4R consists of IL-4R α and γ c. IL-4 activates STAT6 by JAK 1 and JAK3 as well as the ERK1/2 pathway

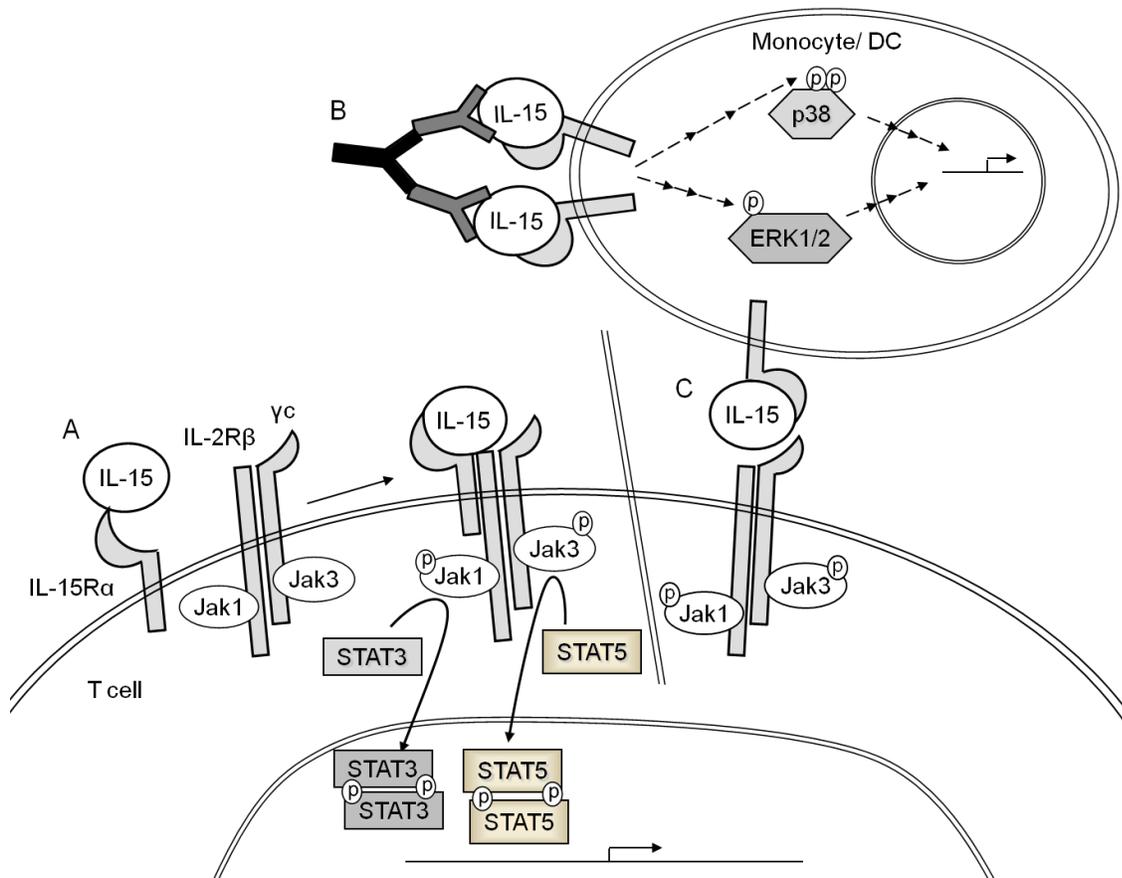


Figure 1-2. Diagram of the three IL-15 signaling mechanisms. A) Classical IL-15R-mediated activation of STAT3 and STAT5. IL-15R α , IL-2R β , and γ c chains indicated. B) IL-15 reverse signaling that occurs on monocytic cells leading to activation of p38 and ERK1/2 MAPK pathways. Membrane-bound IL-15 may or may not be attached to membrane by engagement with IL-15R α as depicted here. C) IL-15 trans-presentation from DC to NK cell or T cell which likely results in STAT3 or STAT5 activation

CHAPTER 2 CHARACTERIZATION OF HUMAN MONOCYTE-DERIVED IL-15 DCS BY COMPARISON WITH TRADITIONAL IL-4 DCS

Introduction

Dendritic cells (DCs) are mainly characterized by the extent of their abilities to stimulate antigen (Ag)-specific T cell responses (13, 161-163). DCs provide three important signals to activate T cells and induce Ag-specific cytolytic responses (11, 55, 56). The first two signals are Ag presentation and co-stimulation which are essential for Ag-specific T cell activation and proliferation. Production of IL-12 or other IFN- γ -inducing cytokines is the third required signal for the development of cytolytic effector functions by the activated T cells. Monocyte-derived DCs can be generated *in vitro* using a variety of cytokines (22, 30, 31). Since 1994, differentiation of purified monocytes using GM-CSF combined with IL-4 has been the most common method for generating the large numbers of DCs necessary for clinical therapies and immune studies (29). Unlike DC differentiated using other cytokines, IL-4 DCs can elicit CD8⁺ effector T cell and Th1 CD4⁺ T cell responses which are the highly desired T cells responses in DC-based immune therapy strategies. (43, 45, 46)

Monocyte-derived IL-4 DCs express high levels of DC-specific surface markers such as CD11c, CD1a, and DC-SIGN. Matured IL-4 DCs produced high amounts of IL-12 as well as up-regulate expression of HLA-DR, CD80, CD86, and CD83 which all have a significant impact in stimulating Ag-specific T cell activities. IL-4 DCs are the only *in vitro*-derived DC subsets capable of stimulating cytolytic Ag-specific CD8⁺ T cell responses.

In 2001, Saikh et al. demonstrated that monocytes cultured in IL-15 differentiate into immune stimulatory DCs comparable with the more traditional IL-4 DCs (44). In

contrast with the more traditionally studied imIL-4 DCs, imIL-15 DCS were capable of stimulating T cell responses however imIL-15 DCs expressed unusually low levels of the common DC surface markers CD1a and/or DC-SIGN (42, 44-46). They also expressed the Langerhans cell-specific marker langerin in three out of four publications that evaluated the expression of langerin on IL-15 DCs (42, 45-47). Maturation of IL-15 DCs was induced by various stimuli depending on the research groups, ranging from LPS alone to cocktails containing prostaglandin E₂ (PGE₂), TNF α , IL-1 β , and IL-6. mL-15 DCs are described to exhibit typical DC morphologically and to be phenotypically alike to the more conventional mL-4DCs. Both DC subsets exhibit typical hooded veil morphology and express comparable levels of MHC class I and HLA-DR (43, 45), CD80 (45, 46), and CD86 (45, 46). Expression of CD83 and CD40 differed between the two DC subsets but no correlation between these markers and the DC function were reported (43, 45-47). The presence of cytokines that promote T cell effector functions IL-12p70, IFN- γ and soluble IL-15 were not consistently detected in IL-15 DC supernatants (43, 45, 46, 164, 165). Nonetheless, IL-15 DCs have been reported to stimulate Ag-specific CD8⁺ T cell responses more efficiently than the traditional IL-4 DCs (43, 45). How IL-15 DCs prime stronger Ag-specific cytolytic CD8⁺ T cell responses compared to IL-4 DCs is not well understood. Dubsky et al. suggested that IL-15 DCs express greater amounts of an unidentified surface proteins that promotes T cell activation during DC-to-T cell interactions (45). In contradiction to Dubsky et al.'s suggestion, the other study comparing anti-melanoma CD8⁺ T cell responses by IL-15 DCs versus IL-4 DCs reported there was 0.2% more NK cells contaminating the IL-15 DC preparations compared to IL-4 DCs. This elevated number of NK cells secreted

large amounts of IFN- γ which was hypothesized to enhance CD8⁺ T cell responses (43).

In this chapter, we assessed the extent of signals 1, 2, and 3 provided by IL-15 DCs compared to IL-4 DCs to determine how IL-15 DCs elicit stronger cytolytic T cell responses. We evaluated signal 1 and 2 based on the expression of molecules involved in Ag presentation and co-stimulation as well as Ag up-take. We measured the strength of signal 3 produced by mIL-15 DCs including IFN- γ and IL-15. And finally, we evaluated the immune stimulatory abilities of IL-15 DCs to elicit CD8⁺ and CD4⁺ cytolytic T cells responses against CMV or HCV.

Materials and Methods

Monocyte Isolation

Buffy coat preparations from healthy donors were obtained from LifeSouth Civitan Blood Center (Gainesville, FL, USA). Peripheral blood mononuclear cells (PBMCs) were isolated by gradient density centrifugation in Ficoll-Hypaque (GE Healthcare Bio-Sciences AB, Piscataway, NJ, USA). Cell viability was determined by trypan blue staining and then cryogenically frozen until needed.

Monocyte-derived DC Differentiation and Maturation

PBMCs were plated at 1×10^7 cells/well in 6-well plates using AIM-V media (Gibco-BRL, Invitrogen, Inc., Carlsbad, CA), then the plates were incubated for 2 hours at 37°C to allow for monocyte attachment. Next, non-adherent PBLs were gently washed away and cryogenically frozen for experiments. Monocytes were cultured in AIM-V media supplemented with 50 ng/mL GM-CSF (BioSource, Invitrogen Inc., Carlsbad, CA) and either 25 ng/mL IL-4 (BioSource) to generate IL-4 DCs or 100 ng/mL IL-15 (Gentaur Molecular, Brussels, Belgium) to generate IL-15 DCs. On the third day fresh AIM-V

media containing the same final concentration of cytokines was added to the wells. After 5 days, imDCs were harvested using 10 mM EDTA-PBS (Sigma-Aldrich, St. Louis, MO) and a cell lifter. ImDCs were either cryogenically stored or cultured with maturation factors. DC maturation was induced by culturing up to 1×10^6 imDCs/well in low adherent 24-well plates with 50 ng/mL TNF α (Gentaur) and 1 μ g/mL LPS (Sigma) in addition to GM-CSF and IL-4 or IL-15 at the same final concentrations as before. After 24 to 48 hours, mDCs were harvested for experimental use.

Monoclonal Antibodies

Fluorophore-conjugated monoclonal antibodies against human CD11c (clone B-ly6, PE), CD25/IL-4R α (clone hIL4R-M57, PE), CD1a (clone HI149, APC), CD40 (clone 5C3, APC), IFN- γ (clone B27, APC), CD86 (clone FUN-1, FITC), CD45RA (clone HI100, FITC), CD107a/LAMP-1 (clone H4A3, FITC), CD14 (clone M5E2, pacific blue), CD3 (clone UCHT1, pacific blue), CD16 (clone 3G8, pacific blue), CD8 (clone SK1, APC-Cy7), CD80 (clone L307.4, CyC), CCR7 (clone 3D12, PE-Cy7), and CD56 (clone B159, PE-Cy7) were purchased from BD Biosciences (San Diego, CA). CD11c (clone BU15, FITC), HLA-DR (clone TÜ36, FITC), HLA class I (clone TÜ149, FITC), CD14 (clone TÜK4, FITC), CD3 (clone S4.1, FITC), CD83 (clone HB15e, APC), CD4 (clone S3.5, PE-TexasRed), and CD8 (clone 3B5, Alexa700) antibodies were obtained from Caltag, Invitrogen, Inc., (Carlsbad, CA). Antibodies against CD11c (clone 3.9, PE-Cy7), PD-L1 (clone MIH1, PE-Cy7), IL-15R α (clone eBioJM7A4, FITC), TLR4 (clone HTA125, PE), CD27 (clone M-T271, APC), DC-SIGN/CD209 (clone eB-h209, APC), and CD28 (clone O323, APC-Alexa750) antibodies were purchased from eBioscience (San Diego, CA).

Flow Cytometry

For analysis of surface proteins, cells were washed with PBS containing 2% fetal bovine serum (FBS) (Gibco), blocked with 10% mouse and human serum mixture for 30 minutes at 4°C, incubated with the desired antibodies for 30 minutes at 4°C, washed again, and then fixed with 2% formaldehyde. Cells stained with isotype-matched antibodies were used for controls. Data was collected and analyzed using BD FACSAria flow cytometer and Flowjo (Tree Star, Inc., Ashland, OR). Isotype value was subtracted from sample value to calculate change in the mean fluorescent index (Δ MFI) or change in percentage of cells expressing a particular protein (Δ percentage). Statistical significance between DC subsets was determined via two-tailed wilcoxon matched-pairs signed rank tests with calculated p values ≤ 0.05 being considered significant.

Antigen Uptake Assay

Donor-matched mIL-4 DCs and mIL-15 DCs were incubated with 10 μ g/mL DQ-ovalbumin (DQ-OVA; Molecular Probes, Invitrogen, Inc.) in AIM-V media for 30 minutes at 37°C. Controls were incubated on ice for 30 minutes with the same concentration of DQ-OVA. mDCs were thoroughly washed and the surface stained as described before with anti-CD11c Ab. Finally the percentages of DQ-OVA⁺ CD11c⁺ mDCs were determined using the BD LSR II or the BD Caliber flow cytometer. Data was analyzed with Flowjo. Statistical significance was evaluated by paired, two-tailed t test with p values ≤ 0.05 being significant.

Multiplex ELISA

ImDCs were matured for 24 hours as previously described. Next, the 24-hour matured DCs were extensively washed and transferred into second 24-well plate in AIM-V media not containing any cytokines at a density of 1×10^6 mDCs/mL. After an

additional 24 hours, the supernatant was collected and frozen. Frozen supernatant samples were sent to Quansys Biosciences (Logan, UT) for their modified multiplex ELISA array service. Each cytokine was analyzed in triplicate.

Semi-quantitative and Quantitative Reverse Transcription-PCR

RNA was isolated with Tri-Reagent following the manufacturer's instructions (Invitrogen). cDNA was generated from 1 µg isolated total RNA using oligo(dT) primers from the cell-to-cDNA II kit as directed by the manufacturer (Ambion, Invitrogen). For the semi-quantitative reverse transcription PCR (RT-PCR), the generated cDNA was serially diluted ranging from no dilution to 1:10, 1:100, and 1:1000 ratios to be used for the RT-PCR template. Reactions consisted of 1 µL template, 12.5 µL PCR mastermix (Promega, Madison, WI), and 2.5 nM of each primer. Reaction conditions were 95°C for the first 10 minutes, then 35 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds, then followed by a final extension step at 72°C for 5 minutes. RT-PCR products were separated on a 2% agarose gel and visualized with ethidium bromide staining. RT-PCR primers were designed to span two adjacent exons in the desired gene transcript. They were purchased from Integrated DNA Technologies, Inc (Coralville, IA). Primer sequences for the cytokines and β-actin were as follows: IFN-γ, 5'-TCAGCTCTGCATCGTTTTGG-3' (sense) and 5'-GTTCCATTATCCGCTACATCTGAA-3' (antisense); TNFα, 5'-TCTTCTCGAACCCCGAGTGA-3' (sense) and 5'-CCTCTGATGGCACCACCAG-3' (antisense); IL-6, 5'-GTAGCCGCCCCACACAGACAGCC-3' (sense) and 5'-GCCATCTTTGGAAGGTTTCAGG-3' (antisense); and β-actin, 5'-ACCTTCTACAATGAGCTGCG-3' and 5'-CCTGGATAGCAACGTACATGG-3' (antisense).

For quantitative SYBR green real time PCR (qRT-PCR), the same cDNA samples and IFN-γ, TNFα, and β-actin primers were used in the qRT-PCR analysis. New IL-6

primers were designed in order to match generate the same amplicon product size as the other three transcripts as required. The new IL-6 primer sequences were 5'-CCACTCACCTCTTCAGAACG-3' (sense) and 5'-TCTGCCAGTGCCTCTTTGC-3' (antisense). Amplification of each gene transcript was completed in triplicate reactions which consisted of 1 μ L template, 12.5 μ L 2x SYBR green qPCR mastermix (SABiosciences, Qiagen, Inc, Valencia, CA), and determined optimal primer concentration. Optimized primer concentrations used in qRT-PCR reactions were determined to be 500 nM of each primer for IFN- γ and IL-6, 300 nM of each primer for TNF α , and 100 nM of each primer for β -actin. Conditions for the qRT-PCR reaction was conducted as suggested by SABioscience using the MX3000P qPCR system (Stratagene, Agilent Technologies, Santa Clara, CA) as 10 minutes at 95°C, followed by 40 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and finally a dissociation curve analysis to confirm amplification of a single amplicon. Also the amplification efficiency for the four genes was verified to be similar to one another before analyzing the relative gene expression levels in DCs. Relative levels of the cytokine transcripts were calculated by the $2^{(-\Delta Ct)}$ method and then normalized using β -actin expression levels.

mDC:PBL Co-culture

Human cytomegalovirus (CMV) matrix phosphoprotein 65 (pp65), hepatitis C virus (HCV) core, and Wilms tumor (WT-1) protein peptide mixtures were obtained from JPT Peptide Technologies, Inc. (Acton, MA). Each peptide mixture consisted of over a hundred 15-mer synthetic peptides which overlap each other to collectively span the entire length of the particular protein.

mIL-4 DCs and mIL-15 DCs were pulsed with 1 $\mu\text{g}/\text{mL}$ β -microglobulin along with either 1 $\mu\text{g}/\text{mL}$ CMV pp65 or 5 $\mu\text{g}/\text{mL}$ HCV core peptide mixtures in AIM-V media for 2-4 hours at 37°C. Next, mDCs were irradiated for 2,000 radians, washed, and mixed with autologous PBLs at a ratio of 1:20 DC to PBLs per well in 96-well U-bottom plate in AIM-V supplemented with 5% human serum. After 3 days, half of the media in the wells was replaced with complete AIM-V media containing 5% human serum and the final concentration of the cytokines 12.5 U/mL IL-2, 5 ng/mL IL-7, and 20 ng/mL IL-15 (Gentaur). One half of the media was replenished with fresh complete AIM-V containing human serum and cytokines every two days. As the stimulated PBLs began to proliferate, they were transferred to plates with larger sized wells as needed. PBL proliferation was assessed by trypan blue staining in which the average number of viable cells from two wells of the co-culture were determined on the days indicated. T cell immune responses against CMV pp65 and HCV core proteins were analyzed on day 14 of the co-culture using T cell antigen recall assay as described later. T cell and NK cell subsets were identified by their specific surface marker phenotypes on the 15th day of the co-culture by flow cytometry.

Antigen Recall Assay

PBLs co-cultured with mDCs were re-stimulated with the CMV pp65/HCV core Ag (specific peptide) or the WT-1 tumor Ag (non-specific peptide). Ag-specific induction of CD3⁺ T cells was determined by measuring IFN- γ production and CD107 degranulation. On day 13 of the DC:PBL co-culture, PBLs were washed and cultured without cytokines overnight to decrease non-specific T cell immune activity. The following day, PBLs were re-stimulated at a ratio of 1:20 DC:PBLs using the autologous mDCs pulsed with the

CMV pp65/HVC core or WT-1 peptides in a 96-well U-bottom plate for a total of 5 hours at 37°C in AIM-V media. Non-stimulated PBLs were used as controls. Anti-CD107a antibody was added to each sample from the beginning of the assay. After the first hour, 6 µg/mL monensin A (Sigma) was added then incubated at 37°C for the remaining 4 hours. CD3, CD4, and CD8 were stained on the cell surface as explained previously. Cells were fixed and permeabilized with BD Cytotfix/Cytoperm solution at 4°C. Next, cells were washed in BD Perm/Wash buffer, stained with anti-IFN-γ antibody for 30 minutes on ice and washed. The data was collected on the BD Aria flow cytometer and analyzed using Flowjo. Ag-specific IFN-γ production and/or CD107a degranulation was calculated by subtracting the percentage of CD3⁺ T cells responding to the WT-1 peptide from those reacting to the original peptide. Statistical significance was determined by two-tailed wilcoxon matched-pairs signed rank tests with *p* values ≤ 0.05 considered to be significant.

Results

Cellular Morphology During DC Differentiation and Maturation

ImDCs were derived by culturing monocytes in GM-CSF and IL-4 (for IL-4 DCs) or IL-15 (for IL-15 DCs) for 5 days and morphology was analyzed by light microscopy. The imIL-4 DCs were lightly attached, or floating oval shaped cells with irregular edges and a few protrusions radiating from the main cell body (Figure 2-1A). However, nearly all of the imIL-15 DCs remained firmly adhered to the plate during differentiation and seemed slightly larger. imIL-15 DCs usually resembled either thin, spindle-like shaped cells with small dendrites at the ends or had a fried egg appearance.

On day 5 imDCs were harvested, washed, and cultured with LPS and TNFα along with GM-CSF and IL-4 or IL-15 for an additional 24 hours to induce maturation. mL-4

DCs and mIL-15 DCs no longer exhibit their own distinctive morphological differences. Both mIL-4 DCs and mIL-15 DCs are thin and elongated adherent cells with small dendrite protrusions (Figure 2-1B).

Surface Marker Phenotype of imDCs

T cell activation and requires three signals: Ag presentation (signal 1), co-stimulation (signal 2), and production of IL-12 (signal 3). Since the presence of IL-4 or IL-15 affected DC morphology, we investigated how the cytokines impacted expression of DC surface markers. First we confirmed DC differentiation from the precursor monocytes which is indicated by the down-regulation of the monocytic surface marker CD14 and the expression of DC-specific markers such as DC-SIGN. We observed that both imIL-4 DCs and imIL-15 DCs display low levels of CD14 and express DC-SIGN (Figures 2-3C and 2-3D). Interestingly, imIL-15 DCs express significantly lower amounts of DC-SIGN ($n=7$, $p=0.02$) compared to the conventional IL-4 DCs. Ag presentation and co-stimulation molecule expression on CD11c⁺ immature and mature IL-4 DCs and IL-15 DC were evaluated. We found that in relation to imIL-4 DCs, MHC class I molecules (HLA I) were expressed at significantly greater levels on imIL-15 DCs ($p=0.02$, $n=8$) and it was detected on a greater number of CD11c⁺ imIL-15 DCs ($p=0.008$) (Figures 2-2A and 2-2B) The expression levels of the lipid Ag presenting molecule CD1a was significantly reduced on imIL-15 DCs compared to imIL-4DCs ($p=0.004$, $n=9$). It was also expressed on the surface of less imIL-15 DCs than imIL-4 DCs ($p=0.008$). HLA-DR expression was not particularly affected by generating imDCs with IL-15 and GM-CSF instead of IL-4 and GM-CSF. Co-stimulatory molecules CD80, CD83, and PD-L1 as well as the chemokines receptor CCR7 were expressed at similar levels on imIL-4 DCs and imIL-15 DCs (Figures 2-2C and 2-2D). For the other co-stimulatory molecules, imIL-4

DCs expressed significantly more CD86 ($p=0.03$, $n=6$) but less CD40 ($p=0.04$, $n=8$) in contrast with imIL-15 DCs. We also compared the surface expression of TLR4, IL-4R α , and IL-15R α between imIL-4 DCs and imIL-15 DCs to determine whether a particular DC subset would be more responsive to IL-4, IL-15, or LPS during subsequent maturation protocols. We found that the three surface receptors were detected at similar levels on both of the imDC subsets (Figures 2-3A and 2-3B).

Surface Marker Phenotype of mDCs

Addition of LPS and TNF α for about 48 hour to induce DC maturation caused the expression levels of HLA-DR, CD83, and CD86 as well as the number of CD11c⁺ DCs expressing the markers increased on IL-4 DCs and IL-15 DCs which confirmed the maturation status of mIL-4 DCs and mIL-15 DCs (Figures 2-4C and 2-4D). In contrast with mIL-4 DCs, mIL-15 DCs did not up-regulate CCR7 which is another DC maturation marker that directs mDC migration to secondary lymphoid organs in vivo (imIL-15 DC median Δ MFI=484.7 versus mIL-15 DC median Δ MFI=288) (Figures 2-3C and 2-5C) (166). However, the number of CCR7⁺ mDCs did increase compared to imDCs (imIL-15 DC median%=18% versus mIL-15 DC median%=30.3. imIL-4 DC median%=17.9 versus mIL-4 DC median%= 41.5). Donor-matched mIL-4 DCs exhibit the typical elevated expression CCR7 levels compared to imIL-4 DCs (imIL-4 DC median Δ MFI=296.5 versus mIL-4DC median Δ MFI =684.4). Additionally, compared with mIL-4 DCs, DC-SIGN expression was significantly decreased ($p=0.03$, $n=7$) and detected on less CD11c⁺ mIL-15 DCs ($p=0.08$) (Figures 2-5C and 2-5D). Another commonly used DC surface marker, CD1a was significantly reduced on mIL-15 DCs compared to mIL-4 DCs ($p=0.02$, $n=7$) and expressed on a considerably lower percentage of mIL-15 DCs than on mIL-4 DCs ($p=0.03$) (Figures 2-4A and 2-4B).

mIL-4 DCs and mIL-15 DCs express similar levels of HLA class I molecules and HLA-DR suggesting that mIL-15 DCs present peptide Ags at the same efficiency with the more conventional mIL-4 DCs (Figures 2-4A and 2-4B). The co-stimulatory molecules CD80 and CD83 were individually detected on lower numbers of CD11c⁺ mIL-15 DCs in relation to mIL-4 DCs however they were expressed on both of the mDC subsets at similar amounts (Figures 2-4C and 2-4D). The other co-stimulatory molecule CD86 ($p=0.008$, $n=8$) and the negative T cell activation regulator PD-L1 ($p=0.02$, $n=8$) were significantly reduced on mIL-15 DCs compared to mIL-4 DCs. Finally the expression of CD40, IL-4R α , IL-15R α , and TLR4 were not significantly different between the mIL-4 DCs and the mIL-15 DCs (Figures 2-4C, 2-4D, 2-5A, and 2-5B).

Antigen Uptake by mDCs

The ability of the mIL-4 DCs and mIL-15 DCs to present Ags to elicit lymphocyte immune responses was further assessed by a flow cytometry-based Ag uptake assay. mDCs were incubated with ovalbumin (OVA) that had been conjugated to a fluorophore and then the percentages of CD11c⁺ mDCs that endocytosed the OVA -fluorophore conjugate were determined. On average, mIL-15 DCs were more efficient at internalizing OVA than their donor-matched mIL-4 DCs; however, the difference between the mDC subsets was not found to be statistically significant (Figure 2-6).

Production of IL-12 and Pro-inflammatory Cytokines by mDCs

The third signal necessary for the development of cytotoxic effector functions in T cells is presence of IL-12 (55, 167, 168). Other pro-inflammatory as well as anti-inflammatory cytokines secreted by mDCs such as IL-1, IL-6, IL-15, IFN- γ , TNF α , IL-4, and IL-10 can also influence T cell effector functions or maintenance of certain T cell functional subsets (169-174). We compared the production of these cytokines by mIL-4

DCs to those secreted by mIL-15 DCs by ELISA. In both donors, mIL-15 DCs produced slightly more IL-12p70 than mIL-4 DCs (Table 2-1). mIL-15 DCs also secreted more pro-inflammatory cytokines IL-1 α , IL-1 β , IL-6, IL-15, IFN- γ , and TNF α compared to mIL-4 DCs. IL-4 was produced at low amount by both DC subsets but the amounts of IL-10 produced by a particular DC subset depended on the donor. These results suggest that production of more IL-12p70 and pro-inflammatory cytokines by mIL-15 DCs than mIL-4 DCs may help promote more cytolytic responses by T cells primed by mIL-15 DCs.

Pro-inflammatory Cytokine Transcript Regulation

The four pro-inflammatory cytokines IFN- γ , TNF α , IL-15, and IL-6 were secreted at much higher amounts by IL-15 DCs in comparison with mIL-4 DCs (Table 2-1). Differential production of IFN- γ , TNF α , IL-15, and IL-6 may be influenced by the continual presence of IL-15 or IL-4 during mDC generation. These four cytokines are regulated at multiple levels ranging from transcriptional to post-translational (75, 95, 104, 175, 176). IL-4 can inhibit transcription of IL-6 in monocytic cell lines and IFN- γ in T cells (172, 177). On the other hand, IL-15 plays a role in promoting IL-6 and TNF α production in monocytes (178). We investigated whether the four cytokines were differentially regulated at the transcriptional level in the two DC subsets during DC differentiation or maturation. To reduce potential donor variations, we pooled RNA from three donors before comparing cytokine mRNA levels in the original monocyte precursors, mIL-4 DCs, and mIL-15 DCs. Potential differences in cytokine transcript levels were first compared semi-quantitatively using serially diluted cDNA and reverse transcription PCR (RT-PCR). Our results showed IFN- γ and IL-6 transcripts were more notably more abundant in mIL-15 DCs compared to mIL-4 DCs and monocytes (Figure 2-7A). TNF α transcript levels appeared to be marginally greater in mIL-15 DCs and

monocytes than in mIL-4 DCs. Both of the IL-15 isoforms, the shorter IL-15SSP that encodes the non-secreted protein and the longer IL-15LSP transcript that encodes the secreted protein, were detected in the monocytes, mIL-4 DCs, and mIL-15 DCs at nearly the same levels. Hence, it is likely that the higher secretion of IL-15 by mIL-15 DCs than mIL-4 DCs caused by post-transcriptional regulation (75, 104).

To quantify differences in IFN- γ , TNF α , and IL-6 transcript levels in mIL-4 DCs and mIL-15 DCs we used SYBR green quantitative real time PCR (qRT-PCR). Conditions for the qRT-PCR were optimized and the amplification efficiency for each primer set was matched to the other three primer sets (data not shown). Cytokine expression were normalized to β -actin transcript levels and then the relative expression levels were calculated using the $2^{(-\Delta Ct)}$ method. We confirmed that IFN- γ , TNF α , and IL-6 transcripts were more abundant in mIL-15 DCs compared to donor-matched mIL-4 DCs (Figure 2-7B). IFN- γ mRNA levels are at least 330-fold greater in mIL-15 DCs compared to mIL-4 DCs. TNF α transcripts were at least 14-fold higher in mIL-15 DCs than mIL-4 DCs. Finally, IL-6 transcript level was found to be 20-fold higher in mIL-15 DCs in comparison with mIL-4 DCs. Therefore, the differences in IFN- γ , TNF α , and IL-6 production in the two mDC subsets are regulated at least to some degree by either gene transcription or stabilization (or destabilization) of the cytokine mRNA transcripts.

CMV-specific Memory Immune Responses by CD3⁺ T Cells

We investigated whether mIL-15 DCs are more efficient than the conventional mIL-4 DCs at eliciting cytolytic CD3⁺ T cell immune responses against human cytomegalovirus (CMV). PBLs were stimulated with autologous mDCs pulsed with CMV pp65 for 14 days and then re-stimulated with the relevant mDCs. Since T cell activation can be characterized by clonal proliferation, we first determined the extent of mIL-15

DC- versus mIL-4 DC-induced T cell stimulation by comparing the rate at which the lymphocytes expanded through the co-culture. Over the duration of the DC:PBL co-cultures, the number of PBLs steadily increased over the 16 days following either stimulation with mIL-4 DCs or mIL-15 DCs. The expansion rate of the lymphocytes that were co-cultured with mIL-15 DCs showed no difference from the lymphocytes that were primed with donor-matched mIL-4 DCs which suggests that mIL-15 DCs and mIL-4 DCs induce comparable levels of T cell activation to one another (Figure 2-7A).

To determine whether mIL-15 DCs elicit stronger cytolytic T cell responses against CMV pp65 than mIL-4 DCs, CD3⁺ T cell IFN- γ production (IFN- γ ⁺) and CD107a, a vesicle membrane protein, translocation to the cell surface (CD107a⁺) were measured separately or in combination with each other (Figure 2-8B). Using a series of Ag recall assays, the cultured PBLs were re-stimulated with the same autologous mDC subset and then the extent of the Ag specific reactions are assessed. CMV pp65 specificity was calculated by subtracting the percentage of T cells reacting to the non-specific control from the CMV pp65-responding cells for CD8⁺ and CD4⁺ T cell populations.

We observed that akin to mIL-4 DCs, mIL-15 DCs is able to elicit CMV-specific cytolytic T cells responses from CD4⁺ T cells as well as the more commonly investigated CD8⁺ T cells. Furthermore, stimulation by either mIL-15 DCs or mIL-4 DCs results in more CD8⁺ T cells exhibiting IFN- γ ⁺, CD107a⁺, and IFN- γ ⁺ CD107a⁺ responses against CMV in comparison with their CD4⁺ T cell counterparts. Since this trend did not appear to be linked with a particular mDC subset suggests that the increased CD8⁺ T cell populations are associated with the CMV pp65 peptides. Concerning the extent of CMV T cell responses, compared with the donor-matched mIL-

4 DCs, lymphocytes co-cultured with mIL-15 DCs showed significantly higher cytolytic responses to CMV for CD8⁺ T cells as well as CD4⁺ T cells.

HCV-specific Primary Immune Responses by CD3⁺ T cells

Next, we investigated potential differences in the extent of primary T cell cytolytic responses against hepatitis C virus (HCV) induced by mIL-15 DCs versus the conventional mIL-4 DCs. The DC:PBL co-cultures and Ag recall assays using HCV core peptide mixtures were completed in parallel with the previously described CMV pp65 peptide experiments. We first compared the expansion rate of the lymphocytes primed with the HCV-pulsed mIL-15 DCs and the mIL-4 DCs over the duration of the co-culture. Again we observed that PBLs cultured with mIL-4 DCs proliferated at the same rate as those stimulated with mIL-15 DCs (Figure 2-9A). In comparing the distribution of CD8⁺ and CD4⁺ T cells, we found the percentage of HCV-responsive CD8⁺ T cells to be nearly the same as CD4⁺ T cells. Thus the previously noted preference for CD8⁺ T cells over the CD4⁺ T cells in the CMV experiments was likely a peptide-dependent phenomenon. As expected there were less CD3⁺ T cells with HCV-specific cytolytic responses compared to the large number of T cells responding to CMV pp65 peptides. Nonetheless, there was a marked difference in the number of CD8⁺ and CD4⁺ T cells that exhibited IFN- γ ⁺, CD107a⁺, or both immune activities against HCV following stimulation by mIL-15 DCs compared with mIL-4 DCs (Figure 2-9B). The mIL-15 DC-primed CD8⁺ T cells showed significantly greater HCV-specific IFN- γ responses than those CD8⁺ T cells cultured with mIL-4 DCs. Furthermore, there were significantly more CD4⁺ T cells producing IFN- γ and undergoing CD107a degranulation after being primed by mIL-15 DCs than their donor-matched mIL-4 DCs.

Effect of mIL-15 DC Stimulation on Distribution of T Cell Subset Populations

Next we evaluated the possible effect of the two mDC subsets on the distribution of the memory and effector T cell subset populations in both CD8⁺ and CD4⁺ T cells following mDC co-culture. In several murine studies IL-15 produced by DCs has been reported to play a role in promoting or maintaining the CD8⁺ memory T cell populations (97, 179-181). Thus we investigated whether human mIL-15 DCs, which we have shown to secrete relatively high levels of IL-15, may also support the CD8⁺ memory T cell populations. We assessed the distribution of the three major CD3⁺ T cell functional subsets at the end of the DC:PBL co-cultures. Central memory (T_{cm}), effector memory (T_{em}), and effector (T_{eff}) T cells were identified from the CD8⁺ CD3⁺ T cells or the CD4⁺ CD3⁺ T cells based the expression of five surface markers which have been discussed in prior T cell reviews (52, 53). T_{cm} cells are defined as CD45RA⁻ CD27⁺ CD28⁺ CD62L⁺ CCR7⁺ while T_{em} are identified by CD45RA⁻ CD27⁺ CD28⁺ CD62L⁻ CCR7⁻. CD8⁺ and CD4⁺ T_{eff} cells have slightly different phenotypes. CD8⁺ T_{eff} cells are CD45RA^{+/+} CD27⁻ CD28⁻ CD62L⁻ CCR7⁻ whereas CD4⁺ T_{eff} cells do not express any of the five markers. Only about half of the CD3⁺-gated T cells from our mDC-primed lymphocyte cultures were determined to be T_{cm}, T_{em}, or T_{eff} cells due to our stringent surface phenotype requirements for T cell subset classification (data not shown). The type of peptide, whether it was CMV pp65 (Figure 2-10A) or HCV (Figure 2-10B), did not affect the memory and effector T cell subset distribution. In Figure 2-10, we show for the first time that culture with human mIL-15 DCs does not actively support the CD8⁺ memory T cells in relation to their donor-matched mIL-4 DCs. Also T cell priming by mIL-15 DCs does not promote memory T cell subsets in the CD4⁺ T cell population compared with mIL-4DCs. Actually mIL-15 DCs stimulation results in decreased

numbers of Tcm and Tcm cells in nearly all the comparisons with mIL-4 DC lymphocyte co-cultures. Teff cell populations in the CD8⁺ and CD4⁺ T cells were much larger in mIL-15 DC-stimulated cultures in contrast with the mIL-4 DC-primed cultures, especially for the CD8⁺ T cells. The relative increase of Teff cell percentages detected in mIL-15 DC-stimulated CD8⁺ and CD4⁺ T cells corresponds with the greater number of T cells exhibiting CMV- or HCV-specific cytolytic responses. Thus, in contrast with the suggestions from murine DC studies, human IL-15 DCs promote CD8⁺ and CD4⁺ Teff cells and they do not promote memory T cells populations in either CD8⁺ or CD4⁺ T cells. Furthermore, the preference towards Teff cells in IL-15 DC lymphocyte co-cultures correlates with the increased number of Ag-specific IFN- γ ⁺ and/or CD107a⁺ T cells when compared with the donor-matched IL-4 DC stimulated lymphocytes.

Effect of Stimulation by IL-15 DCs versus IL-4 DCs on NK Cells

IL-15 is an essential component in the development and activation of NK cells (73, 74, 95). In addition to their innate cytolytic activities, activated NK cells modulate DC functions and promote T cell activity (182, 183). Generation of IL-15 DCs has been shown to increase the number of residual NK cells found after monocyte purification. The increased number of contaminating NK cells in IL-15 DC preparations then subsequently enhance T cell stimulation (43). Therefore we investigated whether our culture conditions for generating mIL-15 DCs also modulates NK cell expansion and activity. First we compared the percentage of CD3⁻ CD56⁺ NK cells in the lymphocyte size-gated populations following stimulation by mIL-4 DCs and mIL-15 DCs in CMV- and HCV-primed lymphocytes (Figures 2-11A and 2-11B). Stimulation by mIL-15 DCs or mIL-4 DCs did not affect the number of NK cells. In 2 out of 6 donors evaluated, there were high levels of NK cells in the mIL-15 DC stimulated lymphocytes compared with

mIL-4 DC primed lymphocytes which may have skewed the median NK cell percentages slightly. Since the surface phenotype of human NK cells has been directly associated with their effector activity (58, 63), we finally assessed whether mIL-15 DC stimulation affected the NK cell activity in CMV- and HCV-primed lymphocytes (Figures 2-11C and 2-11D). CD3⁻ CD56⁺ NK cells were separated in subpopulations based on their CD56 expression level (dim or bright) and CD16 expression. CD56^{bright} CD16⁻ NK cells are considered to be immunoregulatory due to their high production of cytokines including IFN- γ and TNF α and relative meager cytotoxicity abilities upon activation. On the other hand, CD56^{dim} CD16⁺ NK cells secrete only low levels of cytokines but exhibit high cytotoxic functions against target cells (58, 59, 63). Co-cultured with mIL-15 DCs or mIL-4 DCs did not affect the distribution of the NK cell subpopulations which means that the subset of stimulating mDCs did not affect NK cell functions. We confirmed that mDC subset did not affect the cytolytic activity of NK cells from an overall lymphocyte co-culture (Appendix A).

Discussion

IL-15 DCs are the first monocyte-derived DC subset other than the more conventionally used IL-4 DCs that are capable of priming Ag-specific CD8⁺ T cell responses. Furthermore IL-15 DCs were reported to be more efficient at priming cytolytic CD8⁺ T cells responses against melanoma Ags than the more classically utilized IL-4 DCs (43, 45, 46). Similarly, we also demonstrated that IL-15 DCs are more potent tools for stimulating CMV- and HCV-specific cytolytic CD8⁺ and CD4⁺ T cell responses compared to IL-4 DCs (Figures 2-7 and 2-8). In this report, we characterized the physical attributes and immune stimulatory functions of the monocyte-derived IL-15 DCs in relation to the more conventional IL-4 DCs.

We first evaluated the surface phenotype of immature and mature IL-15 DCs to determine their extent of DC differentiation and maturation state as well as expression of signal 1 and 2. DC differentiation is evaluated based on decreased expression of monocyte surface markers and presence of DC-specific markers whereas the extent of maturation is determined by its potential immune stimulatory abilities after activation. Typically maturation is defined up-regulation of signal 1 and 2 related molecules (HLA-DR and the classic co-stimulatory molecules CD80, CD83, and CD86), lymphoid tissue homing receptors (CCR7), and decreased Ag up-take capacity. In our flow cytometry analyses, we focused on the monocyte-derived DC populations that were the appropriate size for a myeloid cell and expressed high levels of CD11c which is a commonly used myeloid DC marker. We confirmed that CD11c⁺-gated IL-15 DCs and IL-4 DCs differentiated into DCs based on the decreased CD14 levels (Figures 2-1 and 2-3). However, the expression of the DC-specific surface markers CD1a and DC-SIGN on IL-15 DCs were considerably lower compared to IL-4 DCs suggesting that IL-15 DCs may be less differentiated than IL-4 DCs (Figures 2-2 and 2-4). IL-15 DCs were previously shown to express the Langerhans cell (LC)-specific marker langerin (42, 43, 45). However, in our hands, we observed that langerin transcript levels decreased in differentiating IL-15 DCs whereas langerin mRNA was abundantly expressed in IL-4 DCs (data not shown). After maturation, CD83, CD86, and CCR7 levels increased on CD11c⁺ IL-4 DCs but at best the expression of these markers were only mildly increased on the surface of mIL-15 DCs (Figures 2-2, 2-3, 2-4, and 2-5). The decreased expression levels of DC- and maturation-specific markers suggest that IL-15 DCs exhibited a less differentiated or less mature DC phenotype than the traditional IL-4

DCs. Overall the relatively lower CD1a, DC-SIGN, co-stimulatory molecules, and CCR7 expression indicate IL-15 DCs, respectively, present fewer lipid Ags, have less stable physical interactions with naïve T cells, be less efficient at providing signal 2, and have decreased homing to secondary lymphoid organs. Nevertheless, the lack of “classical” DC surface markers suggests that IL-15 DCs may be a significantly unique DC subset from IL-4 DCs and the “traditional” DC markers established studying IL-4 DCs may not be applicable to IL-15 DCs.

Ag presentation and co-stimulation For our assessment of signal 1 “strength”, we found that the expression of HLA class I and HLA-DR molecules on mIL-15 DCs were similar compared to those detected on mIL-4 DCs (Figure 2-4). Since peptides are loaded onto HLA molecules before being transported to the cell surface, the concentration of surface HLA molecules indicates amount of Ag being presented. Hence there were no differences in peptide Ag presentation abilities between the mDC subsets. In addition to the ability to present Ag, we also compared their capacity to uptake soluble Ag such as viral peptides or OVA. Our results indicate that CD11c⁺ mIL-15 DCs endocytose OVA Ag more efficiently than mIL-4 DCs (Figure 2-6). Previous literature had also described HLA-DR expression on either immature or mature IL-15 DCs to be alike to IL-4 DC counterpart however it was also reported that both mDC subsets equally uptake dextran Ag (43, 46, 47).

We considered the abilities of the mDC subsets at providing signal 2 to induce T cell responses by comparing expression of a handful of co-stimulatory molecules on mDCs. Most of the co-stimulatory molecules evaluated were detected at similar levels on mIL-15 DCs and mIL-4 DCs including CD80, CD83, and CD40 (Figure 2-4). Unlike

some of the previous IL-15 DCs reports, we did not observe extensive decreased expression of CD83 on mIL-15 DCs in relation to mIL-4 DCs as previously described (45-47). Only PD-L1 and CD86 were significantly reduced on CD11c⁺ mIL-15 DCs compared to mIL-4 DCs which was surprising since PD-L1 and CD86 have been shown to be positively regulated by IL-15 stimulation on monocytes and monocyte-derived IL-15 DCs respectively (47, 184, 185). Nonetheless, reduced levels of CD86 or CD80 and lower affinity Ags have been demonstrated to increase IFN- γ production by activated T cells without affecting their proliferation; thus, the restricted amount of CD86 and MHC class I expression on mIL-15 DCs may be advantageous in stimulating IFN- γ production in T cells. (17, 186). Overall, the lack of up-regulation of HLA-DR and co-stimulatory molecules as well as the relatively high Ag uptake levels suggest that “mature” IL-15 DCs exhibit a surface phenotype and behaviors more attributed to immature DCs.

Finally the third important signal for T cell activation, often called “signal 3”, is the production of cytokines by the DCs. The classical signal 3 provided by DCs is IL-12 production. IL-12 secreted by activated DCs and induces IFN- γ production by T cells via direct activation of the STAT4 signaling pathway. In contrast to the prior IL-15 DC characterizations (43, 45, 46), our LPS-matured mIL-15 DCs secreted somewhat higher concentrations of IL-12p70 than mIL-4 DCs (Table 2-1). We also observed that mIL-15 DCs also produced greater amounts of IL-1 α , IL-1 β , IL-6, and TNF α in comparison to IL-4 DCs which is consistent with previous IL-15 DC descriptions (43, 45, 46). These inflammatory cytokines are known to influence T cell activation or their activities along with the traditional “signal 3”; thus the elevated levels of these cytokines may give mIL-15 DCs an advantage in eliciting T cell responses (169, 171, 187). In addition to more

inflammatory cytokines, IFN- γ and IL-15 were more highly secreted by mIL-15 DCs compared to mIL-4 DCs, even though the cytokines were undetectable in the mDC supernatants by Dubsky et al. and Anguille et al. (45, 46). Higher IFN- γ secretion by IL-15 DCs may also play a role in elevated IL-12p70 production by IL-15 DCs (164). IFN- γ and IL-6 mRNA levels in monocytes and in mIL-4DCs were very similar however in mIL-15 DCs the IFN- γ and IL-6 transcripts are up-regulated as the monocytes differentiate into mIL-15 DCs (Figure 2-7A). For qRT-PCR analysis, new IL-6 primers were designed to meet the more stringent qRT-PCR requirements. IL-15 is regulated through multiple transcriptional and post-transcriptional mechanisms. It is mainly regulated at post-transcriptionally via translation and surface translocation. It is stored intracellularly thus it is even regulated at a secretion level (105)

We did not observe any noticeable IFN- γ -driven up-regulation of MHC class I and II on mIL-15 DCs that would have probably enhanced mIL-15 DC T cell Ag priming (188). It is likely that the high production of IL-1 β by IL-15 DCs may impair transcription of the CIITA gene and hence inhibit IFN- γ -induced MHC class II up-regulation (189). Nonetheless, culturing naïve T cells with IFN- γ and IL-4 neutralizing antibodies is a commonly used to induce IFN- γ -producing Th1 CD4⁺ T cells *in vitro* and the presence of IFN- γ is well known to suppress IL-4 transcription in T cells (190). Hence increased levels of IFN- γ secreted by mIL-15 DCs may help polarize co-cultured T cells to demonstrate IFN- γ ⁺ Th1 responses. Higher concentrations of IFN- γ may also be involved in the elevated amounts of IL-15 detected in the supernatant which has been shown to occur on a dose-dependent basis in NK cells, T cells, and monocytes (69, 95). On the other hand, production or trans-presentation of IL-15 by DCs activates NK cells,

maintains memory CD8⁺ T cell populations as well as promotes CD8⁺ T cells immune responses (98, 191, 192). IL-15 trans-presentation by IL-15R α to NK and T cells requires coordinated expression of both IL-15R α and IL-15 from the same DC (73, 75, 79) IL-15R α is also an essential component in regulation of IL-15 secretion (75, 104, 193). IL-15R α binds the IL-15 precursor protein within the endoplasmic reticulum or early Golgi and then acts like a chaperone protein until the IL-15 precursor is processed. Both IL-15R α and IL-15 proteins are finally translocated together to the cell surface where it's could be released or trans-presented. Since mIL-15 DCs secrete higher amounts of IL-15 than mIL-4 DCs, they likely also trans-present more IL-15 and subsequently effect NK and T cell immune responses.

IL-15 DCs have been shown to prime stronger melanoma-specific CD8⁺ T cell responses than IL-4 DCs (43, 45). Rather than using HLA-A0201* immunodominant melanoma peptides, we used CMV and HCV peptides to elicit primary and memory T cell responses specifically. The Centers for Disease Control and Prevention (CDC) cites a 2006 survey that up to 2% of American adults are infected by HCV, while approximately 50-80% adults by the age of 40 are estimated to have been chronically infected with CMV (194). Thus, we used HCV or CMV peptides known to stimulate strong immune responses to induce primary or memory T cell responses respectively. We demonstrate for the first time that mIL-15 DCs are more efficient than donor-matched mIL-4 DCs at priming CMV- and HCV-specific cytolytic responses from autologous CD8⁺ T cells as well as CD4⁺ T cells (Figures 2-8 and 2-9). We demonstrate that stimulation a particular mDC subset does not affect the lymphocyte proliferation rate nor does it affect the final number of NK cells and distribution of NK cell subsets at

the end of the co-culture (Figures 2-8, 2-9, and 2-11). However, mIL-15 DC stimulation tends to promote CD8⁺ effector T cells (Teff) over memory T cell subsets (Tcm and Tem) whereas mIL-4 DCs favor CD8⁺ memory T cell subsets over effector T cells (Figure 2-10). Due to the stringency of using eight surface markers to determine each of the T cell subsets, only about one half of either the CD8⁺- or CD4⁺-gated CD3⁺ T cells were able to be classified into one of the three subsets. Nonetheless, the relatively high numbers of Teff and Tem T cells following mIL-15 DC-stimulation likely corresponds with the increased percentage of IFN- γ ⁺ T cells primed with mIL-15 DCs versus mIL-4 DCs (Figures 2-8, 2-9, and 2-10).

Dissimilarities noted between our IL-15 DC characterization results and the previous reported findings likely stem from our protocol differences. For example, different monocyte purification protocols (plastic adherence versus antibody-based magnetic bead separation kits) may affect the purity of isolated monocytes and/or quality of the monocytes. Hardy et al. found that even small amounts of NK cells that remain after monocyte purification may affect subsequent T cell responses (43). The extent of the DC-primed T cell responses may also be affected by HLA serotypes among donors and the relative compatibility of CMV pp65 or HCV core peptides with the donor's TCR (i.e. CMV pp65 is the immunodominant peptide for HLA-A*0201 serotype). Unlike Dubsy et al. and Hardy et al., we did not use HLA-matched donors in our study. Thus we would likely see a larger range in the percentages of responding T cells (43, 45). Also, we also used a more dilute DC:PBL ratio for our co-cultures and Ag recall assays than described by other groups. Furthermore, differences in surface marker expression may be caused by our IL-15 DCs being differentiated with less IL-15 (100

ng/mL) than the other groups (200 ng/mL). Additionally culturing DCs in serum-free media can reduce the expression levels of certain surface markers such as CD1a (7). This may explain the unusually low CD1a expression on our IL-15 DCs as noted in Figures 2-2 and 2-4 when compared with the previous IL-15 DC descriptions from groups who cultured their DCs in RPMI media containing varying concentration of human serum (2.5-10%). Finally, our IL-15 DCs characterization results differed from prior studies simply because our conditions to identify “DC populations” in our flow cytometry analysis were more stringent. We evaluated cells that were of the general myeloid cell-size which also expressed high levels of CD11c. Due to the inherent heterogeneity of primary cell cultures, the percentages of CD11c⁺ DCs from the myeloid cell size-gated populations ranged from 60-98% depending on the particular donor.

In our study, we assessed the abilities of human monocyte-derived IL-15 DCs to provide the three necessary signals to effectively initiate T cell activation and prime their Ag-specific cytolytic effector responses in comparison to IL-4 DCs. While both mIL-15 DCs and mIL-4 DCs seemed to provide comparable levels of Ag presentation and co-stimulation, mIL-15 DCs secreted much more pro-inflammatory cytokines and IL-12p70. The gene transcripts of the more highly expressed pro-inflammatory cytokines IFN- γ , TNF α , and IL-6 by mIL-15 DCs were also more abundant in mIL-15 DCs than mIL-4 DCs. Additionally, mIL-15 DCs primed CMV pp65- and HCV core-specific CD4⁺ and CD8⁺ T cell exhibited greater cytolytic responses than the traditional IL-4 DCs. mIL-15 DC. Furthermore, mIL-15 DC stimulation tended to favor the T cell subsets which are associated with cytolytic activities, the CD8⁺ effector and CD4⁺ effector memory and effector T cell subsets.

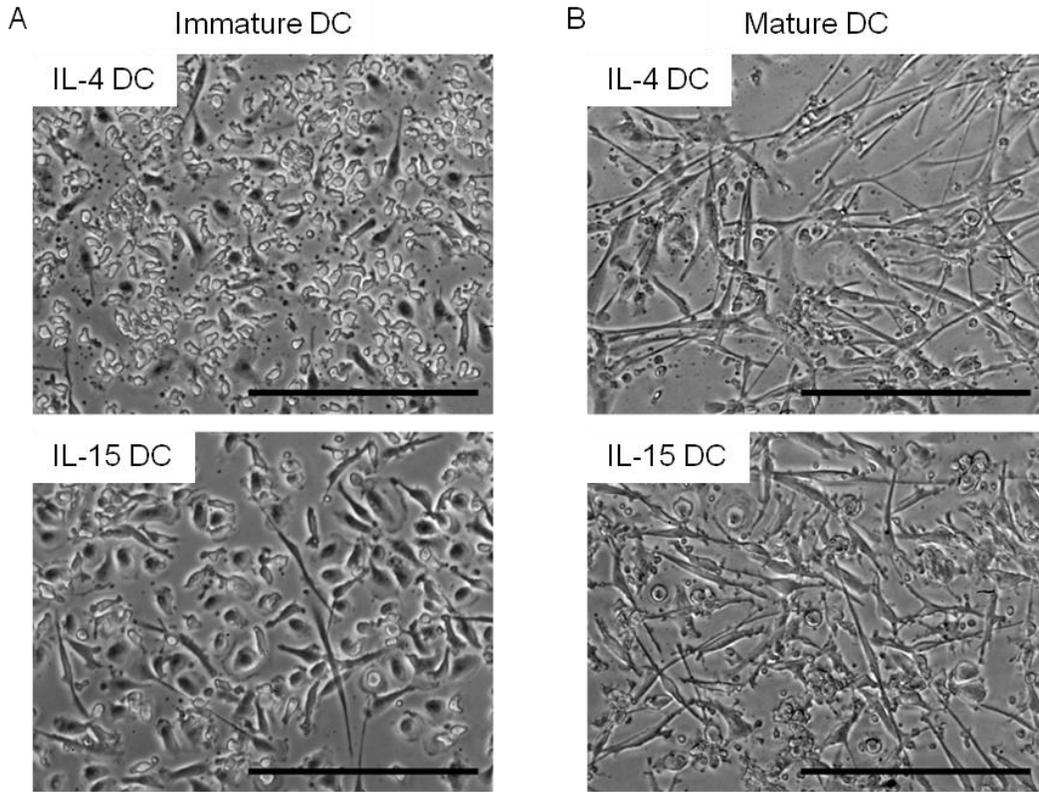


Figure 2-1. Cellular morphology of immature and mature DCs generated in the presence of IL-4 or IL-15. Images shown are representative images of PB monocyte-derived DCs in culture (10x). A) Immature DCs after differentiation from monocytes for 5 days. B) DCs matured for about 36 hours. Scale bar in lower right corner indicates 0.1 mm

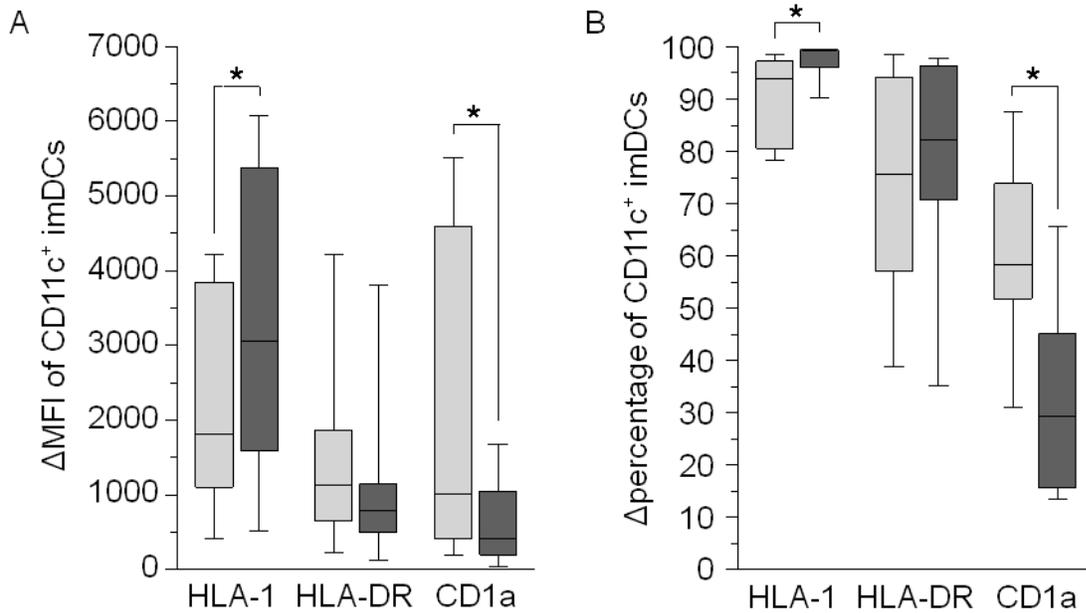


Figure 2-2. Expression of Ag presentation and co-stimulation molecules on the imDC subsets. A-B) Expression of Ag presentation molecules on imIL-4 DCs (light grey bars) and imIL-15 DC (dark grey bars). $n=8-9$. C-D) Expression of lymphocyte co-stimulatory molecules. $n=5-9$. A and C) Change in the mean fluorescence index (Δ MFI) of CD11c⁺-gated imDCs after subtracting isotype values. B and D) Change in percentage (Δ percentage) of CD11c⁺-gated imDC after subtracting isotype values. Statistically significant differences between IL-4 DCs and IL-15 DCs are indicated. Significance calculated by wilcoxon matched-pairs signed rank test with the determined p value ≤ 0.05 considered significant

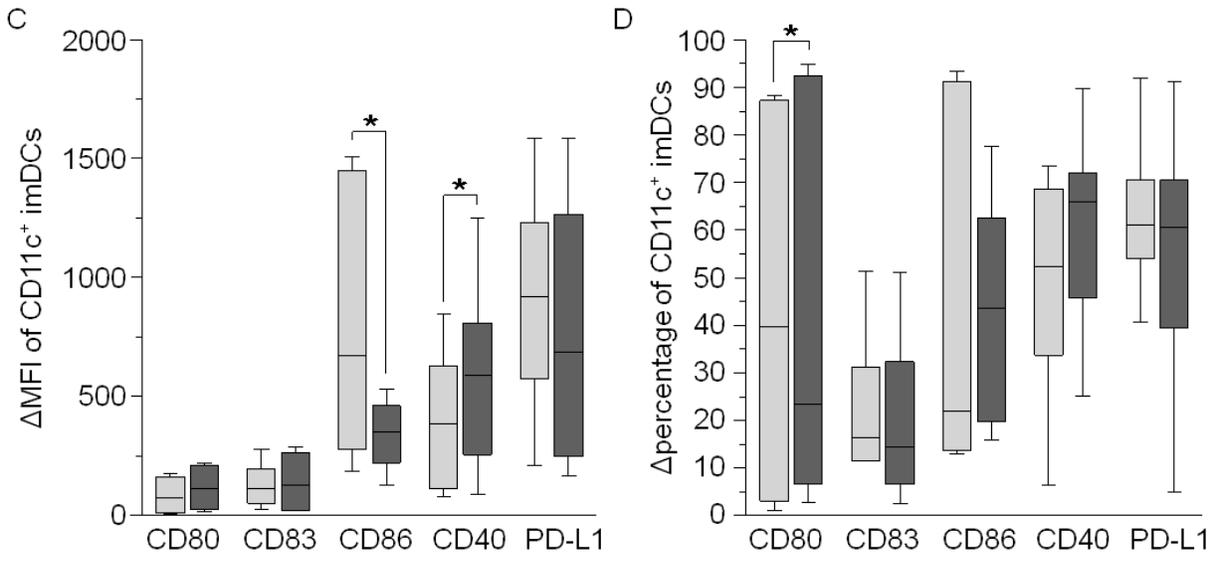


Figure 2-2. Continued

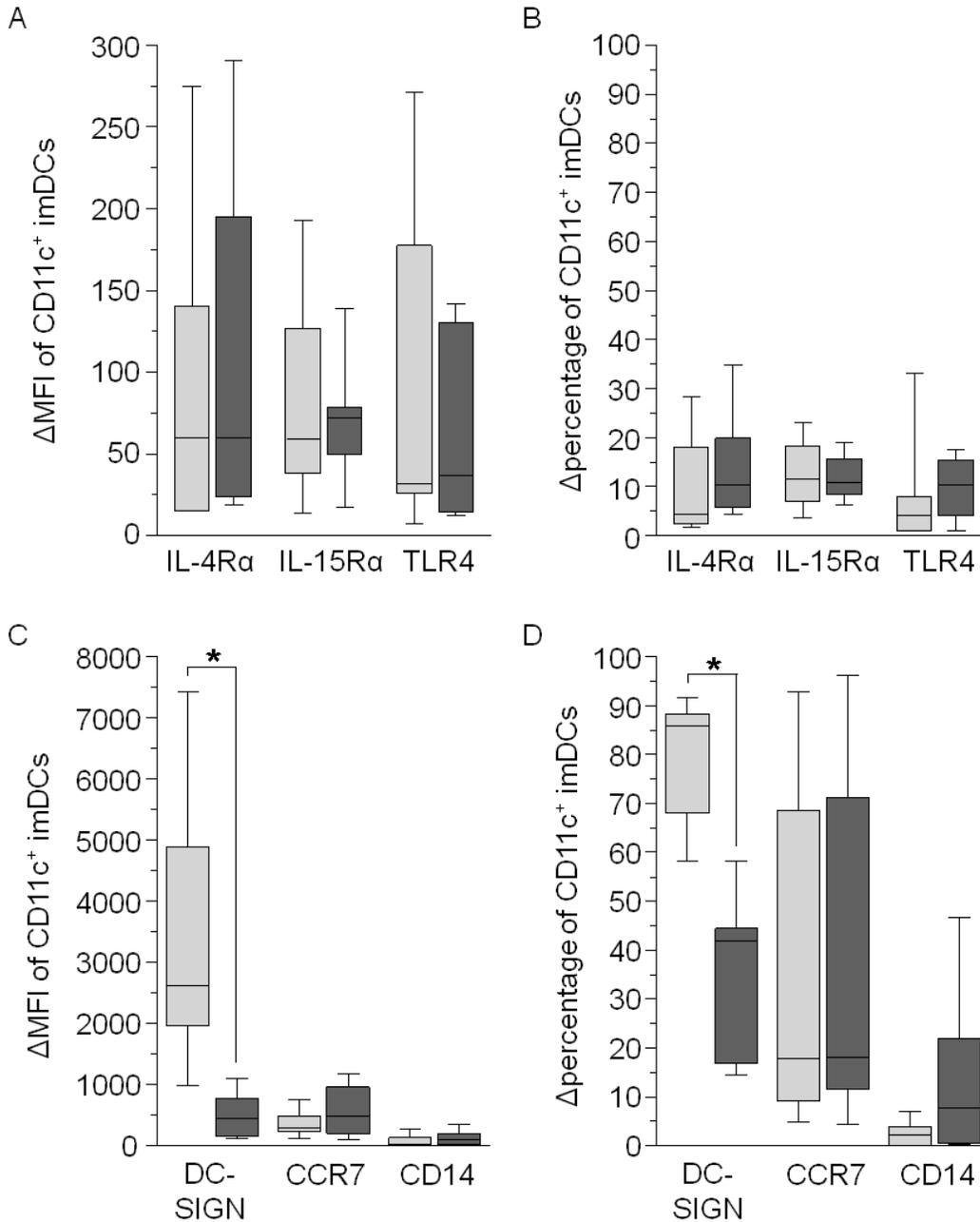


Figure 2-3. Expression of receptors involved in DC differentiation/maturation and additional molecules regulating co-stimulation and motility on imDC subsets. A-B) Receptors Δ MFI and Δ percentage of CD11c⁺-gated imIL-4 DCs (light grey bars) and CD11c⁺-gated imIL-15 DCs (dark grey bars). $n=6-8$. C-D) DC differentiation and maturation markers Δ MFI and Δ percentage of CD11c⁺ imDC. $n=7-9$. A and C) Δ MFI of CD11c⁺-gated imDCs. B and D) Δ percentage of CD11c⁺-gated imDCs. Statistically significant differences between IL-4 DCs and IL-15 DCs are indicated. Significance calculated by wilcoxon matched-pairs signed rank test with the determined p value ≤ 0.05 considered significant

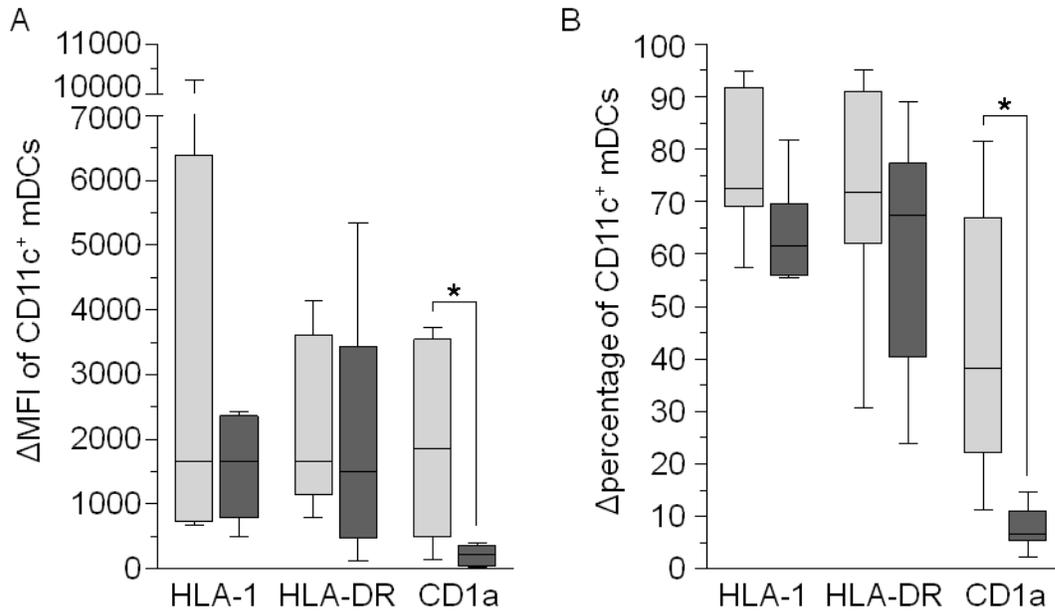


Figure 2-4. Expression of Ag presentation and co-stimulatory molecules on mDC subsets. A-B) Δ MFI and Δ percentage of Ag presentation molecules expressed on CD11c⁺-gated mIL-4 DCs (light grey bars) and CD11c⁺-gated mIL-15 DCs (dark grey bars). $n=7-8$. C-D) Δ MFI and Δ percentage of co-stimulatory molecules on CD11c⁺ mDCs. $n=7-9$. A and C) Δ MFI of CD11c⁺-gated mDCs. B and D) Δ percentage of CD11c⁺-gated mDCs. Statistically significant differences between IL-4 DCs and IL-15 DCs are indicated. Significance calculated by wilcoxon matched-pairs signed rank test with the determined p value ≤ 0.05 considered significant

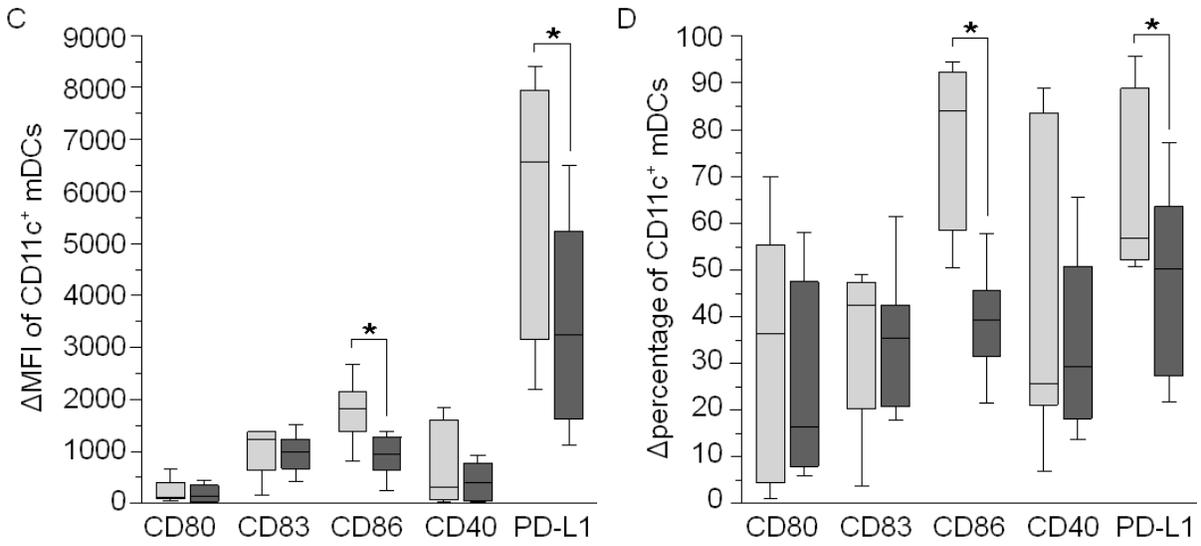


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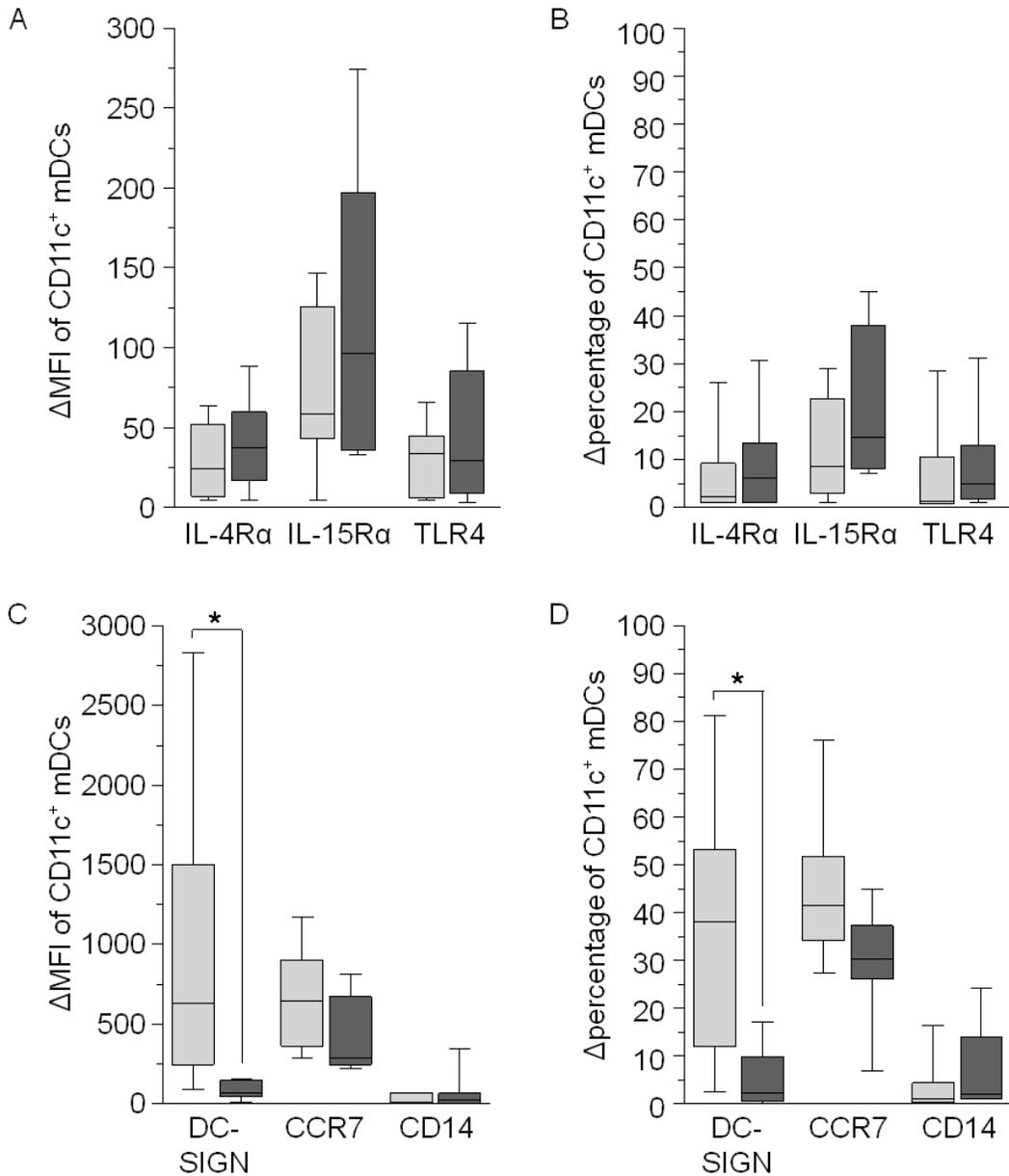


Figure 2-5. Expression of receptors involved in DC differentiation/ maturation and additional molecules involved in regulating co-stimulation and motility in mDC subsets. A-B) Δ MFI and Δ percentage on CD11c⁺-gated mIL-4 DCs (light grey bars) and CD11c⁺-gated mIL-15 DCs (dark grey bars). $n=9$. C-D) Δ MFI and Δ percentage of DC differentiation and maturation markers on CD11c⁺ mDCs. $n=7-8$. A and C) Δ MFI of CD11c⁺-gated mDCs. B and D) Δ percentage of CD11c⁺-gated mDCs. Statistically significant differences between IL-4 DCs and IL-15 DCs are indicated. Significance calculated by wilcoxon matched-pairs signed rank test with the determined p value ≤ 0.05 is considered to be significant

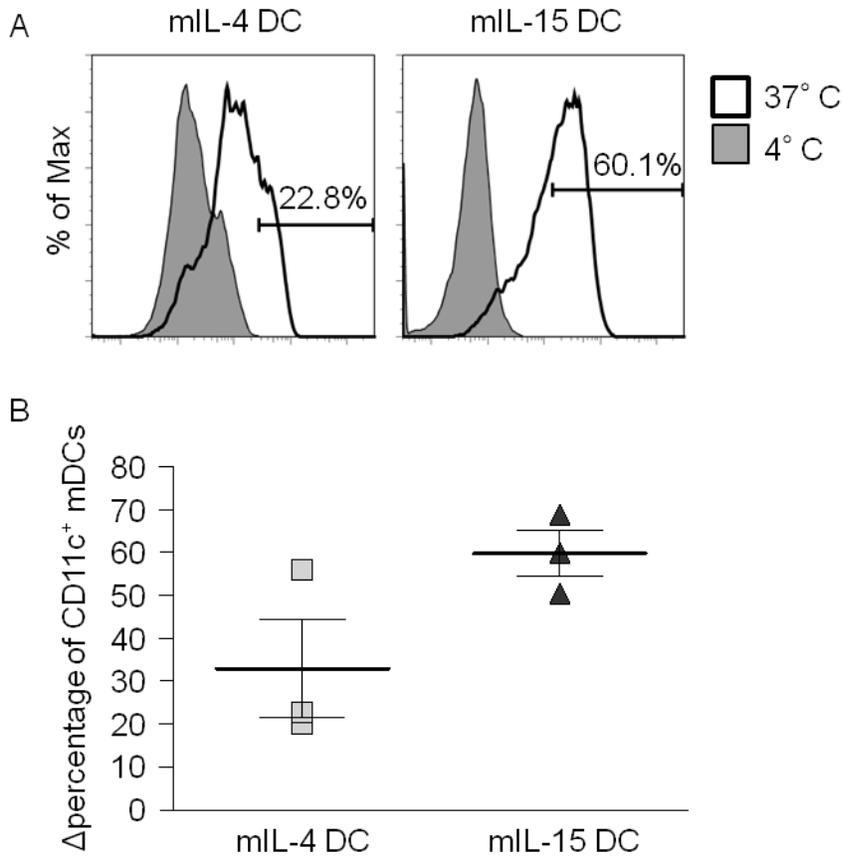


Figure 2-6. Comparison of antigen up-take capacities by mDC subsets. mDCs were incubated with OVA-DQ at 37°C or 4°C, then stained with anti-CD11c antibody and analyzed by flow cytometry. A) Percentage of CD11c⁺ OVA⁺ mDCs after incubation at 37°C (empty line) or 4°C control (shaded line). Histogram data shown is representative of 3 donors. B) Δpercentage of OVA⁺ CD11c⁺ mDCs from 3 donors. OVA uptake was calculated by subtracting the percentage of CD11c⁺ mDCs that non-specifically internalized OVA-DQ at 4°C from the percentage of OVA⁺ CD11c⁺ mDCs incubated at 37°C. Average percentages of mDCs ± SEM are indicated. Paired t-tests found the difference between the CD11c⁺ mDC subsets to not be significant

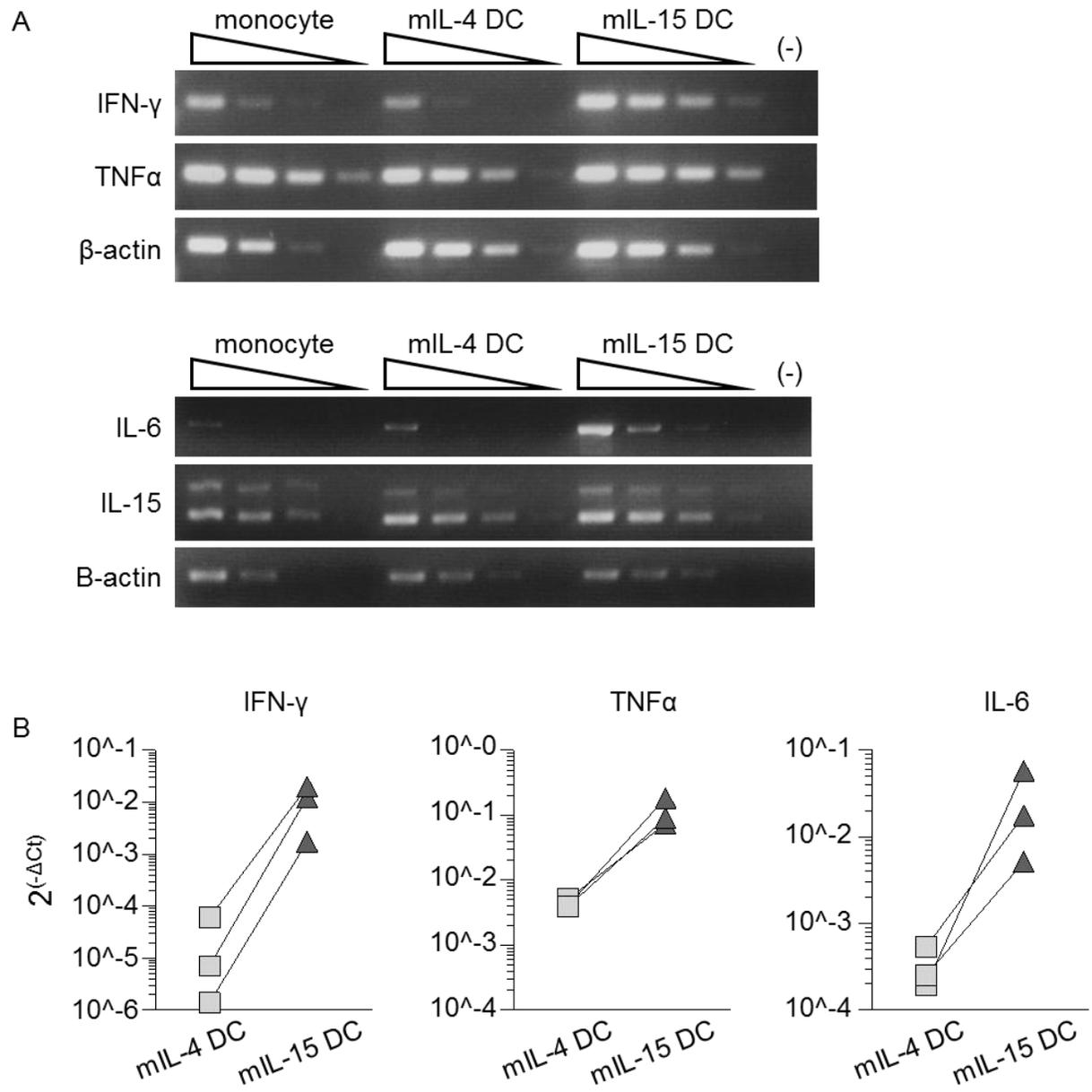


Figure 2-7. Differential regulation and expression of IFN- γ , TNF α and IL-6 transcripts in DC subsets. A) semi-quantitative RT-PCR analysis of IFN- γ , TNF α , IL-6, IL-15, and β -actin transcript levels in monocytes and mDCs. cDNA generated from RNA pooled from 3 donors was serially diluted (no dilution, 1:10, 1:100, and 1:1000). Non-template control is indicated by dash (-). B) quantitative SYBR green RT-PCR analysis of IFN- γ , TNF α , and IL-6 gene expression in mDCs using cDNA generated from the same pooled donors and 2 additional donors. Relative cytokine gene expression is normalized using the β -actin transcript expression levels. mL-4 DC is indicated by the light grey square while the donor-matched mL-15 DC is shown by dark grey triangle. mDCs prepared from the individual donors are connected by the lines

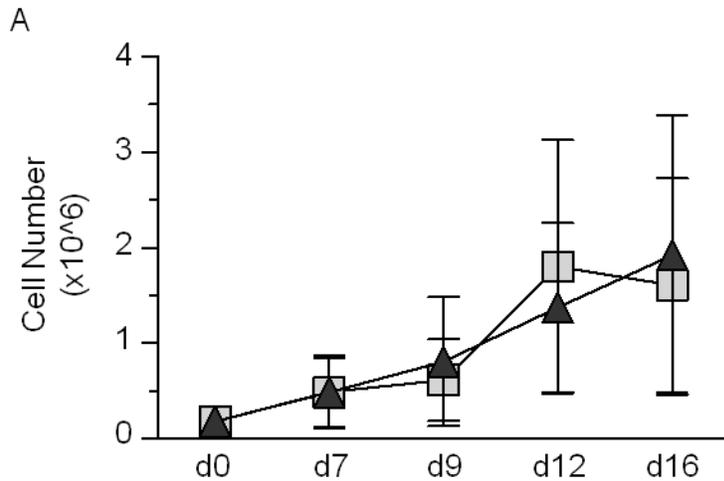


Figure 2-8. Antigen-specific memory T cell responses against CMV pp65. A) The number of PBLs from the first day of the DC:PBL co-culture (d0) through the 16th day (d16) was determined by trypan blue staining. mIL-4 DC-primed PBLs are indicated by light grey squares and dark triangles represent PBLs co-cultured with mIL-15 DCs. The average number of cells \pm SEM is indicated. $n=4$. B) Percentages of CD4⁺- or CD8⁺-gated CD3⁺ T cells that produce IFN- γ (IFN- γ ⁺) and/or degranulate CD107 (CD107⁺). CMV-specific responses were calculated by subtracting the percentage of non-specific response from the percentage of CMV pp65 responses. Light squares represents the percentage mIL-4 DC-primed CD3⁺ T cells (+IL-4 DC) while dark triangles show mIL-15 DC-primed T cells (+IL-15 DC). Median percentage of responding T cells are indicated by the bar. Donor-paired responses are indicated by the line connecting the squares to triangles. $n=6$. Statistically significant differences between IL-4 DCs and IL-15 DCs are indicated. Significance calculated by wilcoxon matched-pairs signed rank test with the determined p value ≤ 0.05 considered significant

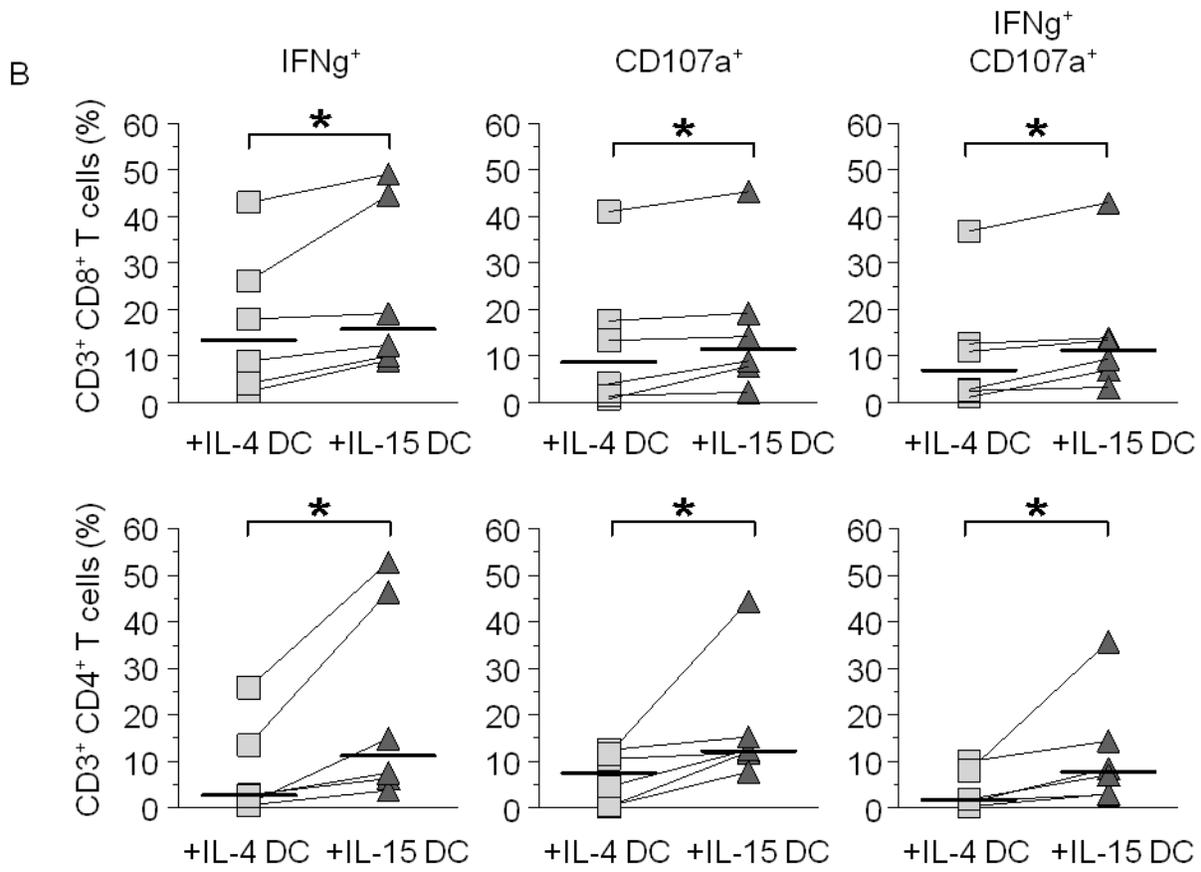


Figure 2-8. Continued

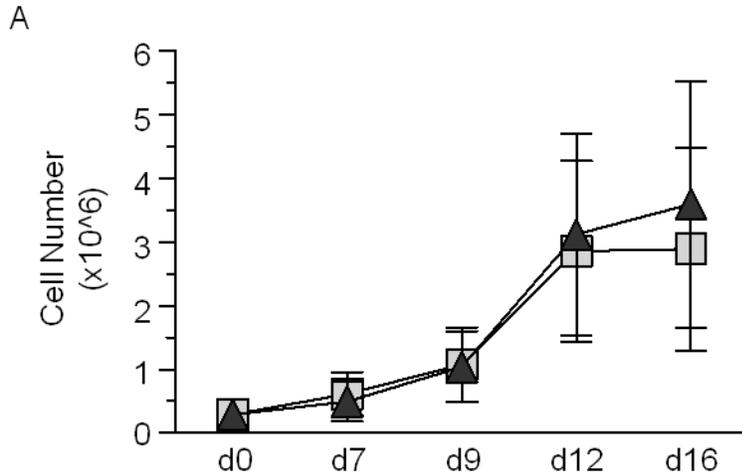


Figure 2-9. Antigen-specific primary T cell responses against HCV core. A) The number of PBLs from the first day of the DC:PBL co-culture (d0) through the 16th day (d16) was determined by trypan blue staining. The average cell number \pm SEM is indicated. $n=4$. B) Percentages of CD4⁺- or CD8⁺-gated CD3⁺ T cells producing IFN- γ (IFN- γ ⁺) and/or CD107 degranulation (CD107⁺). HCV-specific responses were calculated by subtracting the non-specific responses from the HCV-specific responses. Light squares represents the mIL-4 DC-primed CD3⁺ T cells (+IL-4 DC) while dark triangles show mIL-15 DCs-stimulated T cells (+IL-15 DC). Median percentage of responding T cells are indicated by the bar. Donor-paired responses are indicated by the line connecting the squares to triangles. $n=6$. Statistically significant differences between IL-4 DCs and IL-15 DCs are indicated. Significance calculated by wilcoxon matched-pairs signed rank test with the determined p value ≤ 0.05 considered significant

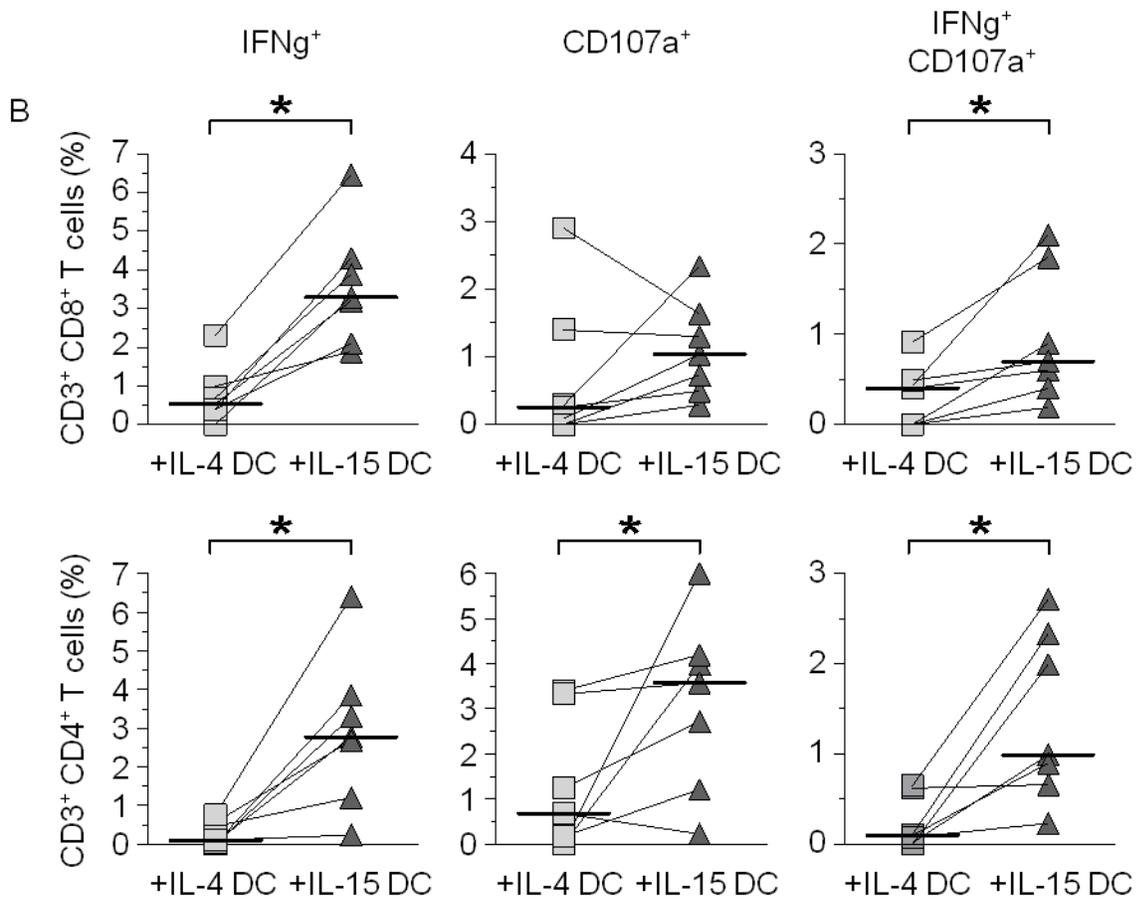


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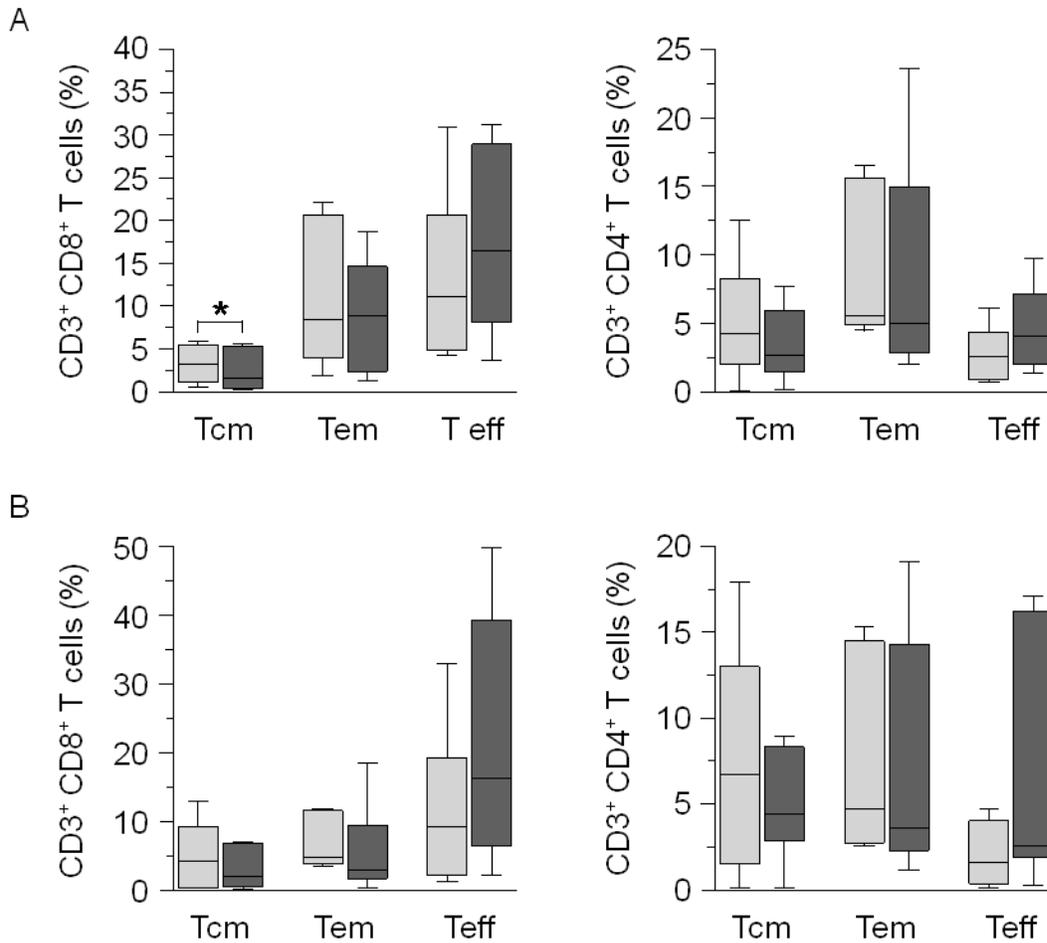


Figure 2-10. Distribution of CD4⁺ and CD8⁺ T cell subsets after stimulation with Ag-pulsed mDCs on day 16-17 of DC:PBL co-culture. Central memory (Tcm), effector memory (Tem), and effector (Teff) CD3⁺ T cells were identified using five characteristic T cell functional surface markers in addition to CD3, CD4, and CD8 by flow cytometry. Light grey bars indicate the percentage of either CD8⁺ - or CD4⁺-gated CD3⁺ T cells primed by mIL-4 DCs. Dark grey bars represent T cells stimulated with mIL-15 DCs. A) T cells primed against CMV pp65. *n*=6. B) T cells stimulated against HCV core. *n*=6. Significance calculated by wilcoxon matched-pairs signed rank test with the determined *p* value ≤ 0.05 considered significant

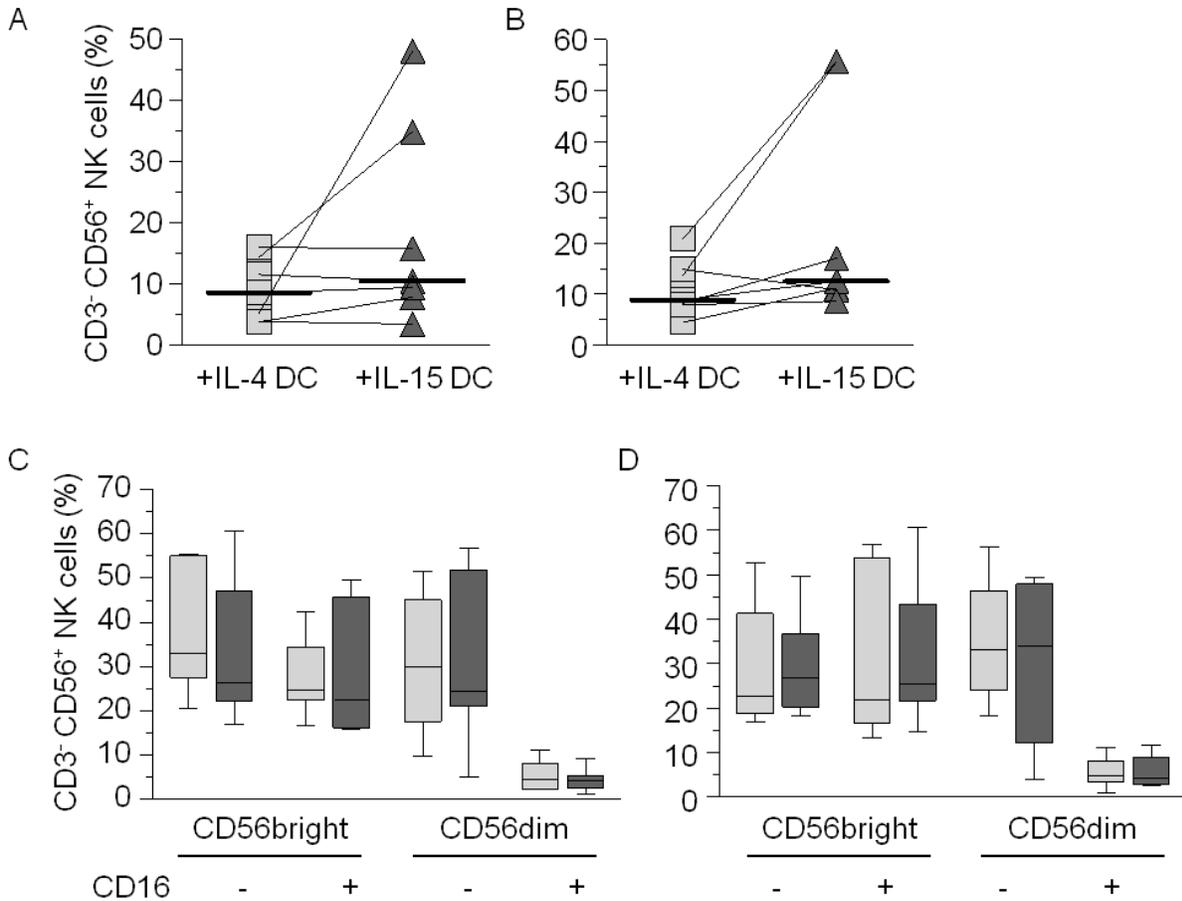


Figure 2-11. NK cell populations in DC-stimulated PBLs are not affected by the subset of DC used to prime PBLs. NK cell populations (CD3⁻ CD56⁺ cells from the lymphocyte cell population) were evaluated at the end of the PBL co-culture using mIL-4 DCs (light grey squares or bars) or mIL-15 DCs (dark triangles or bars). Donor-matched DC subsets are indicated by the line connecting the shapes. The median NK cell percentages are represented by the dark bars. A) Percentage of NK cells detected in the lymphocyte-size population after primed against CMV pp65. $n=6$. B) Percentage of NK cells in the lymphocyte-size population after primed against HCV core. $n=6$. C) Distribution of NK cell subsets in CMV-primed lymphocytes. $n=5$. D) Distribution of NK cell subsets in HCV-primed lymphocytes. $n=5$. No statistical significance was found between mIL-4 DC and mIL-15 DC stimulated lymphocyte cultures. Significance calculated by wilcoxon matched-pairs signed rank test with the determined p value ≤ 0.05 considered significant

Table 2-1. Comparison of cytokine production by mDCs

Cytokine	Donor A (pg/mL)		Donor B (pg/mL)	
	mIL-4 DC	mIL-15 DC	mIL-4 DC	mIL-15 DC
IL-12p70	6 ± 0.3	16 ± 1.5	2 ± 0.3	25 ± 2.1
Pro-inflammatory:				
IL-1 α	135 ± 5.3	332 ± 30	32 ± 0.4	1,228 ± 37
IL-1 β	338 ± 28	601 ± 54	92 ± 3.2	2,696 ± 90
IL-6	652 ± 56	1,664 ± 34	52 ± 2.3	8,036 ± 311
IL-15	25 ± 3.5	2,358 ± 147	23 ± 2.2	248 ± 27
IFN- γ	<1	2,332 ± 185	<1	1,905 ± 35
TNF α	149 ± 16	1,438 ± 41	72 ± 2.5	3,152 ± 104
Anti-inflammatory:				
IL-4	3 ± 0.5	4 ± 0.1	3 ± 0.3	2 ± 0.3
IL-10	67.8 ± 2.3	6.7 ± 0.6	52.5 ± 0.6	113.5 ± 9.8

Results are expressed as average concentration ± SEM (pg/mL).

CHAPTER 3
IDENTIFICATION OF DIFFERENTIALLY ACTIVATED STAT OR MAPK SIGNALING
PATHWAYS IN IL-4 DCS VERSUS IL-15 DCS AND THEIR EFFECT ON DC
MATURATION

Introduction

The molecular signaling events down-stream of IL-4 and IL-15 have been characterized individually in NK and T cells. Yet when these two cytokines are combined separately with GM-CSF, they generate two monocyte-derived DC subsets that display their own distinctive surface phenotype and exhibit different abilities to stimulate Ag-specific T cell responses. The molecular events regulated by GM-CSF and IL-15 versus GM-CSF and IL-4 that drive monocytes to differentiate into DCs and the modulation of their immunostimulatory functions are unknown.

As described previously in chapter 1, the binding of IL-4 and IL-15 to their receptor complexes predominantly activates the STAT6 and STAT5 pathways respectively in NK and T cells (86, 98, 146). In other cell types, IL-4 can activate ERK1/2 or p38 whereas IL-15 stimulation can induce pSTAT3. Membrane-bound IL-15 on IL-15-producing cells can also be trans-presented to nearby NK and T cells to likely activate STAT5 in those lymphocytes (75, 98, 100, 195, 196). Cross-linkage of membrane-bound IL-15 in IL-15 reverse signaling can also directly lead the phosphorylation and activation of the p38, ERK1/2, FAK, and Rac3 proteins (98, 105, 107-110, 196).

In contrast to the IL-4R and IL-15R, the receptor for GM-CSF (GM-CSFR) functions as a hexameric complex consisting of 2 GM-CSFR α chains, 2 common beta chains (β c), and 2 GM-CSF molecules (197). The GM-CSFR α chain specifically binds the GM-CSF molecule while β c is associated with JAK2 and a SH2 domain containing (Shc) adaptor protein and functions as the major signaling subunit (156). The hexameric

configuration of the GM-CSFR complex allows the two β c-associated JAK proteins to trans-phosphorylate the receptors leading to the activation of STAT5 and/or ERK1/2.

There have been only few studies that have demonstrated direct causation of STAT or MAPK activities on myeloid DC immune functions. For example, IL-4-induced pSTAT6 up-regulates CD86 and DC-SIGN or suppresses TNF α and IL-6 production (8, 177, 198, 199). In murine DC studies, STAT3 is essential for differentiation of bone marrow-derived DCs using Fms-like tyrosine kinase 3 ligand (Flt3L) but not for GM-SCF-mediated DC differentiation (200). Additional murine conditional STAT3 KO studies concluded that ablation of STAT3 did not affect the number of CD11c⁺ DCs but inhibited the up-regulation of DC maturation-related proteins (HLA class II, CD86, IFN- γ , TNF α) and subsequently the abilities to stimulate T cell responses was reduced compared to wild type (139, 201). Inhibition of the up-stream kinase (JAK2) and the IL-6 KO murine model studies also showed the same results (138, 201). Activation of ERK1/2 and p38 in monocytes by IL-15 reverse signaling was shown to induce TNF α , IL-6, and IL-8 production (107, 109). Additionally activated p38 in DCs up-regulates expression of co-stimulatory molecules (158, 202-204).

In order to effectively study the cytokine-induced molecular signaling involved in DC differentiation and maturation, an effective DC tumor cell model would allow for rapid and reproducible testing. Despite the lack of an established human DC cell line, we evaluated the potential use of three commonly used monocyte-like tumor cell lines to study the cytokine-induced molecular events. These monocyte-like cell lines U937, THP-1, and HL-60 can be differentiate into macrophages with PMA treatment. Unfortunately these human tumor monocytic cell lines cannot differentiate into

immunostimulatory functional DCs which may limit their usefulness in studying signal pathways (205, 206). Nonetheless these cell lines would provide an unlimited source of cells and eliminate possible donor-to-donor variation.

In this chapter, we identified that IL-4 DCs and IL-15 DCs exhibit their own distinct signaling profiles. These differentially activated pathways appear to directly affect DC phenotype and cytokine production. Additionally, we show that monocytic cell lines are not an ideal model for studying the more complex molecular signaling pathways involved in human DC differentiation and maturation, but they may function as a more simplistic model for IL-4 and GM-CSF signaling mechanisms in DCs.

Materials and Methods

Monocyte-derived DCs and Monocytic Cell Lines

Immature and mature IL-4 and IL-15 DCs were generated as described previously. For signaling studies, DCs were not cryogenically stored during experiment. Human monocytic cell lines U937, HL-60, and THP-1 were purchased from ATCC (Manassas, VA) and maintained in complete RPMI media containing 10% FBS.

To detect phosphorylated signaling proteins in the monocytic cell lines, cells were serum starved overnight to decrease potentially high basal pSTAT levels that would mask detection of cytokine-induced responses. Serum-starved cells were stimulated with 25 ng/mL IL-4, 100 ng/mL IL-15, 50 ng/mL GM-CSF, IL4+GM-CSF, IL-15+GM-CSF, 50 µg/mL IL-6, 5 mM H₂O₂, 50 µM phorbol-12-myristate-13-acetate (PMA, Sigma), or 25 µg/mL anisomycin (Sigma) for 15 minutes at 37°C. For staining of the basal level of phosphorylated proteins in DCs, freshly harvested DCs were briefly rested in AIM-V media at 37°C for 1 - 2 hours as recommended by BD before fixation. To investigate direct activation of signaling proteins by IL-4 or IL-15 during DC maturation, 2-3x10⁵

imDCs were cultured with the maturation mixture (50 ng/mL GM-CSF, 1 mg/mL LPS, and 25 ng/mL TNF α) along with one of the following for 30 minutes at 37°C: 25 ng/mL IL-4, 100 ng/mL IL-15, or both IL-4 and IL-15.

Monoclonal Antibodies and Inhibitors

Antibodies against the phosphorylated proteins STAT3 pY705 (clone 4/P-STAT3, pacific blue), STAT5 pY694 (clone 47/Stat5, Alexa488), STAT6 pY641 (clone 18/Stat6, Alexa647), p38 MAPK pT180/pY18 (clone 36/p38, pacific blue), ERK1/2 pT202/pY204 (clone 20A, Alexa647) as well as CD11c (clone B-ly6, PE), CD11c (clone B-ly6, APC), GM-CSFR α /CD116 (clone M5D12, FITC), CD25/IL-4R α (clone hIL4R-M57, PE), and IFN- γ (clone B27, APC) were from BD. Anti-TNF α (clone MP9-20A4, APC) was from Caltag. Anti-IL-15R α (clone eBioJM7A4, FITC) was purchased from eBioscience and anti-IL-15 (clone 34559, PE) was obtained from R&D systems (Minneapolis, MN). The inhibitors for p38 MAPK (SB203580) and STAT3 (stattic) were purchased from Tocris Bioscience (R&D Systems, Inc., Minneapolis, MN).

Phospho-protein Flow Cytometry

To crosslink membrane-bound IL-15 to either the IL-15R α or the cell membrane, freshly harvested DCs were immediately fixed in 2% paraformaldehyde (PFA) final concentration for 10 minutes at 37°C before proceeding with the usual surface staining protocol. Staining for surface markers was described in the previous chapter.

Intracellular phosphorylated proteins were stained according to the recommendations made by Krutzik and Nolan's publication as well as BD (207). For basal signaling levels harvested DCs were rested for 2 hours without cytokines to allow recovery from the prior manual manipulations before fixation. Monocytic cell lines were serum starved overnight and then stimulated for 15 minutes at 37°C before fixation. For

determining the direct effects of IL-4 or IL-15 during DC maturation, harvested imDCs were cultured with maturation mixture along with IL-4, IL-15, or IL-4 and IL-15 for 30 minutes at 37°C before fixation. All of the cells were fixed with a final concentration of 2% PFA for 10 minutes at 37°C, permeabilized with cold 90% methanol for 1 hour on ice or overnight at -20°C, washed, and stained with anti-phospho-protein antibodies for 1 hour at room temperature. Anti-CD11c antibody was also added when staining DCs. Matched isotype antibodies were not used as controls for intracellular phospho-protein staining due their high background. Non-stimulated cells and non-stained cells were used to determine the phospho-protein background and autofluorescence. Data was collected on the BD LSRII flow cytometer and analyzed with Flowjo. Δ MFI and Δ percentage of phospho-protein⁺ cells were determined by subtracting the non-specific autofluorescence value from the sample value. Statistical significance was determined via two-tailed wilcoxon matched-pairs signed rank test or the two-tailed t test when there were only a small number of donors. The calculated *p* values ≤ 0.05 were considered to be significant.

DC Intracellular Cytokine Flow Cytometry

DCs matured as described previously for 24 hours. Next the donor-matched mDCs from 8 individuals were collected, washed, transferred into low adhesion plates in AIM-V media without cytokines, and treated with 10 μ M SB203580, 30 μ M static, or 0.3% DMSO in the presence of 1 μ L/mL BD GolgiPlug for 6 hours at 37°C. GolgiPlug was also added to the no treatment control consisting of 5 donors. The following described steps were completed on ice. mDCs were washed, blocked with 10% human-mouse serum for 30 minutes, stained with anti-CD11c antibody for 30 minutes, fixed and permeabilized with BD Fix/Perm Buffer, stained with anti-IFN- γ or anti-TNF α for 1 hour.

Data was collected on the BD LSRII flow cytometer and analyzed with Flowjo. Δ MF1 and Δ percentage of IFN- γ^+ or TNF α^+ from the CD11c $^+$ -gated mDCs were determined. Statistical significance was determined using the two-tailed wilcoxon matched-pairs signed rank test in which calculated p values ≥ 0.05 were considered to be significant.

Results

Expression of Surface Cytokine Receptors on DCs

Expression of IL-4R α , IL-15R α , and GM-CSFR α were assessed on immature and mature IL-4 DCs as well as IL-15 DCs prepared from a new donor group. GM-CSFR α was the highest expressed cytokine receptor on both immature and mature DCs (Figure 3-1). Expression of GM-CSFR α was significantly higher levels on imIL-15 DCs in comparison with imIL-4 DCs ($p=0.03$, $n=6$) (Figure 3-1A). GM-CSFR α expression seemed to be down-regulated with DC maturation, especially mIL-15 DCs. Consequently GM-CSFR α expression was determined to be much higher on mIL-4 DCs and detected on a significantly larger number of mIL-4 DCs in relation to mIL-15 DCs ($p=0.03$, $n=6$) (Figures 3-1C and 3-1D). Additionally, the expression levels of IL-4R α and IL-15R α on the surface of immature and mature IL-4 DCs were similar to those determined on the corresponding IL-15 DC populations. The numbers of IL-4 DCs that expressed either IL-4R α or IL-15R α were also comparable to number of IL-15 DCs expressing IL-4R α or IL-15R α .

In addition to cytokine receptors, we also assessed the relative level of IL-15 on membrane surface (membrane IL-15) which would signify the DCs are trans-presenting IL-15 and/or involved in IL-15 reverse signaling. IL-15 trans-presentation by DCs has recently been determined to have an essential role in modifying T cell immune activities as well as NK cell development and activation whereas IL-15 reverse signaling in

monocytes leads to inflammatory cytokine production (72, 97, 105, 107-109, 208-211). In order to detect IL-15 on the cell membrane, cells were treated with PFA to crosslink IL-15 to its receptor or the cell membrane. The levels of membrane IL-15 on immature and mature CD11c⁺ IL-15 DCs were much higher compared to the amounts of membrane IL-15 found on the surface of the IL-4 DCs (imDCs, $p=0.03$, $n=6$) (mDCs, $p=0.06$, $n=6$). IL-15 was also detected on the membrane of a greater percentage of CD11c⁺ IL-15 DCs than IL-4 DCs (imDCs, $p=0.03$, $n=6$) (mDCs, $p=0.06$, $n=6$).

Identifying the IL-4 DC and the IL-15 DC Basal Signaling Profiles

Replacing the traditional GM-CSF and IL-4 combination with GM-CSF and IL-15 likely has significant repercussions on the molecular signaling event in generation of monocyte-derived DCs. Using phospho-protein flow cytometry, we measured the basal activity levels of the STAT and MAPK pathways responsive to IL-4, IL-15, or GM-CSF in order to determine the signaling profiles for IL-4 DCs and IL-15 DCs. IL-4 has been strongly linked with the STAT6 pathway but it also induces pp38 and pERK1/2 (86, 146). ERK1/2 is also activated by GM-CSF but the primary signaling mechanism for GM-CSF is considered to be STAT5 (148, 212-214). IL-15 stimulation predominantly activates STAT5 as well, along with the STAT3 pathway (123, 192, 215-217). Thus we assessed the pSTAT6, pSTAT5, pSTAT3, pp38, and pERK1/2 signaling pathways in the DCs. Out of the five signaling pathways evaluated, STAT6, STAT5, and ERK1/2 were found to be more active in CD11c⁺ IL-4 DCs compared to IL-15 DCs regardless of the DC maturation status (Figures 3-2 and 3-3). The levels of pSTAT6 (imDC, $p=0.03$, $n=6$) (mDC, $p=0.01$, $n=9$) and pERK1/2 (imDC, $p=0.06$, $n=5$) (mDC, $p=0.02$, $n=7$) were considerably greater in immature and mature IL-4 DCs compared to IL-15 DC populations. Furthermore the percentages of the CD11c⁺ IL-4 DCs exhibiting pSTAT6

(imDC, $p=0.03$, $n=6$) (mDC, $p=0.03$, $n=9$) or pERK1/2 (imDCs, $p=0.06$, $n=5$) (mDCs, $p=0.05$, $n=7$) were higher compared to IL-15 DCs.

Interestingly, there were significantly lower numbers of immature and mature IL-15 DCs that exhibited activated STAT5 compared to IL-4 DCs (imDC, $p=0.0005$, $n=12$) (mDC, $p=0.02$, $n=10$). Furthermore the levels of phosphorylated STAT5 in both immature and mature IL-15 DCs were significantly less than those in the donor-matched IL-4 DCs (imDC: $p=0.0005$, $n=12$) (mDC, $p=0.03$, $n=10$). Relative quantification of the STAT5A and STAT5B transcripts via SYBR green qRT-PCR during monocyte-to-mDC differentiation demonstrated that the two STAT5 transcripts were detected at similar levels in IL-4 DCs and IL-15 DCs (Appendix D). This indicates that the relatively lower pSTAT5 activity in IL-15 DCs is not caused by a limited availability of STAT5 but STAT5 is simply not becoming activated in IL-15 DCs. We found pSTAT3 ($n=6$) and pp38 ($n=5$) to be nearly the same level in imIL-4 DCs and imIL-15 DCs. Following DC maturation, the pSTAT3 ($\Delta\text{MFI} \sim 18$ to ~ 125) and the pp38 ($\Delta\text{MFI} \sim 18$ to ~ 83) levels were mildly elevated in CD11⁺ mIL-15 DCs whereas the activated proteins are barely detectable in the mIL-4 DC population. In addition to the level of pSTAT3 ($p=0.008$, $n=8$) and pp38 ($p=0.02$, $n=7$) being significantly different between the mDC subsets, there were more mIL-15 DCs exhibiting pSTAT3 ($p=0.02$) and pp38 ($p=0.02$) activity than mIL-4 DCs.

In summary, IL-15 DCs exhibit much less STAT6, STAT5, and ERK1/2 signaling than in comparison to their donor-matched IL-4 DCs. STAT3 and p38 pathways become up-regulated by DC maturation exclusively in IL-15 DCs which suggests that STAT3 and p38 may have influence mIL-15 DC phenotype or functions in a subset-specific

manner. Next we assess which signaling mechanism(s) are directly activated by IL-4, IL-15, and GM-CSF during DC differentiation and/or maturation.

Cytokine Receptor Expression on Monocytic Cell Lines

Monocytic tumor cells lines, though they cannot successfully differentiate into APCs with DC phenotype and function, are frequently used as a cellular model for human monocyte and macrophage molecular studies (205, 206). To determine whether we could utilize the monocytic cell lines U937, THP-1, and HL-60 to study IL-4, IL-15, and GM-CSF signaling, we first examined the expression of the cytokine receptors on the cell lines. IL-4R α , IL-15R α , and GM-CSFR α were detected on the surface of all of the monocytic cell lines (Figure 3-4). Of the three cytokine receptors, GM-CSFR α was the highest expressed receptor and IL-4R α was the least expressed receptor.

Additionally, we found that compared to the other two monocytic cell lines, HL-60 cells expressed the lowest amounts of surface IL-4R α which may in relation to the other two monocytic cell lines. Thus, U937 and THP-1 cell lines readily expressed the three receptors suggesting that they could be used to evaluate the molecular signaling pathways involved in differentiation of IL-4 DCs and IL-15 DCs.

Optimization of Phosphorylated Protein Detection Using Monocytic Cell Lines

STAT protein activation is very transient. To determine the optimal time to detect phosphorylated STAT proteins after cytokine stimulation, we evaluated pSTAT6, pSTAT5, and pSTAT3 in time course experiments by stimulating U937 cells for 15 minutes, 30 minutes, 1 hour, and 48 hours. We observed pSTAT6 and pSTAT5 levels to be up-regulated within 15 minutes after adding IL-4 or GM-CSF respectively (Figure 3-5). pSTAT6 and pSTAT5 proteins in U937 cells remained up-regulated for 15-30 minutes post-stimulation. By the first hour following GM-CSF-stimulation, elevated

pSTAT5 began to notably decrease and then 48 hours post-stimulation pSTAT5 was nearly the same level as the non-stimulated control. We analyzed cytokine-induced pSTAT protein responses in the following experiments by the first 30 minutes after stimulation.

Direct Induction of STAT and MAPK Signaling in Monocytic Cell Lines by IL-4 or IL-15

Next we evaluated the STAT and MAPK signaling pathways classically associated with IL-4, IL-15, or GM-CSF stimulation in the two candidate monocytic cell lines U937 and THP-1 via phospho-protein flow cytometry. Our results showed that IL-4 stimulation directly activated STAT6 without affecting the other STAT pathways in U937 and THP-1 cells (Figure 3-6). STAT3 was responsive to IL-4, IL-15, or GM-CSF stimulation. pSTAT3 was only increased in the presence of IL-6, one of the main activators for the STAT3 pathway. Similarly the pp38 or pERK1/2 pathways were not up-regulated by IL-4, IL-15, or GM-CSF (data not shown). However treatment with the known positive stimulators PMA or anisomycin increased pERK1/2 and pp38 levels (Appendix C). The positive control results indicated the p38 and ERK1/2 pathways in the cancer cell lines simply did not respond to IL-4 stimulation. The presence of GM-CSF alone or GM-CSF combined with IL-4 or IL-15 induced STAT5 activation in U937 and THP-1 cell lines. Interestingly, the addition of IL-15 alone did not induce up-regulation of pSTAT5 or pSTAT3 in either monocytic cell line.

In summary, U937 and THP-1 cells exhibited strong IL-4-STAT6 and GM-CSF-STAT5 responses. However U937 and THP-1 cell lines did not demonstrate any known molecular signaling responses to IL-15 stimulation. Hence, these cell lines are likely not a good model system to study IL-15 signaling mechanisms in differentiating DCs. These

results also suggest that monocytic cells do not respond to IL-15 stimulation even though they express IL-15R α or that IL-15 activates different signaling pathways than the traditional STAT5 and STAT3 pathways identified in lymphocytes.

Direct Activation of STAT and MAPK Pathways During DC Maturation in the Presence of IL-4 or IL-15

To confirm the absence of pSTAT5 or pSTAT3 in DCs stimulated with IL-15 and whether STAT3 and/or p38 are directly activated during IL-15 DC maturation, we analyzed the IL-4 or IL-15 molecular signaling events during DC maturation. imDCs were cultured with maturation factors (GM-CSF, TNF α , and LPS) along with IL-4, IL-15, or IL-4 and IL-15 for 30 minutes before assessing pSTAT and pMAPK levels in CD11c⁺ DCs. First, we compared the imIL-4 DCs (Figure 3-7, indicated by squares) cultured with maturation factors and IL-4 alone (imIL-4 DC+IL-4) versus imIL-4 DCs matured in the presence of IL-15 alone (imIL-4 DC+IL-15) or IL-4 and IL-15 (imIL-4 DC+IL-4+IL-15). When IL-4 is used in IL-4 DC maturation, pSTAT6 is significantly up-regulated in relation to the imIL-4 DC+IL-15 condition in which IL-4 was not added (imIL-4 DC+IL-4, $p=0.003$) (imIL-4 DC+IL-4+IL-15, $p=0.02$). The percentage of IL-4 DCs exhibiting pSTAT6 also greatly increased when matured with IL-4 (imIL-4 DC+IL-4, $p=0.006$) (imIL-4 DC+IL-4+IL-15, $p=0.008$). The other STAT and MAPK pathways were not differentially activated in imIL-4 DCs by culturing the cells with IL-4 and/or IL-15.

Next, imIL-15 DCs (Figure 3-7, indicated by triangles) cultured with maturation factors along with IL-4 alone (imIL-15 DC+IL-4) or IL-4 and IL-15 (imIL-15 DC+IL-4+IL-15) were compared to the imIL-15 DCs cultured with maturation factors along with IL-15 (imIL-15 DC+IL-15). Again, pSTAT6 levels increased only in the presence of IL-4 (imIL-15 DC+IL-4, $p=0.008$; imIL-15 DC+IL-4+IL-15, $p=0.003$). There were also more

pSTAT6⁺ imIL-15 DCs when cultured with IL-4 than in its absence (imIL-15 DC+IL-4 and imIL-15+IL-4+IL-15, $p=0.001$). Finally, consistent with the maturing IL-4 DCs responses, maturation in the presence of IL-4 and/or IL-15 did not lead to up-regulation of pSTAT5, pSTAT3, pERK1/2, or pp38 levels in the CD11c⁺ IL-15 DCs.

We also evaluated pSTAT or pMAPK responses between IL-4 DCs and IL-15 DCs. Despite the relative lack of up-regulated pSTAT6, there was a significant difference in the pSTAT6 levels in imIL-4 DC+IL-15 and imIL-15 DC+IL-15 ($p=0.008$). There was also significantly more pSTAT6⁺ imIL-4DC+IL-15 cells than in the imIL-15 DC+IL-15 population ($p=0.01$). These results suggest that imIL-4 DCs are continuously secreting low levels of IL-4 or they are predisposed to have a higher basal pSTAT6 than imIL-15 DCs. Stimulated imIL-4 DCs also exhibited slightly higher averages of pSTAT5, pSTAT3, pERK1/2, and pp38 than their imIL-15 DC counterparts but the differences were not statistically significant. Overall, the up-regulation of pSTAT6 or the lack of phospho-protein responses by IL-4 and/or IL-15 were the same in maturing imIL-4 DCs and maturing imIL-15 DCs. The level of phospho-protein following the addition of both IL-4 and IL-15 showed the same results for stimulation by only IL-4. Hence the presence of IL-15 in maturing IL-4 DCs or IL-15 DCs does not directly activate any of the evaluated STAT or MAPK pathways including STAT3 or p38 MAPK.

Effect of STAT3 or p38 MAPK Inhibition on IFN- γ and TNF α Production in mDCs

We have previously shown that STAT3 and p38 MAPK are more active in mIL-15 DCs than in mIL-4 DCs and that pSTAT3 and pp38 level are elevated during IL-15 DC maturation (Figures 3-2 and 3-3). We have also shown that mIL-15 DCs produce higher levels of IFN- γ and TNF α mRNA transcripts and proteins (Figure 2-7). p38 activity promotes TNF α production. Inhibition of p38 by the inhibitor SB203580 or dominant-

negative p38 leads to decreased production of TNF α and IFN- γ (203). Using commercial inhibitors and intracellular cytokine flow cytometry, we investigated whether pSTAT3 or p38 promote IFN- γ and/or TNF α in mIL-15 DCs. Mimicking the ELISA protocol, DCs were matured for 24 hours, thoroughly washed, cultured with p38 MAPK inhibitor (SB203580) or STAT3 inhibitor (stattic) along with BFA for 6 hours, and then intracellular IFN- γ or TNF α was assessed in CD11c⁺-gated mDCs. SB203580 is a commonly used p38 inhibitor that specially inhibits the p38 α and p38 β isoforms kinase activities (158). SB203580 binds to the ATP pocket of the enzyme and thus sterically hinders its kinase activity. Stattic is a relatively novel STAT3 inhibitor that can act specifically against STAT3 without affecting other major signaling pathways (218). Optimal concentrations of inhibitors were determined using THP-1 cells and PBMCs (data not shown). SB203580 treatment did not affect the production of IFN- γ or TNF α in either mIL-4 DCs or mIL-15 DCs (Figures 3-8 and 3-9). Additionally, inhibition of pSTAT3 activity did not notably affect TNF α production in mDCs. However, IFN- γ production was significantly down-regulated in CD11c⁺ mIL-4 DCs ($p=0.008$, $n=8$) and mIL-15 DCs ($p=0.04$, $n=8$) treated with stattic compared to the DMSO controls. pSTAT3 inhibition also reduced the percentages of IFN- γ ⁺ mIL-4 DCs ($p=0.02$) as well as the IFN- γ ⁺ mIL-15 DCs ($p=0.02$).

Discussion

The cytokine-regulated signaling mechanisms that drive the differentiation of monocytes into immunostimulatory DCs are not well understood. Individually the binding of the cytokines IL-4, IL-15, or GM-CSF to their respective receptor complex predominantly activates the STAT6, STAT5/STAT3, or STAT5/ERK1/2 pathways respectively (86, 98, 148, 156). Depending on the cell type, IL-4 can also activate the

ERK1/2 or p38 pathways (86, 146). Monocyte-derived DCs generated with IL-4, IL-15, or GM-CSF alone have impaired immunostimulatory functions compared to DCs prepared with GM-CSF combined with either IL-4 or IL-15 (33, 37, 38). Thus the molecular mechanisms regulated by pairing of GM-CSF and IL-4 or IL-15 plays a significant role in determining the extent of the DCs' abilities to induce and modify T cell responses. However, what molecular mechanisms are being regulated in human monocytes and *in vitro* generated DCs is unknown. In this chapter, we identified the molecular signaling events that are directed by GM-CSF and IL-4 or GM-CSF and IL-15 during DC maturation that likely play a role in DC functions. We also demonstrated for the first time that human monocytic cells and DCs do not activate STAT5 or STAT3 in response to IL-15.

To determine the activity of the five STAT and MAPK pathways classically linked with IL-4, IL-15, and/or GM-CSF in IL-4 DCs and IL-15 DCs, we assessed the activity of STAT6, STAT5, STAT3, ERK1/2 and p38 pathways. We first verified addition of IL-4 or IL-15 at same concentration used to generate our DCs activated STAT6, STAT5, and/or STAT3 pathways in CD3⁺ T cells (Appendix B). Using phospho-protein flow cytometry, we confirmed IL-4 activated STAT6 and IL-15 induced pSTAT5 in T cells. The other pathways, STAT3, p38, and ERK1/2, were not responsive to either IL-4 or IL-15 stimulation, but they were activated to their respective positive controls.

Next we evaluated the basal STAT and MAPK activity levels in immature and mature DCs. We found the pSTAT6, pSTAT5, and pERK1/2 proteins were consistently more abundant in CD11c⁺ IL-4 DCs than compared to IL-15 DCs regardless of the DC maturation state (Figures 3-2 and 3-3). GM-CSF and IL-4 can activate ERK1/2 and

STAT6 respectively, thus the elevated pERK1/2 and pSTAT6 protein levels in IL-4 DCs are probably due the presence of IL-4 during generation of IL-4 DCs (86, 117, 119, 156). Additionally, we demonstrated that pSTAT6 is directly up-regulated in either imIL-4 DCs or imIL-15 DCs in the presence of IL-4 (Figure 3-7). Along the same line, GM-CSF and IL-15 can also activate STAT5 and both DC subsets were generated in the presence of GM-CSF. Therefore it we interesting that CD11c⁺ IL-4 DCs exhibited significantly higher pSTAT5 levels in comparison to IL-15 DCs (Figures 3-2 and 3-3). We did not notice any dramatic differences in the relative expression of STAT5A and STAT5B mRNA transcripts in IL-4 DCs and IL-15 DCs that could explain the unexpected absence of pSTAT5 from CD 11c⁺ IL-15 DCs (Appendix D). Considering that the expression of IL-15R α was nearly equal on both imDCs and that GM-CSFR α was more abundant on the imIL-15 DCs than on imIL-4 DCs the higher pSTAT5 in imIL-4 DCs was even more surprising (Figure 3-1).

One potential reason for lower pSTAT5 levels in IL-15 DCs compared to IL-4 DCs could potentially be the transient nature of STAT protein activation. The tyrosine phosphorylation of STAT proteins is a highly regulated process involving tyrosine kinases as well as multiple tyrosine dephosphorylases and tyrosine kinase inhibitors (115, 219). In a time course experiment we noted that GM-CSF-stimulated pSTAT5 levels peaked around 15-30 minutes after adding the cytokine, and then the elevated pSTAT5 levels began to decrease (Figure 3-5). The pSTAT5 levels were reduced by about half the maximum level about an hour after GM-CSF stimulation. Therefore measuring the phosphorylated STAT protein levels 36-48 hours after adding GM-CSF and IL-15 in the freshly harvested mDCs would likely be too late to determine a direct

correlation between cytokine stimulation and an increase in pSTAT5 or pSTAT3. Therefore in order to the optimal time to quantify phospho-protein levels, we evaluated the immediate pSTAT5 and pSTAT3 responses in U937 cells, THP-1 cells, imIL-4 DCs, and imIL-15 DCs after a brief IL-15 stimulation. We did not observe any pSTAT5 or pSTAT3 increases in response to IL-15 stimulation even though the different cell types were shown to express IL-15R α and pSTAT5 up-regulation which were observed in the PBL controls (Figures 3-1, 3-4, 3-5, 3-6, and 3-7). As a result, we are the first to demonstrate that IL-15 does not directly activate the classical STAT5 or STAT3 pathways in monocytic cell lines or monocytic-derived DCs (Figures 3-5, 3-6, and 3-7).

In addition to the differential activation of the STAT5, STAT6, and ERK1/2 pathways between the DC subsets, we noticed that the p38 and STAT3 activity levels were enhanced specifically in IL-15 DCs after maturation (Figures 3-2 and 3-3). This would suggest that the p38 and STAT3 pathways may be involved in regulating the different phenotypes and behaviors exhibited between the mDC subsets. However, our results indicate that IL-4 or IL-15 stimulation of the monocytic cell lines did not activate p38 or STAT3 (Figures 3-5 and 3-6). Additionally, we evaluated whether or not p38 or STAT3 activation only occurred in maturing DCs. imIL-4 DCs and imIL-15 DCs were briefly cultured with maturation factors (GM-CSF, LPS, TNF α) in the presence of IL-4 or IL-15, but we did not observe up-regulation of pp38 or pSTAT3 (Figure 3-7).

Potentially the up-regulation of pSTAT3 in mIL-15 DCs could be due to the greater production of IL-6 by mIL-15 DCs and/or relative responsiveness of mIL-4 DCs or mIL-15 DCs to the presence of IL-6 (Figure 2-7 and Table 2-1). To support this in a pilot experiment, mDCs were briefly cultured with IL-6 and then assessed for changes in

pSTAT3 levels using IL-6-stimulated PBLs as controls. We found that after IL-6 stimulation, CD11c⁺ mIL-4 DCs only showed a minor increase in pSTAT3 whereas pSTAT3 was noticeably up-regulated in mIL-15 DCs (data not shown). IL-6 stimulation of mIL-4 DCs prepared from another donor and confirmed the lack of pSTAT3 response compared to PBL controls.

Increased pp38 activity levels in mIL-15 DCs compared to mIL-4 DCs may be one of the molecular mechanisms regulating immunostimulatory functions of mIL-15 DCs. In mature murine DCs, greater p38 MAPK activity promotes DC maturation by increasing co-stimulatory molecule expression and cytokine production (150, 155, 202). pp38 induces production of IL-12, IL-6, and TNF α as well as enhances IFN- γ transcription and subsequent protein production (220-222). Thus the significantly higher pp38 levels that we noted in human mIL-15 DCs compared to mIL-4 DCs could play a role in the relatively higher amounts of IL-6, TNF α , and IFN- γ secreted by the mIL-15 DCs (Figure 3-5 and Table 2-1). To determine whether the increased pp38 levels in mIL-15 DCs influenced the TNF α or IFN- γ production, we treated mDCs with the commonly used p38 inhibitor SB203580 and measured cytokine production by intracellular cytokine flow cytometry. p38 activity promotes the translation of the TNF α gene by increasing the mRNA stability but it does not have much effect on transcription of the TNF α mRNA (158, 204, 223). Therefore if the TNF α production is directly affected by p38, addition of SB203580 should reduce intracellular TNF α levels. Although intracellular cytokine production in DCs is not usually measured by flow cytometry, this method allowed us to evaluate cytokine production from a defined CD11c⁺-gated DC population. Attempting to mimic the previous ELISA assay, imDCs were matured for 24 hours before determining

cytokine production (Table 2-1). These mDCs were cultured for 6 hours in the presence of BFA and SB203580 and then analyzed for intracellular TNF α or IFN- γ in CD11c⁺ DCs. Consistent with our prior results from the ELISA and TNF α qRT-PCR, the relative amount of TNF α detected in mIL-15 DCs and the number of TNF α ⁺ CD11c⁺ mIL-15 DCs were significantly greater than in mIL-4 DCs (Figures 2-7 and 3-9, Table 2-1). However we found that inhibiting the p38 signaling pathway did not decrease the number of DCs producing TNF α or production of TNF α as reported to occur in other types of cells (Figures 3-8 and 3-9) (158, 224, 225). Inhibiting p38 activity also did not affect the numbers of mIL-4 DCs that were generating IFN- γ or the production of IFN- γ in either of the DC subsets. Evaluation of the relative intracellular IFN- γ levels in mDCs via western blot using whole cell lysates prepared from two individual donors suggested that mIL-15 DC may have more intracellular IFN- γ than mIL-4 DCs but unfortunately the data was not conclusive (data not shown).

The discrepancy between our intracellular IFN- γ flow cytometry results and our previous ELISA and IFN- γ mRNA quantification is likely due not measuring IFN- γ production in DCs at the optimal time point. One of the limitations of intracellular cytokine flow cytometry is that cytokine production can only be measured for short periods of time due to the toxicity of BFA. Moreover, the optimal window of time depends on the particular cytokine and cell type. It is likely that the best time to capture the expected differences IFN- γ production in the DC subsets is not the same time period as previously reported for TNF α (226). Finally, at least one of the benefits of attempting to measure intracellular cytokines by flow cytometry analysis was that we

repeatedly demonstrated CD11c⁺ DCs produce IFN- γ which contradicts Hardy et al.'s conclusions that IL-15 DCs do not produce IFN- γ (43).

To determine whether the presence of IL-15 effects transcription of either the IFN- γ or TNF α gene via an unidentified signaling pathways we also evaluated whether the presence of IL-4, IL-15, or both IL-4 and IL-15 during DC maturation along with the other maturation factors (GM-CSF, LPS, TNF α) affect transcription of the IL-6, TNF α , or IFN- γ genes or the IFN- γ secretion (Appendix E). Although we only assessed two donors, there was no clear trend whether IFN- γ secretion was affected by IL-4 or IL-15 as measured via ELISA. In the qRT-PCR analysis, we did note that the IFN- γ transcript levels in imIL-4 DCs matured in the presence of IL-15 or IL-4+IL-15 were up-regulated compared to imIL-4 DCs matured in only IL-4. Unfortunately when we looked at imIL-15 DCs, we did not see the same pattern. IFN- γ mRNA level for imIL-15 DCs matured with IL-4 or IL-4+IL-15 were lower than imIL-15 DCs matured with IL-15. The effect of IL-4 or IL-15 during DC maturation on transcription of TNF α and IL-6 mRNA levels was also not clear.

STAT3 seems to have a dual role in DC development and maturation. STAT3 is essential for murine DC differentiation but it inhibits murine DC maturation as measured by the lack of co-stimulatory molecule up-regulation and subsequently reduces their efficiency to prime T cell responses (138, 139, 200, 201, 227). It also promotes production of IL-12, IL-6, TNF α , and IFN- γ in murine DCs and since the STAT3 conditional KO DCs are no longer responsive to IL-10 stimulus, pro-inflammatory cytokines are continuously secreted by the DCs. Inhibition of STAT3 in murine DCs compared to non-inhibited wild type controls shows an increase in CD80, CD86, and

MHC class II expression but reduces the capacity for OVA Ag up-take. Thus our previous phenotypical analysis on human IL-15 DCs in chapter 2 with their increased pSTAT3 activities appears to be consistent with the murine DCs with activated STAT3. We demonstrated that inhibition of STAT3 negatively affects IFN- γ production in both CD11c⁺-gated mDC subsets using intracellular cytokine flow cytometry analysis (Figures 3-8 and 3-9). STAT3 inhibition did not affect TNF α production in the mDCs.

Perhaps monocytic cell lines and monocyte-derived DCs may utilize another signaling pathway down-stream of the IL-15R that we have not examined in this study. Direct activation of STAT5 and STAT3 pathways by IL-15 exposure has been established using NK and T cells but IL-15-mediated signaling in monocytic cell lines or DCs has not been confirmed to occur in the same manner (98, 100). Monocytic cell lines and DCs could also use an alternative IL-15 receptor complex similar to mast cells though it is not likely (98). Mast cells express an alternative IL-15 receptor consisting of a novel IL-15RX receptor subunit and the normal IL-15R α chain (228). Although this alternate IL-15 receptor is still not well characterized, it is associated with STAT5 and STAT6 activation and thus likely not the reason for the relative lack of pSTAT5 and pSTAT6 in IL-15-stimulated monocytic cell lines or DCs.

In general, there are three IL-15-mediated signaling mechanisms, IL-15 receptor-mediated, trans-presentation, and reverse signaling (98, 196). IL-15 receptor-mediated is initiated by the classical IL-15 interaction with its heterotrimer receptor which results in STAT5 and STAT3 activation. IL-15 trans-presentation and reverse signaling are important alternative IL-15 signaling mechanisms involving IL-15 that is attached to the surface membrane of DCs and monocytes. Since the trans-presented IL-15 binds to the

IL-2R β - γ dimer on the neighboring NK or T cells, it is likely that the traditional STAT5 and STAT3 pathways become activated but so far no study has shown this to be the case. The mechanism in IL-15 reverse signaling, membrane-bound IL-15 on monocytes and THP-1 cells are cross-linked by anti-IL-15 antibody which activates ERK1/2 and p38 and consequently induces TNF α , IL-6, and IL-8 production (107, 109). Additionally, IL-15 reverse signaling also increased cellular adhesion in monocytes and cancer cells. It is likely that if IL-15 DCs participate in IL-15 trans-presentation, they also activate the reverse signaling pathway. IL-15 DCs exhibit greater amounts of membrane IL-15 and appear to be a more adherent cell type than the IL-4 DCs with less membrane IL-15 (Figures 2-1 and 3-1). Furthermore, IL-15 DCs express high amounts of TNF α and IL-6 mRNA transcripts and protein production compared with IL-4 DCs (Table 2-1).

We attempted to determine whether the higher levels of membrane IL-15 on more IL-15-mediated cell-to-cell contact among IL-15 DCs promotes TNF α production. The membranes of IL-15 DCs were labeled fluorescent dye, co-cultured with non-labeled IL-4 DCs, and then TNF α production was assessed by flow cytometry. Both DC subsets, regardless of maturation status, shared a lot of cell-cell interaction. Unfortunately, this cell-cell interaction involved large amounts of membrane exchange between the membrane-labeled IL-15 DCs and unlabeled IL-4 DCs and caused difficulties in clearly identifying the two DC subsets (data not shown). Due to the complications in studying the molecular signaling mechanisms regulated by IL-15 trans-presentation/reverse signaling in primary APCs, we did not further investigate these alternative IL-15 signaling pathways.

Overall our results show that monocyte-derived IL-15 DCs exhibit a molecular signaling profile that is unique from the one observed in IL-4 DCs and may have implications with their immune functions. The basal activity levels of STAT and MAPK proteins in IL-15 DCs are generally reduced compared to in IL-4 DCs. Phosphorylated STAT6, STAT5, and ERK1/2 proteins were much more abundant in IL-4 DCs compared to IL-15 DCs. We demonstrate that in contradiction to the classical IL-15-STAT5 concept, IL-15R α -expressing monocytic cell lines and monocyte-derived DCs do not respond to IL-15 stimulation by activating STAT5, or even STAT3. Finally, pSTAT3 is up-regulated during DC maturation in only CD11c⁺-gated IL-15 DCs and appears to enhance IFN- γ production by mDCs.

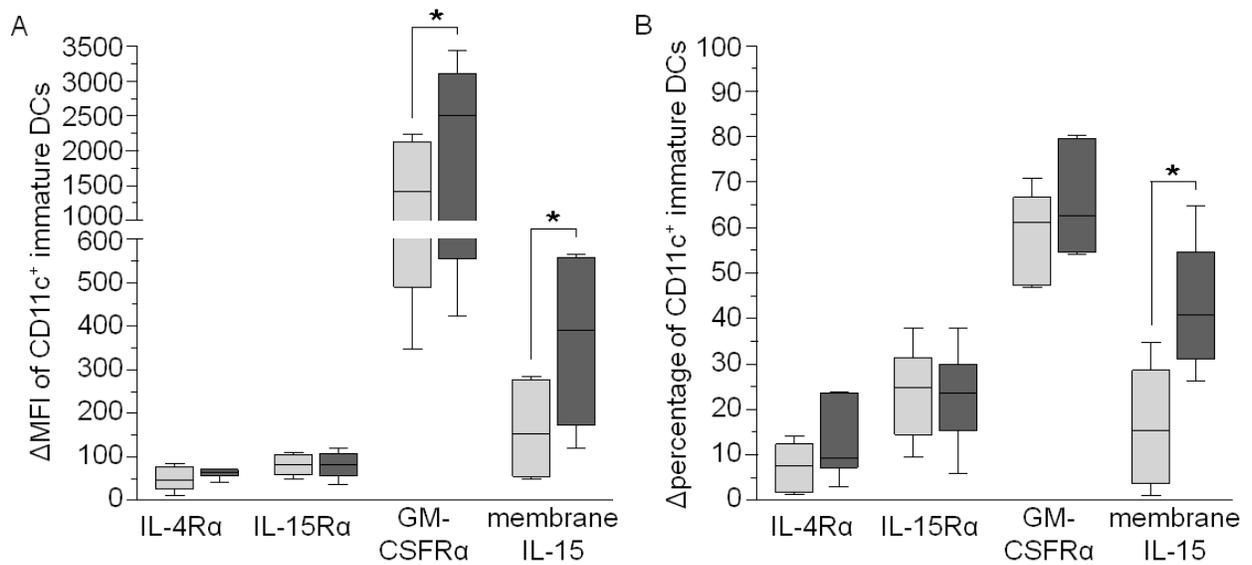


Figure 3-1. Expression of cytokine receptors and membrane-bound IL-15 detected on immature and mature DC subsets. A) Expression of surface protein Δ MFI of CD11c⁺ imIL-4 DCs (light grey bars) and CD11c⁺ imIL-15 DCs (dark grey bars). B) Δ percentage protein⁺ CD11c⁺ imDC subsets. C) Expression of surface protein Δ MFI of CD11c⁺ mIL-4 DCs and mIL-15 DCs. D) Δ percentage of protein⁺ CD11c⁺ mDC subsets. $n=6$. Significance calculated by wilcoxon matched-pairs signed rank test with the determined p value ≤ 0.05 considered significant

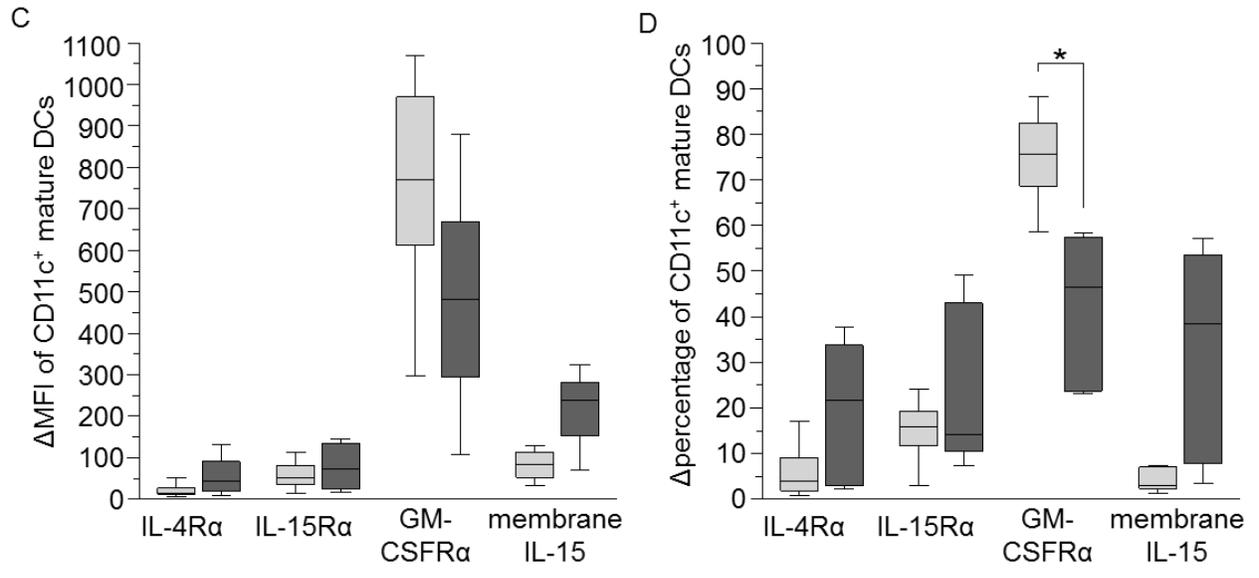


Figure 3-1. Continued

A

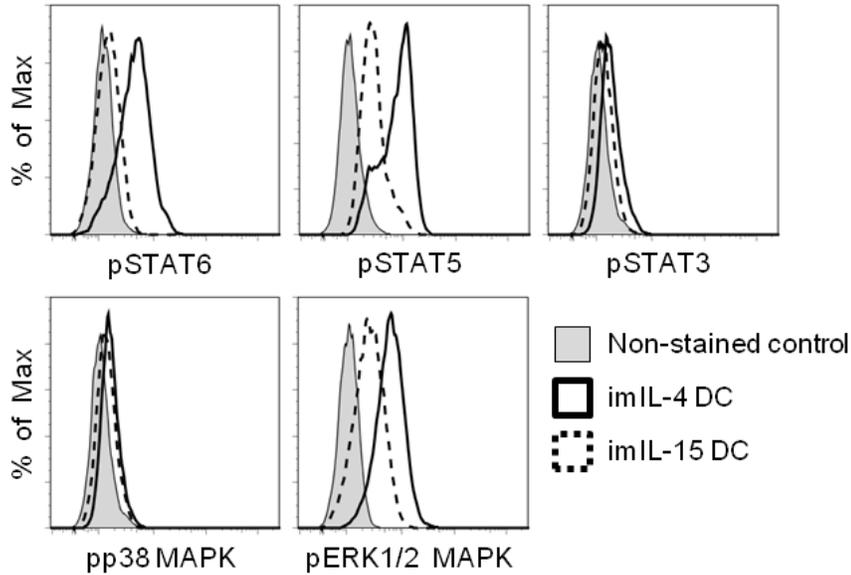


Figure 3-2. Comparison of signaling pathway profiles between the CD11c⁺ imDC subsets. A) Representative histogram data of CD11c⁺-gated imDCs. Grey shaded histograms indicate the non-stained imDC controls. imIL-4 DCs are shown by the solid lines and imIL-15 DCs are indicated by the dashed lines. B) Phospho-protein⁺ Δ MFI of CD11c⁺-gated cells after subtraction of the non-stained mDC values. C) Phospho-protein⁺ Δ percentage of CD11c⁺ imDCs after subtraction of the non-stained imDC values. imIL-4 DCs are shown as light squares and imIL-15 DCs are indicated by dark triangles. Median values are indicated by the bars. $n=5-12$. Significance calculated by wilcoxon matched-pairs signed rank test with the determined p value ≤ 0.05 considered significant

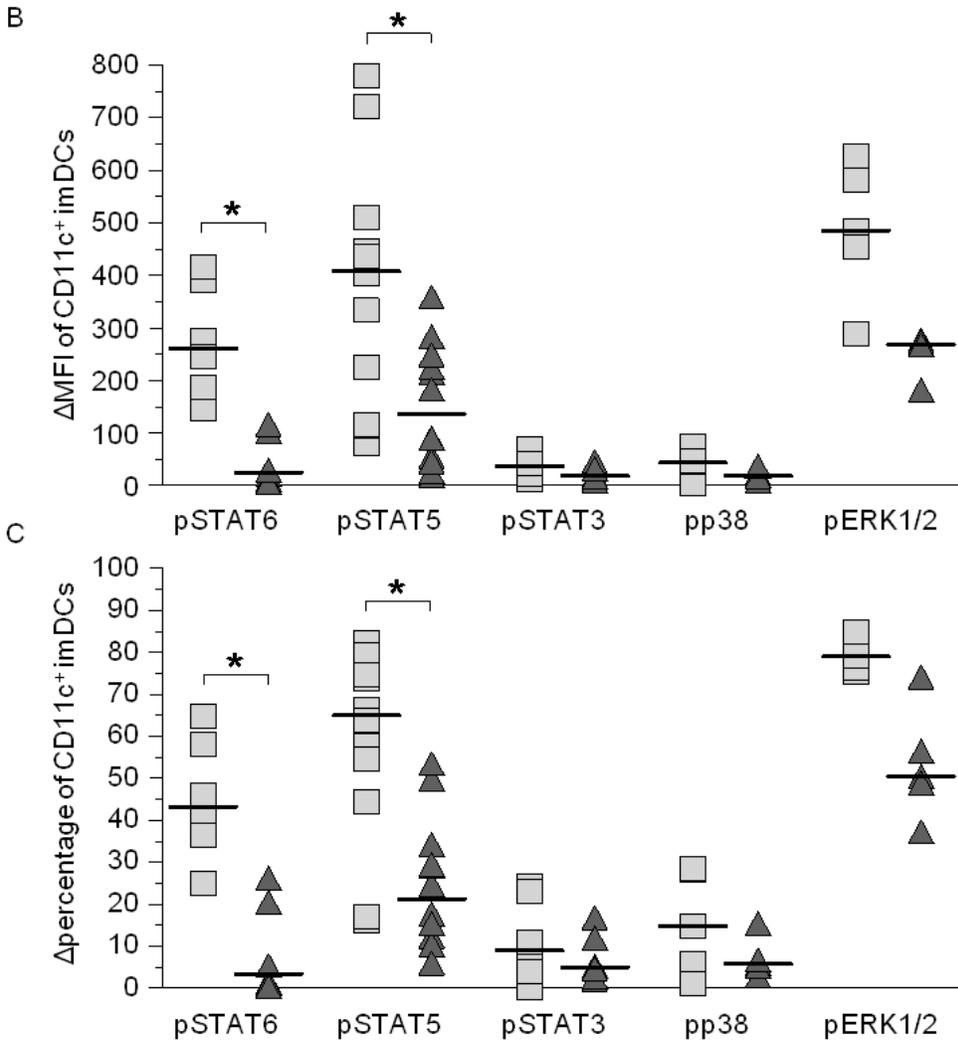


Figure 3-2. Continued

A

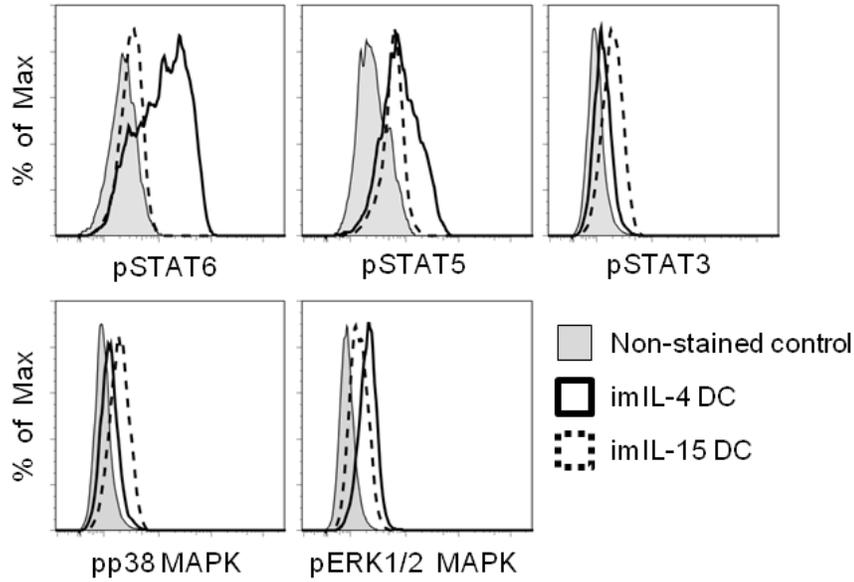


Figure 3-3. Comparison of signaling pathway profiles between the mature CD11c⁺ DCs. A) Representative histogram data of CD11c⁺-gated mDCs. Grey shaded histograms indicate the non-stained mDCs. mIL-4 DCs is shown by the solid lines and mIL-15 DCs are represented by the dashed lines. B) Phospho-protein⁺ Δ MF1 of CD11c⁺-gated cells after subtraction of the non-stained mDC values. C) Phospho-protein⁺ Δ percentage of CD11c⁺ mDCs after subtraction of the non-stained mDC values. mIL-4 DCs are shown as light squares and mIL-15 DCs are indicated by dark triangles. Bar indicates the median values. $n=7-10$. Significance calculated by wilcoxon matched-pairs signed rank test with the determined p value ≤ 0.05 considered significant

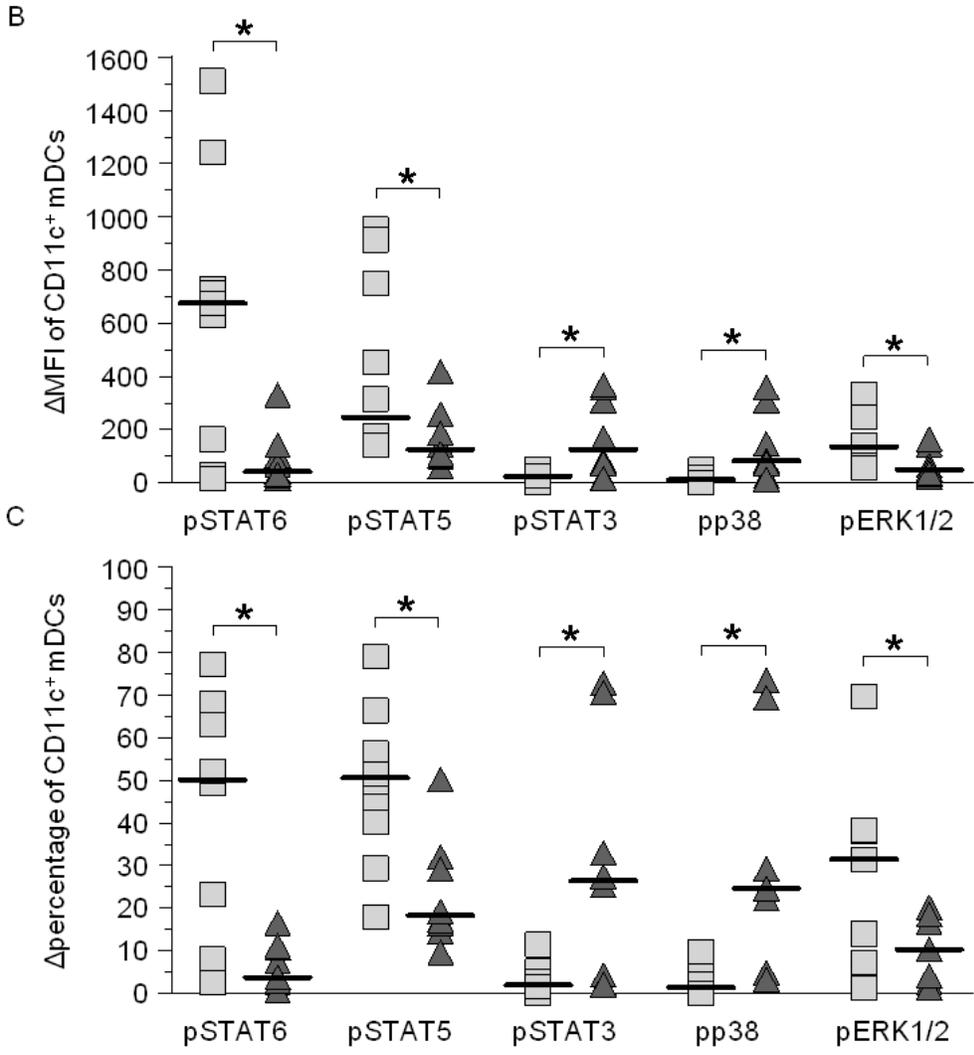


Figure 3-3. Continued

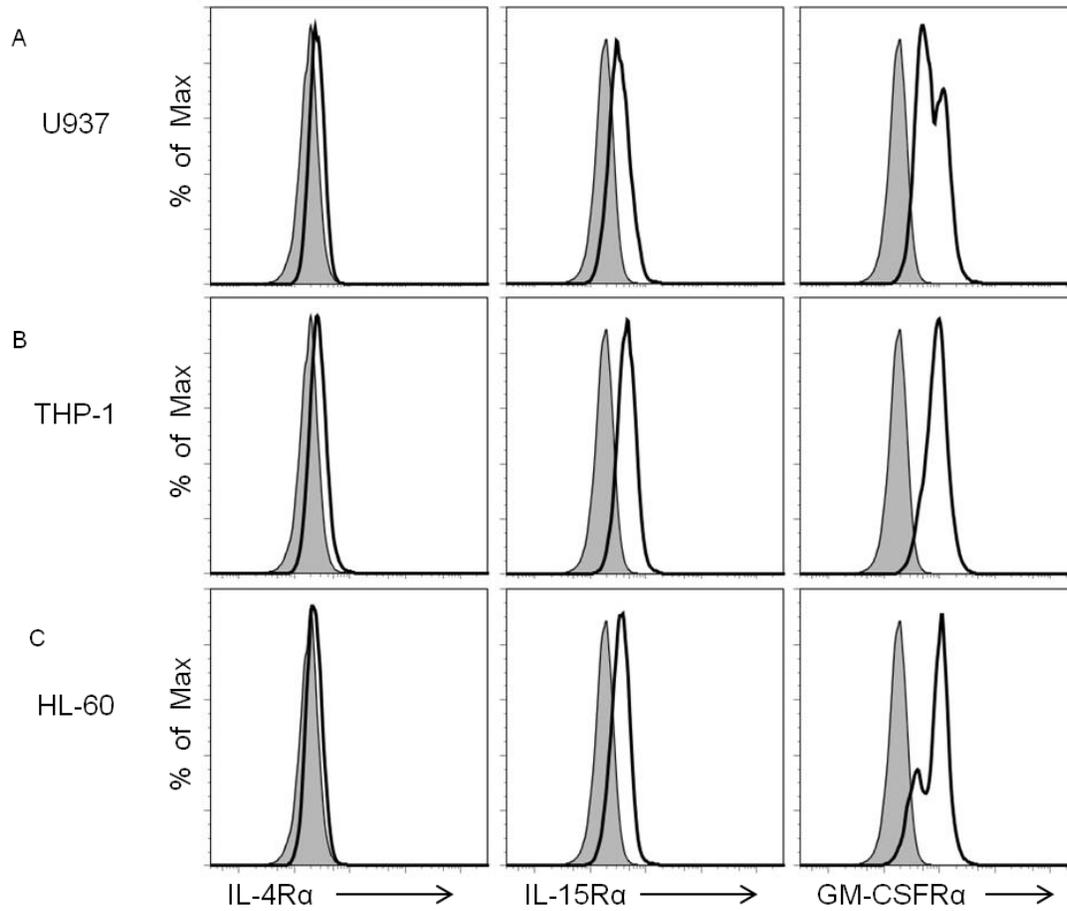


Figure 3-4. Expression of the three cytokine receptors on the monocytic cell lines U937, THP-1, and HL-60. Isotype is represented by grey peak and surface cytokine receptors are indicated by the black line. Histogram data shown here are representative images of at least two independent experiments

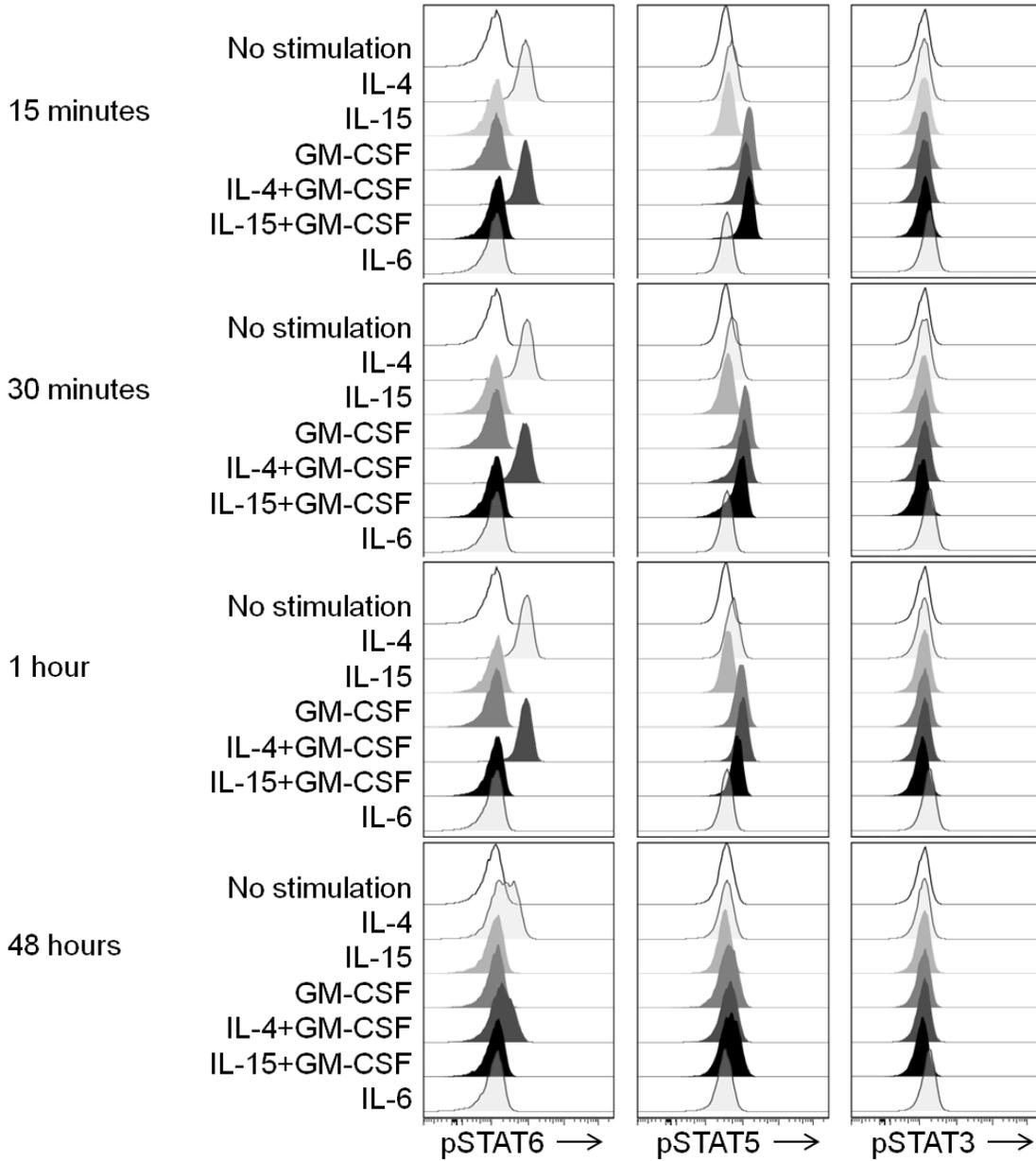


Figure 3-5. Temporal activation of the STAT6, STAT5, and STAT3 pathways in U937 cells by cytokine stimulation. Serum starved U937 cells were stimulated with IL-4, IL-15, GM-CSF, IL-6, or combinations of GM-CSF and IL-4 (IL-4+GM-CSF) or GM-CSF and IL-15 (IL-15+GM-CSF) for the times indicated. Non-stimulated cells were used as controls. Histograms displayed here are representative data of two independent experiments. Temporal pSTAT responses in HL-60 cells were analyzed with similar results

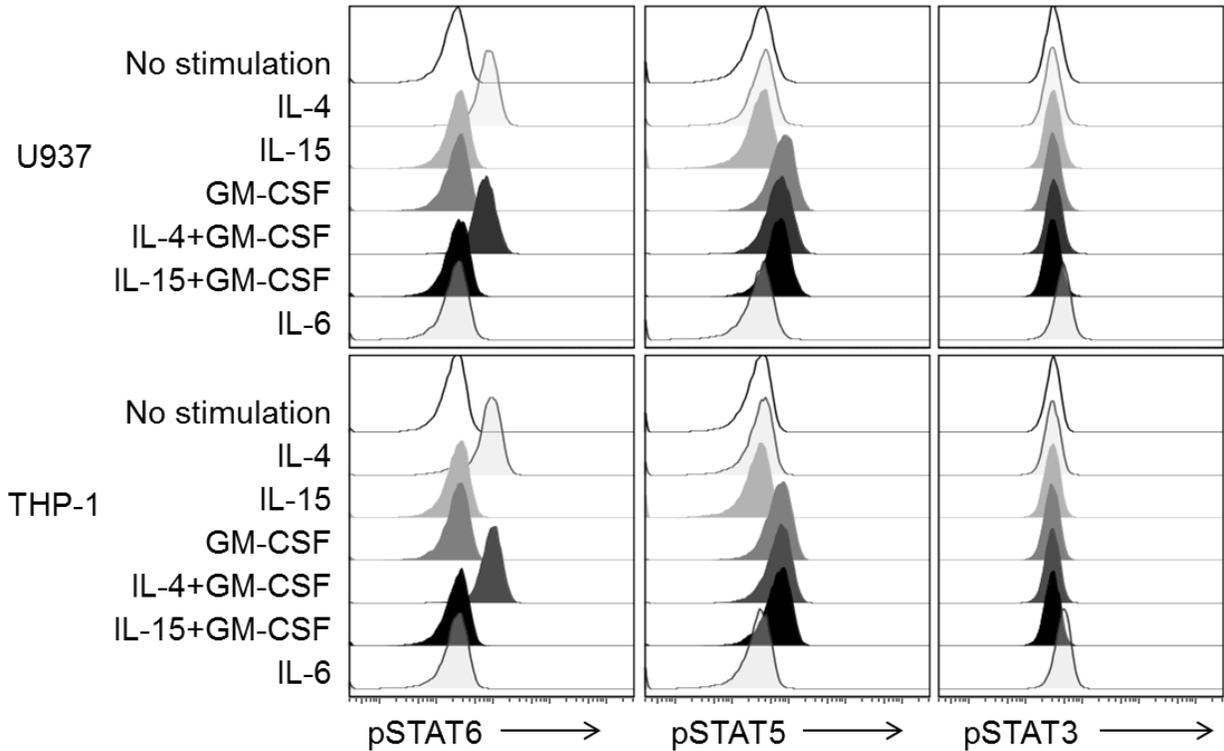


Figure 3-6. Direct activation of the STAT6, STAT5, and STAT3 pathways by IL-15, IL-4, or GM-CSF in U937 and THP-1 cells. Monocytic cell lines were serum starved prior to stimulation. IL-4 and IL-15 were added to the cells alone or in combination with GM-CSF (IL-4+GM-CSF, IL-15+GM-CSF). No stimulation and IL-6-stimulated cells served as controls for the basal signaling levels and for pSTAT3 respectively. Histograms that are shown here are representative data from at least two independent experiments

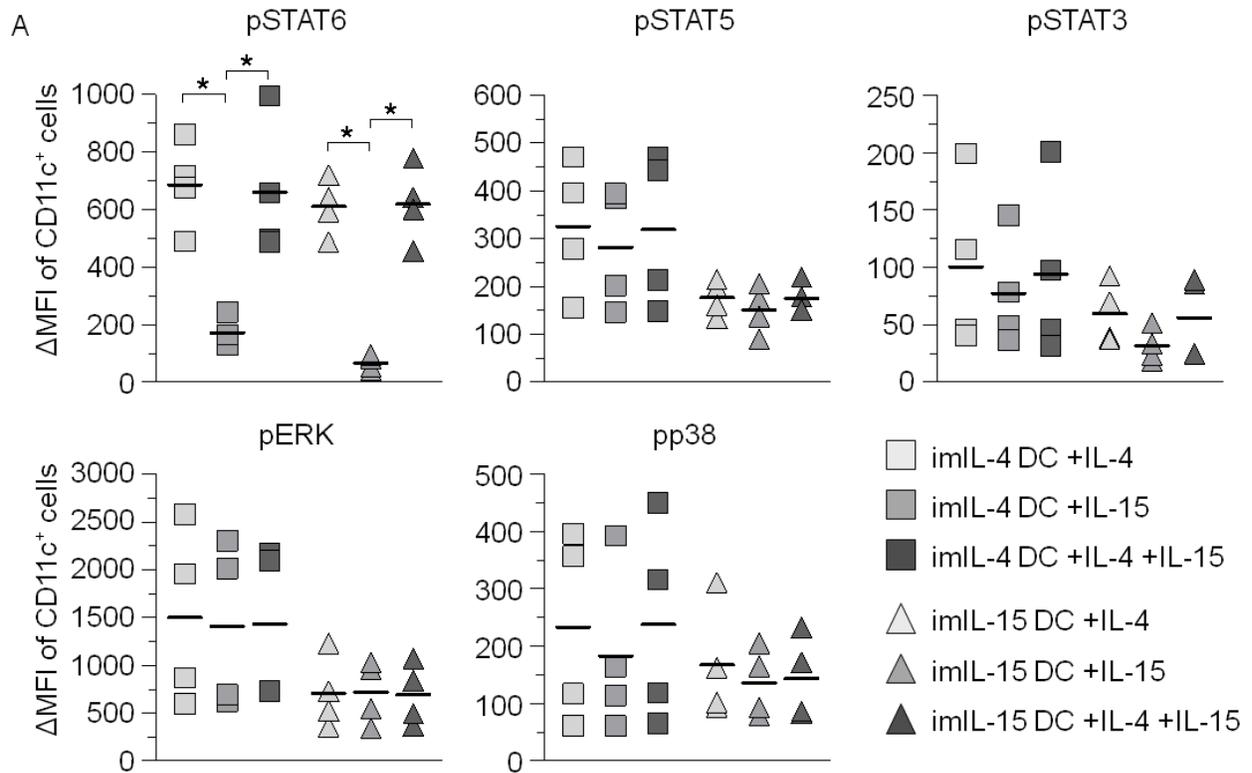


Figure 3-7. Immediate activation of the STAT and MAPK pathways by IL-4 or IL-15 during DC maturation. imIL-4 and imIL-15 DCs were incubated with DC maturation factors (GM-CSF, LPS, and TNF α) in presence of IL-4 alone (+IL-4, light grey), IL-15 alone (+IL-15, medium grey), or in IL-4 combined with IL-15 (+IL-4+IL-15, dark grey) for 30 minutes before assessing pSTAT or pMAPK change in CD11c⁺-gated imDCs. Shaded squares indicate imIL-4 DCs and shaded triangles represent imIL-15 DCs. A) Phospho-protein⁺ Δ MFI of CD11c⁺-gated cells after normalization with non-stained imDCs. B) Phospho-protein⁺ Δ percentage of CD11c⁺ cells after normalizing with non-stained imDCs. Due to the lower n value evaluated, the bar indicates the mean value. $n=4$. Significance calculated by paired t tests with the determined p value ≤ 0.05 considered significant

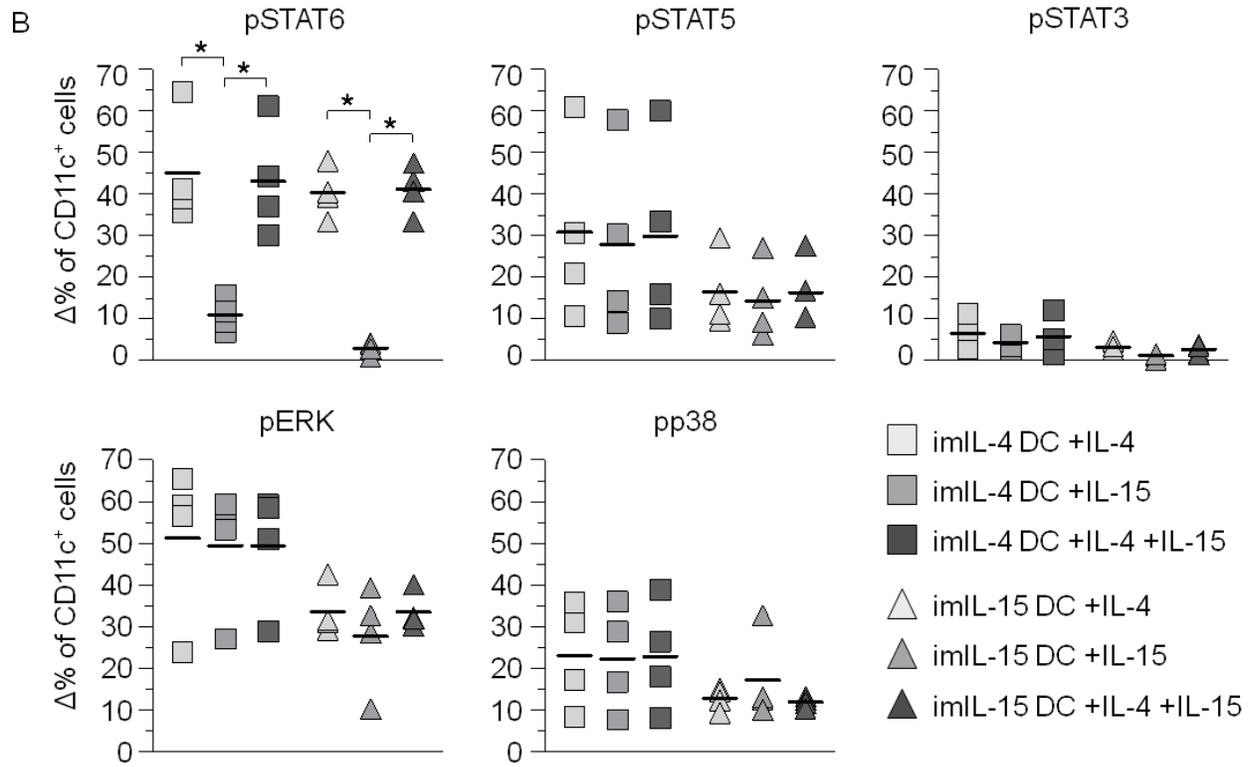


Figure 3-7. Continued

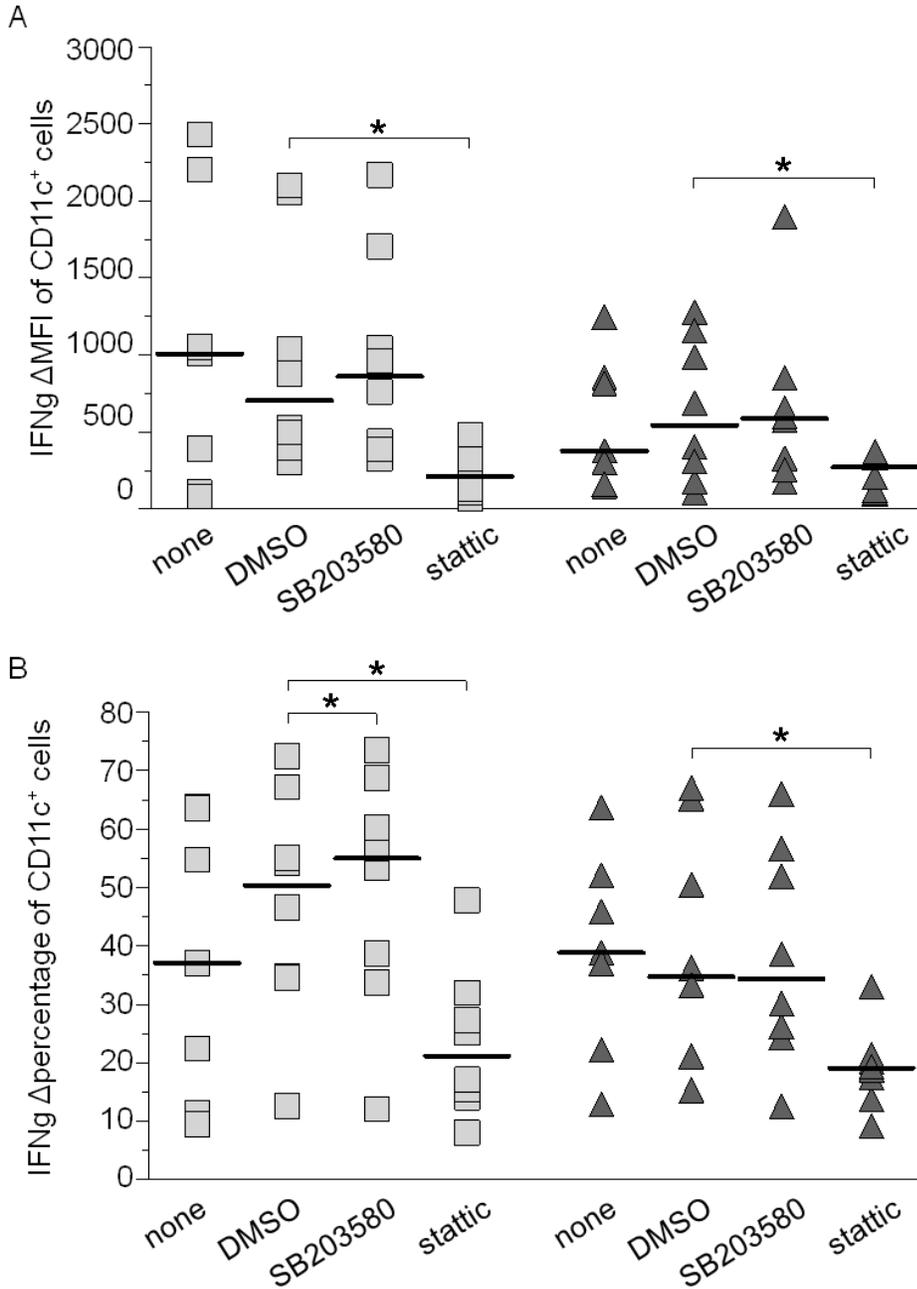


Figure 3-8. STAT3 inhibition down-regulated IFN- γ production however inhibition of p38 did not affect IFN- γ or TNF α production in mDCs. imDCs were matured for 24 hours, washed, and incubated with STAT3 or p38 inhibitors along with BFA for 6 hours. IFN- γ ⁺ or TNF α ⁺ CD11c⁺ DCs were evaluated. CD11c⁺-gated mIL-4 DCs are light grey squares while CD11c⁺ mIL-15 DCs are shown in dark grey squares. Median percentages of IFN- γ ⁺ or TNF α ⁺ mDCs are shown by the bars. $n=7$. Significance calculated by wilcoxon matched-pairs signed rank test with the determined p value ≤ 0.05 considered significant

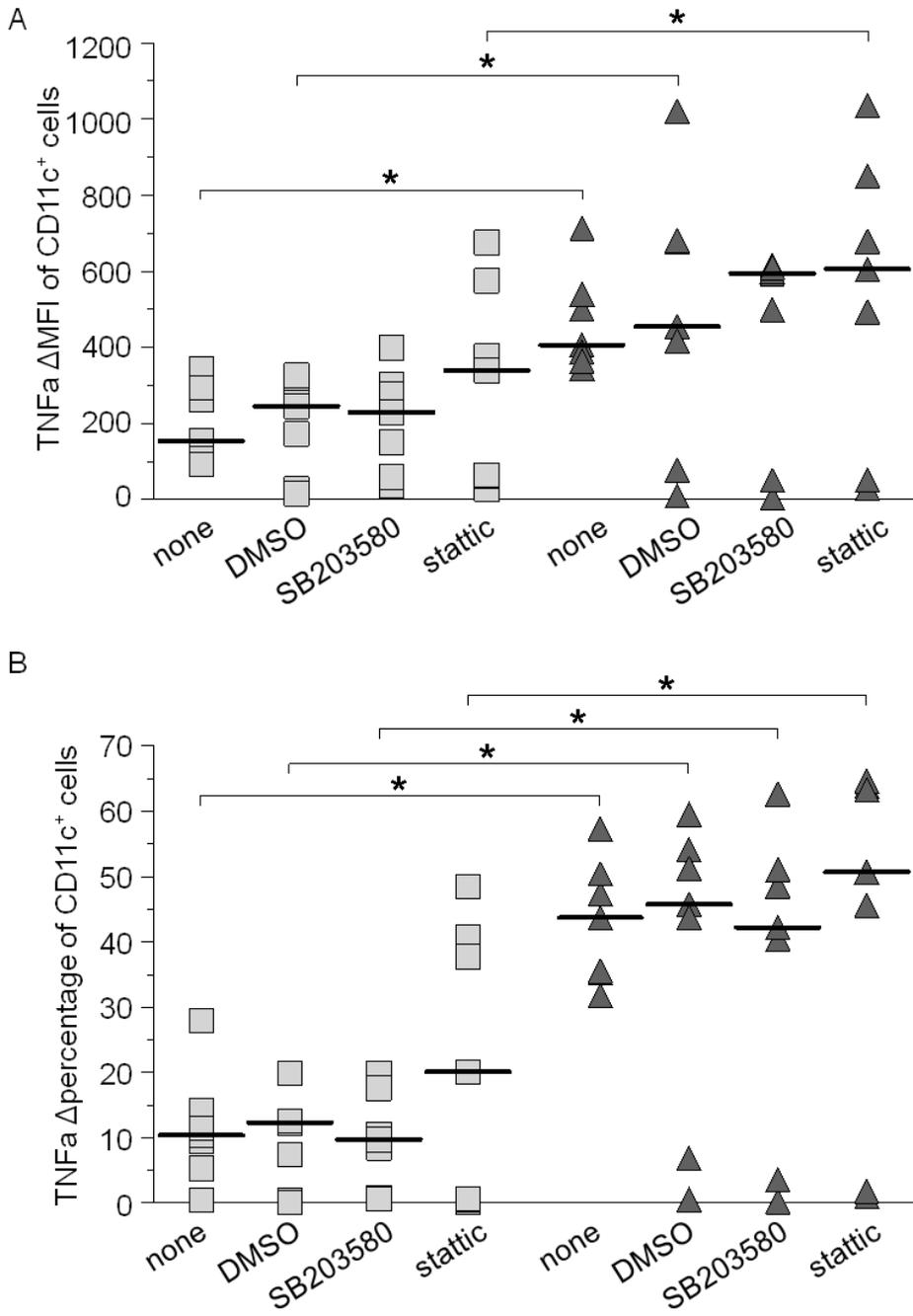


Figure 3-9. Inhibition of STAT3 or p38 had no effect on TNF α production in mDCs. imDCs were matured for 24 hours, washed, and incubated with STAT3 or p38 inhibitors and BFA for 6 hours. IFN- γ ⁺ or TNF α ⁺ CD11c⁺ DCs were evaluated. CD11c⁺-gated mIL-4 DCs are represented by light grey squares while CD11c⁺ mIL-15 DCs are shown by the dark grey squares. Median percentages of the IFN- γ ⁺ or TNF α ⁺ mDCs are indicated by the bars. $n=7$. Significance calculated by wilcoxon matched-pairs signed rank test with the determined p value ≤ 0.05 considered significant

CHAPTER 4 CONCLUSIONS

Findings and Future Directions

Due to their abilities at regulating immune responses and the discovery of how to generate monocyte-derived IL-4 DCs, there has been a strong interest to develop an effective DC vaccine for cancer immune therapy applications. The general therapeutic goal for DC vaccine strategies is the complete eradication of the tumor cells by administering DCs loaded with the tumor/tumor-associated Ag. Unfortunately although the many of the clinical trials using IL-4 DCs to treat cancer patients report immune responses in the patients, the majority of the studies do not objective clinical responses concerning notable decreases in patient's tumor load (19-25). Seeking to overcome this problem, alternative cytokine combinations from the original pairing of GM-CSF and IL-4 that generated immunostimulatory monocyte-derived DC subsets were evaluated (30, 31). The capacity of these DC subsets to elicit Ag-specific cytolytic CD8⁺ T cell responses were usually compared with to the more well-known monocyte-derived IL-4 DCs. Unfortunately these alternative DC subsets either did not undergo maturation which was evaluated by the up-regulate the classical co-stimulatory molecules or only primed CD4⁺ T cell responses without activation of the desired cytolytic CD8⁺ T cell responses. Monocyte-derived DCs prepared with GM-CSF combined with IL-15 were the first DC subset that was more efficient at priming melanoma-specific cytolytic CD8⁺ T cell responses than IL-4 DCs and they induced NK cell proliferation suggesting that IL-15 DCs could be a more potent immune therapeutic tool (43, 45). However, these IL-15 DCs were not well characterized and the descriptions concerning the IL-15 DC surface phenotype and cytokine production were not consistent among the IL-15 DC

studies (42-47). Furthermore, whether IL-15 DCs can induce stronger melanoma Ag-specific T cell responses than the conventional IL-4 DCs via a unique cell-to-cell contact mechanism or secreting a soluble factor to enhance T cell activation remains inconclusive. Of two studies that investigated this mechanism, Dubsky et al. purported IL-15 DCs provide an unidentified cell-to-cell contact mechanism to enhance Ag-specific T cell proliferation, whereas Hardy et al. reported that greater amounts of IFN- γ that helped prime T cell responses (43, 45). Therefore we conducted our own extensive comparative analysis between IL-15 DCs and IL-4 DCs to first assess the expression levels of surface molecules potentially interacting with T cells as well as the production of cytokines from the DC subsets.

In our hands, CD11c⁺-gated IL-15 DCs do not highly express all of the typical DC-specific surface markers such as CD1a and DC-SIGN (Chapter 2). Furthermore, LPS-matured IL-15 DCs do not up-regulate the expression of every surface marker that is traditionally associated with DC maturation like CD86 (Chapter 2). These phenotypical differences from typical IL-4 DCs cause IL-15 DCs to appear physically less differentiated and mature than IL-4 DCs. Consistent with being more phenotypically immature, mIL-15 DCs endocytosed OVA Ags more efficiently than the mIL-4 DCs. They also tended to promote the effector CD8⁺ T cell subset over the memory T cell subsets and induced greater CMV- and HCV-specific cytolytic effector responses from both CD8⁺ and CD4⁺ T cells (Chapter 2).

In contrast with prior IL-15 DC literature, we observed each DC subset exhibited their own distinctive cell morphologies and IL-15 DCs secreted higher amounts of pro-inflammatory cytokines such as IFN- γ , TNF α , and IL-6 (42, 43, 45, 46). In spite of Hardy

et al.'s report that the IFN- γ detected in the IL-15 DC culture supernatant was secreted by the small number of contaminating NK cells and not the DCs, we confirmed by intracellular cytokine flow cytometry analysis that CD11c⁺ IL-15 DCs produce a large amount of IFN- γ (Chapter 3) (43). Also we noted that the amount of IFN- γ mRNA transcript in IL-15 DCs were considerably more abundant in comparison to the level in IL-4 DCs which further supports that the IFN- γ in IL-15 DC culture supernatants is produced by IL-15 DCs, and not from the very small amount of NK cells contaminating the DC cultures (Chapter 2).

It was clear that the presence of GM-CSF and IL-15 and their down-stream molecular signaling mechanisms affected the differentiation of monocyte-derived DCs compared to GM-CSF and IL-4. We attempted to identify which of the STAT or MAPK signaling pathways which are traditionally associated with GM-CSF-, IL-4-, or IL-15-stimulation were uniquely affected in IL-4 DCs versus IL-15 DCs. We found that IL-4 DCs always exhibited higher activity levels of STAT6, ERK1/2, and STAT5 (Chapter 3). The tendency for IL-4 DCs to have more active STAT6 and ERK1/2 pathways can be attributed to the presence of IL-4 which are the dominant molecular signaling pathways activated by IL-4 in other cell types (86).

The absence of a strong up-regulation of the pSTAT5 protein levels in IL-15 stimulated monocytic cell lines and imDCs was surprising. According to the established IL-15 receptor-mediated signaling model, STAT5 is phosphorylated by JAK3 and, in some cell types, STAT3 is activated by JAK1 leading to increased amounts of pSTAT5 and/or pSTAT3 (98). In our study, we demonstrated for the first time that IL-15 stimulation does not affect the STAT5 or STAT3 pathways in monocytic cell lines and

DCs despite these cells expressing IL-15R α (Chapter 3). We showed that the monocyte-derived DCs transcribed the genes for STAT5a, STAT5b, and STAT3 as well as these two STAT pathways are directly activated by other cytokines in monocytic cells and DCs (Chapter 3, Appendixes B and D). The lack of change in the pSTAT5 levels following IL-15 stimulation in monocytic cell lines and DCs could be due to the preferential activation of the STAT5b isoform rather than the STAT5a which was the isoform that we had measured. Other potential reasons for the absence of pSTAT5 up-regulation after adding IL-15 to monocytic cell lines and *in vitro* generated DCs include decreased expression of either the other two IL-15R subunits (IL-2R β and γ c) or the associated JAKs (JAK3 and JAK1). The potential lack of IL-15 receptor chains, JAK1, or JAK3 expression in monocytic cell lines and DCs can easily be determined by western blot or flow cytometry. Also the association of JAK3 and/or JAK1 with the cytoplasmic domains of the IL-2R β and γ c proteins respectively can be verified in the IL-4 DCs and IL-15 DCs by immunoprecipitation.

Although IL-15 stimulation does not directly activate any of the signaling pathways evaluated in our study, the presence of IL-15 during DC differentiation must induce activation of some type of molecular signaling pathways via IL-15R. Blocking IL-15 or IL-15R via neutralizing antibodies targeting either IL-15 or the γ c receptor subunit impaired generation of monocyte-derived IL-15 DCs (42, 47). Also in one of the studies, they showed that the presence of recombinant IL-15 (~50 pg/mL) during differentiation of DCs from monocytes promoted the expression of co-stimulatory molecules (CD80, CD83, CD86) in relation to DCs generated in the absence of IL-15. To further understand how the presence of IL-15 affects DC differentiation and maturation, the

activities of other molecular signaling pathways, including the remaining STAT family proteins STAT1, STAT2, and STAT4, should also be assessed. Other signaling pathways commonly activated by cytokines in many cell types include the third MAPK signaling mechanism c-Jun N-terminal kinase (JNK) pathway as well as the phosphatidylinositol 3-kinase (PI3-K) pathway.

Some of the physical and functional differences noted in the two DC subsets can be directly associated with the continual presence of IL-4 or IL-15. For example, IL-4 inhibits transcription of the TNF α , IL-1 α , IL-1 β , IL-6, IL-8, and IL-15 genes and subsequently the negatively regulates production of the cytokine proteins (86, 89, 199, 220, 229-231). Hence the lower production of TNF α , IL-1 α , IL-1 β , IL-6, and IL-15 by our mIL-4 DCs could be simply due to negative regulation by IL-4 (Chapter 2). Murine DCs treated with IL-15 appears to exhibit similar functions as human monocyte-derived IL-15 DCs. In the murine DCs, the presence of IL-15 was reported to markedly increase IFN- γ production and enhance Ag-specific CD8⁺ T cell proliferation (208, 232).

We also assessed if the molecular signaling profiles of the DC subsets were affected by DC maturation or remained the same. There were no obvious differences in the relative activities of the STAT6, STAT5, and ERK pathways after inducing maturation (Chapter 3). However the p38 and STAT3 pathways were mildly activated in the mIL-15 DCs compared to the imIL-15 DCs and these changes were only observed in the IL-15 DCs. Interestingly, it seems that mIL-4 DCs do not up-regulate pSTAT3 in contrast to mIL-15 DCs because of some unknown impairment in the activation of the STAT3 pathway. The lower pSTAT3 levels in mIL-4 DCs is not caused by the relatively lower levels of IL-6 or other cytokines that strongly activate STAT3 in the mIL-4 DC

culture supernatant as noted in the mDC ELISA results (Chapter 2). In a small pilot study using two individual donors, we found that pSTAT3 levels in mIL-4 DCs were only slightly increased after IL-6 stimulation in comparison to the IL-6-treated mIL-15 DCs and PBL controls (data not shown). The increased STAT3 activity in the mIL-15 DCs may play an important role in modulating the differential immunostimulatory capacities shown between mIL-15 DCs and mIL-4DCs. We later noted that inhibiting STAT3 activity significantly down-regulated the production of IFN- γ in both mDC subsets but did not affect the production TNF α (Chapter 3). Since STAT3 has not been reported to directly regulate IFN- γ in other known IFN- γ -producing types of cells such as Th1 CD4⁺ cells and CD8⁺ T cells, we attempted to verify if the reduction of STAT3 activity in DCs can quantitatively down-regulate secretion of IFN- γ in DCs. Perhaps, STAT3 activity can affect IFN- γ production in DCs in a cell type-specific manner. However we found that incubating the DCs with the only commercially available inhibitor specific for STAT3 seemed to be cytotoxic to DCs. Short-term or longer-term incubation of the IL-4 DCs in IL-15 or IL-6 only induces little to no pSTAT3 up-regulation and had little effect on the IFN- γ gene transcription or IFN- γ secretion (Appendix E and data not shown).

Finally, in the future it would be beneficial to determine whether IL-15 DCs elicit stronger Ag-specific cytolytic T cells responses by a cell-to-cell contact mechanism such as IL-15 trans-presentation or by secreting large amounts of inflammatory cytokines like IFN- γ . The conclusions from the prior two studies investigating how human IL-15 DCs induce more T cell responses than IL-4 DCs contradicted each other (43, 45). Dubsky et al. concluded that IL-15 DCs promote T cell responses due to an unidentified cell-to-cell contact, based on the proliferation of T cells co-cultured with IL-15 DCs compared to a

trans-well experiment involving IL-4 DCs, IL-15 DCs, and the T cells (45). Dubsky et al. hypothesize that this cell-to-cell mechanism could be trans-presentation of IL-15. On the other hand, Hardy et al. concluded that the high concentration of IFN- γ found in the supernatant of IL-15 DCs by contaminating NK cells promote T cells responses (43). Dubsky et al.'s mIL-15 DCs did not secrete IFN- γ and Hardy et al. did not measure production of IL-15 in their mIL-15 DCs.

We assessed if the presence of the pro-inflammatory cytokine IFN- γ , TNF- α , IL-6, IL-15 at the average concentration determined in our prior ELISA helped to promote T cell activation or had no effect (Chapter 2). PBLs were stimulated in a polyclonal manner overnight with anti-CD3/anti-CD28 antibody covered beads in the presence of IFN- γ , TNF α , IL-6, IL-15, or all four combined (Appendix F). Of the four cytokines, IL-6 was the most effective at increasing the number of activated T cells which emphasizes the potential importance of the high production of IL-6 by mIL-15 DCs. It also suggests that the IFN- γ plays a bigger role in polarization of the T cells into Th1 or cytolytic T cells and not in promoting activation of T cells. Additionally, Dubsky et al.'s and our data indicated that the greater amount of membrane-bound IL-15 on IL-15 DCs may have a significant effect on T cell activation/polarization (Chapter 3). To reduce the additional variables of DC-produced cytokines, and differentially expressed surface markers, IL-15 trans-presentation in the future can be studied by using micro-spheres loaded with recombinant IL-15R α and IL-15 proteins as described in previous NK cell publications (107, 209). Reverse signaling for IL-15 can also be evaluated by loading the micro-spheres with anti-IL-15 antibodies alone. Overall, the required mechanisms involved in

human DC development and the regulation of DC immune stimulatory functions is complex and still not well understood.

Clinical Implications

Monocyte-derived IL-15 DCs

The basic DC vaccine strategy involves of loading *in vitro* generated DCs with tumor or tumor-associated Ag and then administering these DCs into patients in order to stimulate an immune response against cells expressing the particular Ag. Human clinical trials have demonstrated that DC vaccines are non-toxic and have little to no side effects in the patients (19, 24, 26). These DC vaccines have demonstrated to be immunogenic *in vivo* in many the clinical trials by a range of methods including tetramer staining, ELISpot, Ag-recall killing assay, and T cell proliferation. Yet significant reduction of the tumor size/mass is rarely observed and the necessity for the development of a more effective DC-based immune therapeutic strategy is widely acknowledged. Monocytes cultured in the presence of GM-CSF and IL-15 differentiate into a rather novel DC subset which elicit greater Ag-specific CD8⁺ T cells responses than “standard” monocyte-derived IL-4 DCs (43, 45, 46). Based on this demonstrated ability, human IL-15 DCs appear to be a promising tool to improve DC-based immunotherapy.

This study demonstrated that human monocyte-derived IL-15 DCs can stimulate stronger CMV- or HCV-specific cytolytic T cell response from either the CD8⁺ T cell or the CD4⁺ T cell populations. Usage of IL-15 DCs to prime T cells responses tended to favor the effector T cell subset over the memory T cell population which may be beneficial for priming more immediate cytolytic T cell responses for currently infected individuals. IL-15 DCs may retain their immunostimulatory functions near tumor

microenvironments with high IL-6 concentrations which normally inhibits mIL-4 DC immune functions. Also, the relatively lower expression of the lymph node homing receptor CCR7 on the surface of mIL-15 DCs suggest that if these mDCs are re-introduced into the patient, mIL-15 DCs will likely remain in a concentrated population near the injection site. In addition to stimulating cytolytic T cells responses, IL-15 DCs may also provide a source IL-15 and TNF α which are important for NK cells. NK cells are another potential tool for cancer immune therapeutic applications (233-235). Unlike T cells which respond to a single Ag, NK cells can become activated by a range of different Ags. Their cytolytic activities against target cells provide a source of tumor Ags for DCs to engulf and present to T cells. They also produce high amounts IFN- γ which promotes Th1 responses and facilitate DC maturation.

Potential Limitations

All IL-15 DC characterization studies, including this one, concur that IL-15 DCs stimulate stronger CD8⁺ T cell responses than IL-4 DCs. However, the method of isolating the monocytes, the culture conditions utilized to differentiate IL-15 DCs (type of cell media, percentage of serum added to cell media, number of days in culture), and the type of stimuli used to mature the DCs can notably affect the expression of surface markers and cytokine production. Although differences in CCR7 expression or cytokine production would not affect the immunostimulatory abilities of IL-15 DCs *in vitro*, these differences could have a large impact on their abilities to mobilize to lymph nodes and/or attract lymphocytes *in vivo*. It is necessary for a “standardized” IL-15 DC culture method to obtain consistent and comparable results among the various groups that are evaluating IL-5 DCs for future immune therapeutically applications.

A few possible drawbacks to using IL-15 DCs in future cancer immune therapy strategies would be that IL-15 DCs are an adherent cell type, only have low expression of CCR7, and they secrete relatively high levels of pro-inflammatory cytokines such as IL-6 and TNF α . IL-15 DCs could attached themselves to tissues near the DC vaccine injection site and, without migrating to the draining lymph nodes, produce large amounts of IL-6 and TNF α . The secretion of pro-inflammatory cytokines in a localized area may have detrimentally effects. Prolonged inflammation can damage tissues and is associated with many inflammatory and autoimmune diseases like rheumatoid arthritis.

Furthermore using a DC subset that produces high levels of IL-6 might not be ideal type of cells to use for treating some types of cancers. Some cancers are known to promote their own proliferation by secreting high amounts of IL-6 on their own such as melanoma, multiple myeloma, and prostate cancer; thus, additional production of IL-6 would likely allow these types of IL-6-responsive cancers to proliferate (120, 133, 135, 236). The production of IL-6 by these cancers has also been shown to suppress cytolytic T cells responses by inhibiting Th1 differentiation as well as inhibiting DC maturation which consequentially induces immune tolerance (138, 139, 171, 201, 237).

APPENDIX A

STIMULATION BY A PARTICULAR DC SUBSET DOES NOT AFFECT NK CELL SUBSET DISTRIBUTION OR CYTOLYTIC FUNCTION

In addition to assessing CMV- or HCV-specific CD3⁺ T cells responses elicited by mIL-15 DCs versus mIL-4 DCs, we evaluated the cytolytic functionality of the NK cells previously detected at the end of the DC:PBL co-culture. Using a non-radioisotope method, cultured NK cells were tested for cytotoxic activity using the NK-sensitive cell line K562 as a target cell. K562 target cells were labeled with 1 μ M CFSE then cultured in a U-bottom 96-well plate along with increasing ratios of the PBLs that had been previously co-cultured with mDCs. The labeled K562 cells and PBLs were co-cultured for 5 hours in AIM-V media with 5% human serum at 37°C. Next, the cells were washed with FACS buffer, a set amount of APC-labeled beads were added per sample immediate before collecting data on the BD Aria FACS machine, and the number of CFSE-labeled was quantified in relation to a defined number of APC-labeled beads (3000 beads per sample). Data was analyzed on the same BD FACSDIVA program used to obtain the data. We evaluated two donors with each effector-to-target ratio completed in either duplicates or triplicates. The percentage of CD3⁻ CD56⁺ NK cells in the DC-primed PBL population was determined in a previous described assay.

Overall, it seems NK cells co-cultured with Ag-pulsed mIL-15 DCs may induce more cell death to their target cells regardless of Ag type. However, since we did use the same number of purified NK cells to compare the effect of mIL-4 DCs and mIL-15 DCs, we cannot disregard the possibility the observed increased lytic function is simply due to the presence of more NK cells

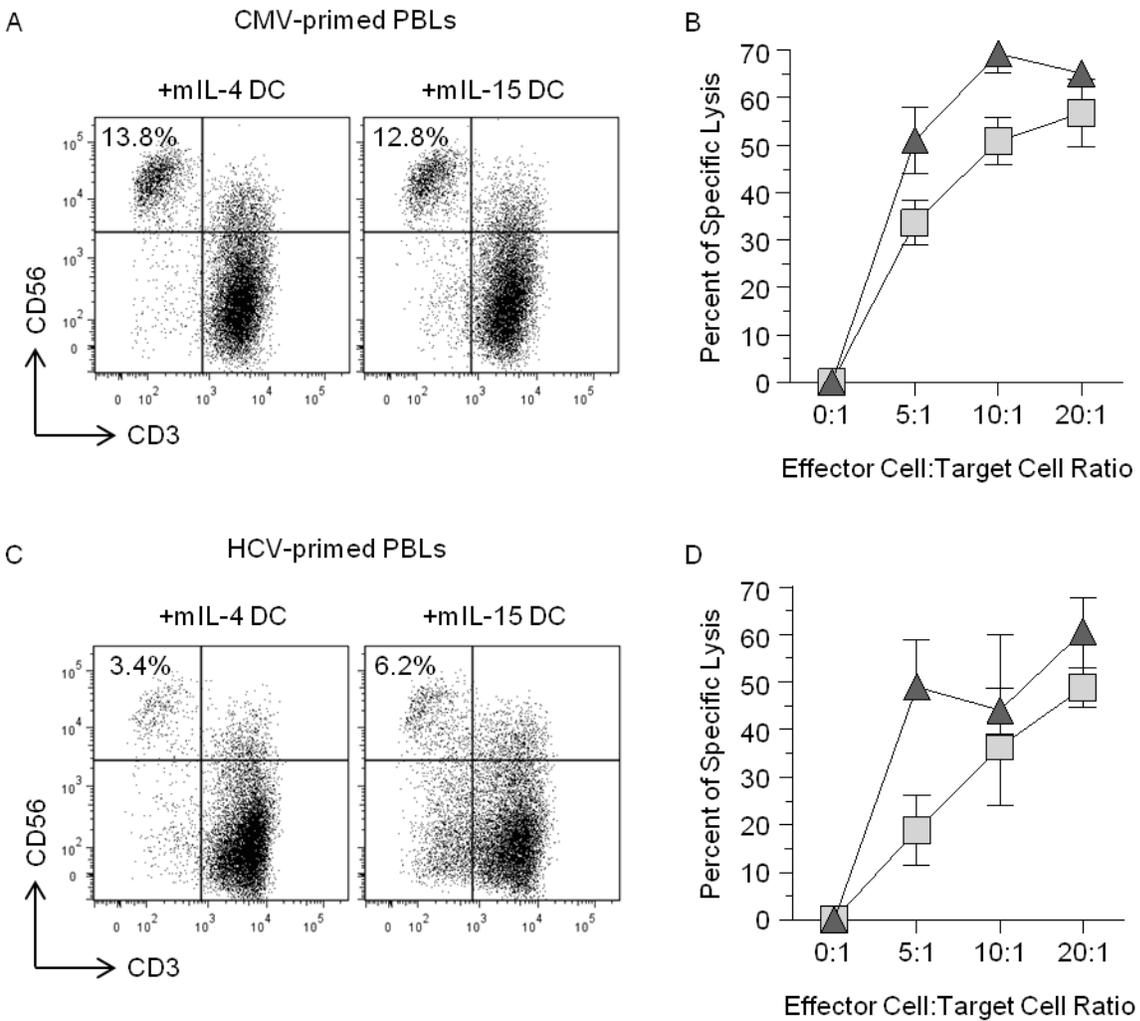


Figure A-1. Evaluation of NK cell lytic function after being co-cultured with mIL-4 DCs or mIL-15 DCs. NK cell surface staining data and NK cell specific lysis data are representative of one out of two donors evaluated. A) Percentage of CD3⁺ CD56⁺ NK cells in the general lymphocyte-gated population after being co-cultured with CMV-pulsed mIL-4 DC (+mIL-4DC) or mIL-15 DC (+mIL-15 DC). B) Determined percentages of specific lysis of the CSFE labeled-K562 target cells. The light squares represent cells exposed to mIL-4 DCs and the dark triangles indicate the cells co-cultured with mIL-15 DCs. Each data point represents the mean values \pm SEM from the particular ratio analyzed in triplicate. C) Percentages of NK cells after PBLs co-cultured with HCV-pulsed mDCs. D) Calculated percentage of specific lysis of CSFE labeled-K562 target cells

APPENDIX B CYTOKINE INDUCED STAT PROTEIN ACTIVATION IN CD3⁺ T CELLS

PBLs were thawed and then stimulated with 25 ng/mL IL-4, 100 ng/mL IL-15, 50 ng/mL GM-CSF, 50 µg/mL IL-6, 5 mM hydrogen peroxide (H₂O₂), or 50 µM phorbol-12-myristate-13-acetate (PMA) for 15 minutes at 37°C. Stimulated cells were fixed with 2% PFA, permeated in 90% methanol overnight at -20°C before washing and staining the cells with phospho-protein antibodies for 1 hour at room temperature. Data was collected on the BD LSRII flow cytometer and analyzed with Flowjo.

Following IL-4 stimulation, CD3⁺-gated T cells increased pSTAT6 without activation of the other STAT pathways. Addition of soluble IL-15 exclusively activated STAT5. STAT3 was not activated by either IL-4 or IL-15 stimulation. GM-CSF did not stimulate any of the three STAT pathways since T cells do not express GM-CSFR α . As expected, the p38 MAPK and ERK1/2 MAPK pathways were not activated by IL-4, IL-15, GM-SF, or IL-6 (data not shown). Up-regulation of pSTAT3 and the two pMAPK proteins were only observed when stimulated with the commonly used positive controls which were IL-6 for pSTAT3, H₂O₂ for pp38, and PMA for pERK1/2. IL-6 is a key activator of the STAT3 pathway in many cell types (120). Activation of the T cell receptor (TCR) signaling pathway leads to phosphorylation of the p38 and ERK1/2 proteins. Both H₂O₂ and PMA can initiate the TCR signaling pathway by, respectively, the induction of oxidative stress or mimicking diacylglycerol (DAG) which a TCR signaling protein necessary for ERK1/2 activation (238, 239).

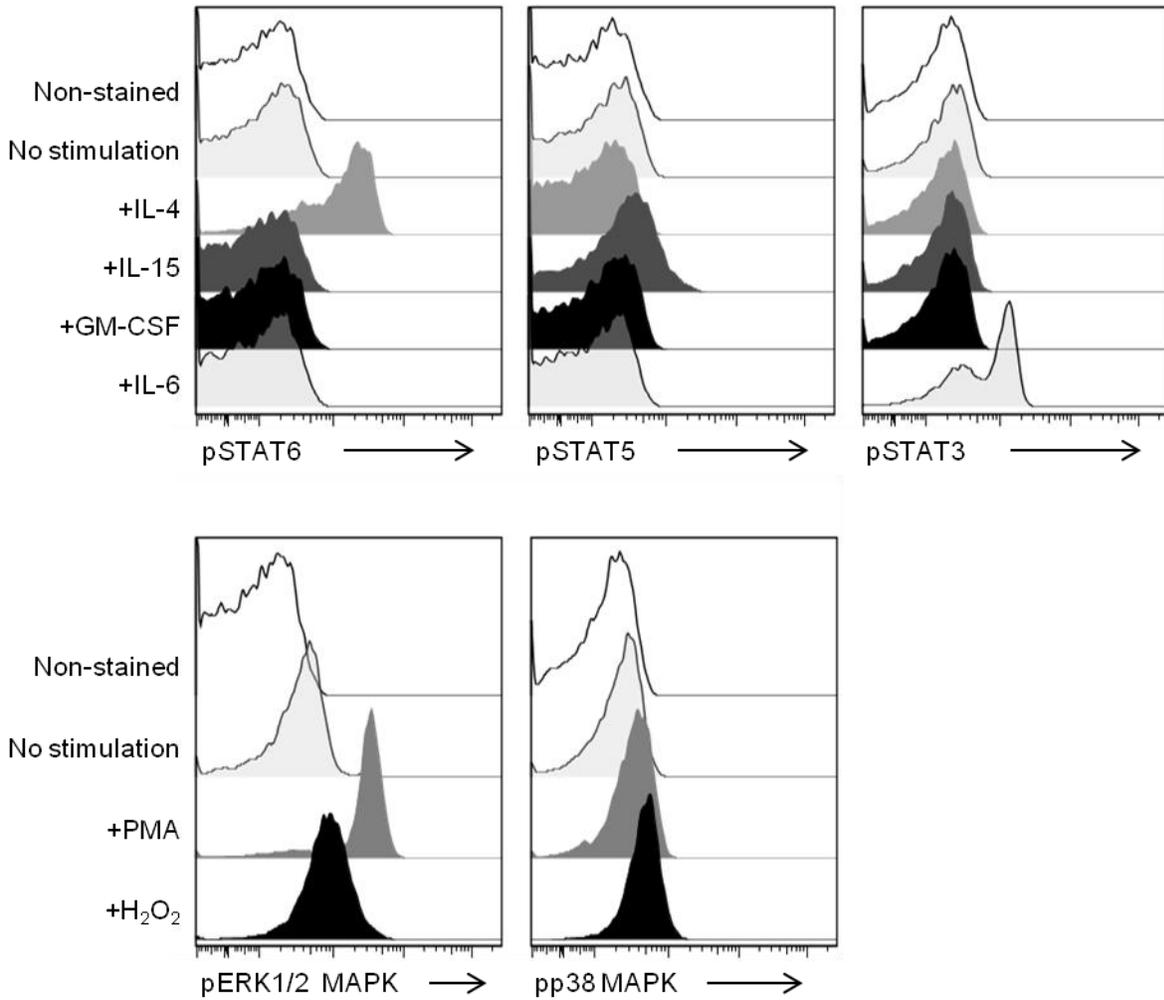


Figure B-1. STAT and MAPK activation in stimulated PBLs. PBLs were stimulated with IL4, IL-15, GM-CSF, IL-6, PMA, or H₂O₂ for 15 minutes. Non-stimulated, non-stained and non-stimulated controls were included to determine background cellular auto-fluorescence and basal protein phosphorylation state. Phosphorylated proteins were detected in CD3⁺-gated T cells. Histograms are representative of cytokine-, phorbol ester-, or oxidative stress-induced activation of STAT or MAPK proteins for at least three independent experiments

APPENDIX C
ACTIVATION OF MAPK SIGNALING PATHWAYS IN MONOCYTIC CELL LINES BY
PMA OR ANISOMYCIN

Serum-starved U937 and THP-1 cells were stimulated with 50 nM PMA or 25 ng/mL anisomycin to determine ERK1/2 or p38 activation respectively. Cells were stimulated for 15 minutes at 37°C. Then the cells were fixed with 2% PFA, permeated in 90% methanol overnight at -20°C before washing and staining the cells with phospho-protein antibodies for 1 hour at room temperature. Data was collected on BD LSRII flow cytometer and analyzed with Flowjo. PMA and anisomycin are the positive stimulatory controls suggested by BD. Of the two monocytic cell lines, it appears that THP-1 cells are more responsive to PMA and anisomycin.

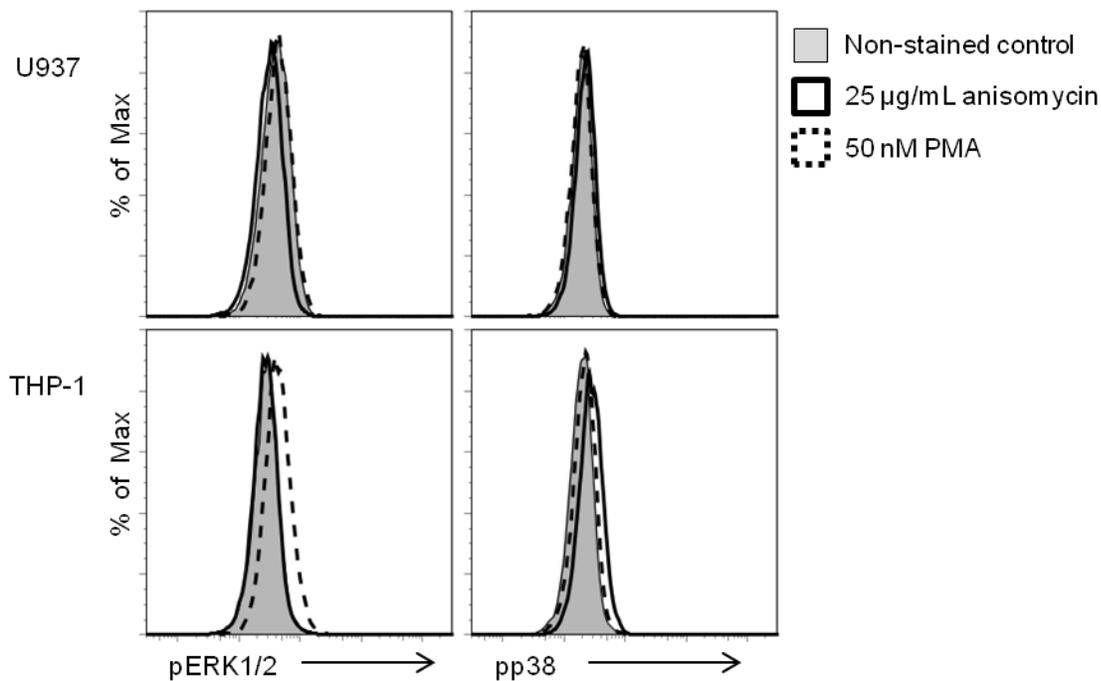


Figure C-1. pERK1/2 and pp38 induction by PMA or anisomycin in monocytic cell lines. U937 and THP cells were stimulated with anisomycin or PMA for 15 minutes at 37°C. Cells were fixed and stained for pERK1/2 or pp38. Non-stimulated, non-stained control used to determine the cellular auto-fluorescence levels are indicated by grey histogram. PMA-stimulated cells are shown by dashed line whereas the anisomycin-stimulated cells are displayed as solid line

APPENDIX D
RELATIVE QUANTITATION OF STAT PROTEIN TRANSCRIPT EXPRESSION
LEVELS DURING DC DIFFERENTIATION AND MATURATION

The human transcription factor and human RT² RNA QC PCR arrays were obtained from SABiosciences (Qiagen, Valencia, CA). Monocyte, imIL-4 DC, imIL-15 DC, mL-4 DC, and mL-15 DC total cellular RNA were extracted and pooled together from 3 donors using TRI Reagent following the manufacturer's protocol (Invitrogen). The pooled RNA was treated with DNase I (Promega) then purified using the RNeasy mini kit (Qiagen). RNA concentration and purity was measured with a NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) and general RNA integrity was evaluated via denaturing agarose gel electrophoresis followed by ethidium bromide staining. cDNA was generated from 1 µg total RNA using the RT² First Strand kit (SABioscience). Following the company's protocols cDNA quality and the transcription factor gene expression were determined with the Human RT² RNA QC PCR array and the Human transcription factor qPCR array respectively. SYBR green-based quantitative PCR (qPCR) data was collected and analyzed using the MX3000P thermal cycler (Agilent Technologies, Inc., Santa Clara, CA).

Overall, STAT3, STAT5A, STAT5B, and STAT6 transcripts were detected in monocytes, imDCs, and mDCs. We found that the transcripts level for STAT3, STAT5A, STAT5B, and STAT6 are very similar as the monocyte under DC differentiation and maturation. In the TF qRT-PCR array, the STAT1 transcript was one of the most differentially expressed TF between IL-4 DCs and IL-15 DCs. The relative difference in the abundance of STAT1 transcripts in IL-4 DCs and IL-15 DCs progressively increases from imDCs to mDCs.

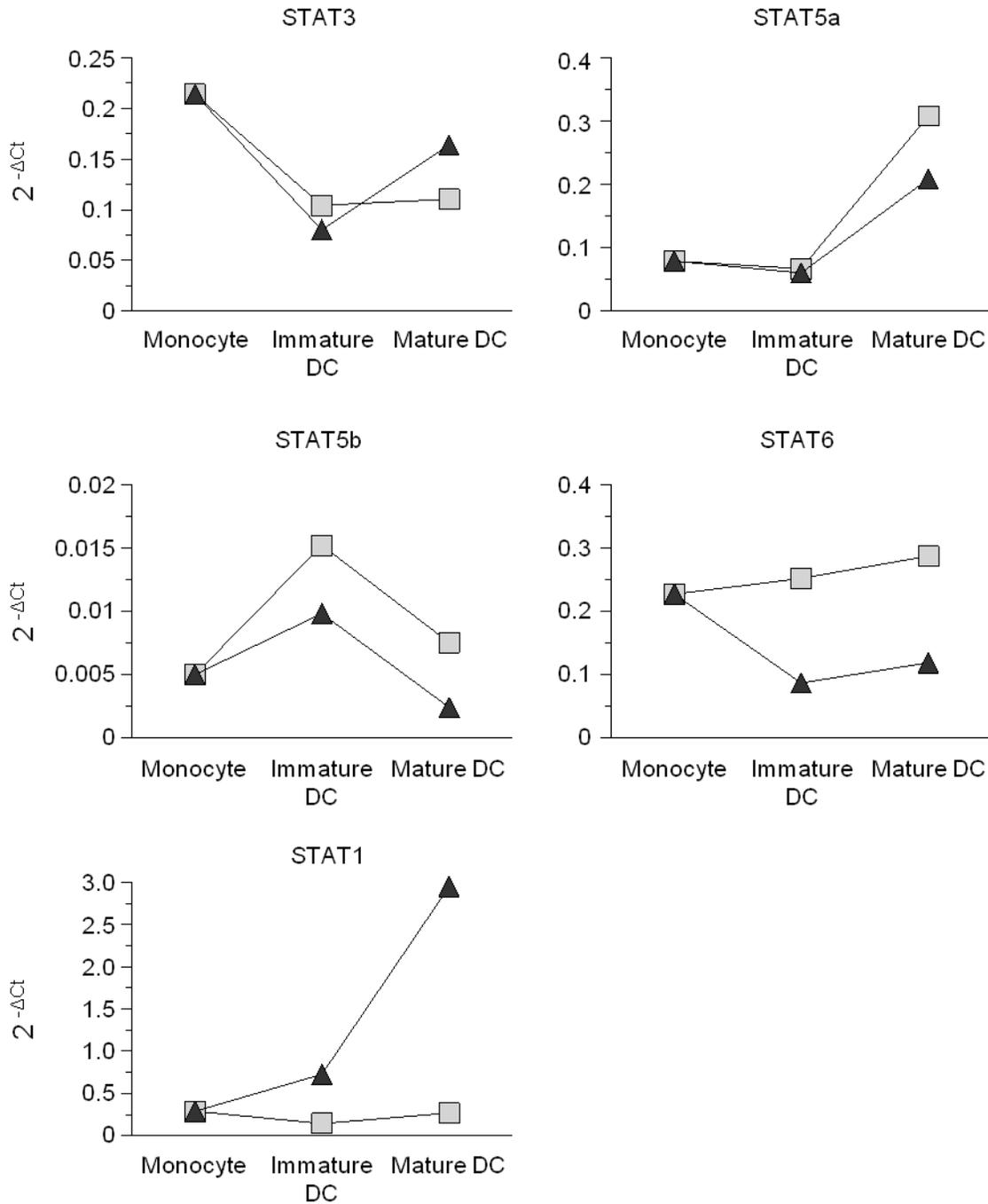


Figure D-1. Quantification of expression of the STAT3, STAT5a, STAT5b, and STAT6 transcript levels in monocytes, imDCs, and mDCs. RNA was pooled from 3 individual donors and used to generate cDNA which was used in the SYBR green-based qPCR arrays. The average expression level of 5 housekeeping genes including GAPDH and β -actin provided in the array were used for normalization as suggested by the manufacturer. The calculated $2^{-\Delta Ct}$ values for IL-4 DCs are shown by the light grey squares while IL-15 DCs are indicated by the dark grey triangles

APPENDIX E
EFFECT OF IL-4 VERSUS IL-15 ON CYTOKINE PRODUCTION BY MATURE DCS

imDCs were matured with LPS, TNF α , GM-CSF, and either IL-4 or IL-15 for 24 hours as in the previous multiplex ELISA. Then the DCs were thoroughly washed and cultured for another 24 hours in AIM-V media without cytokines at the same cell density as before (1×10^6 mDCs/mL). The mDC supernatant was collected and stored at -80°C until analysis. Total cellular RNA from the mDCs was isolated using Tri-Reagent while following the manufacturer's protocol. The SYBR green qRT-PCR analysis of the expression levels of the IFN- γ , TNF α , and IL-6 mRNA transcripts in mDCs was determined and normalized to β -actin mRNA transcript levels as described previously. The secreted IFN- γ concentration in the mDC supernatant was measured by ELISA. The mini human IFN- γ ELISA kit was purchased from PeproTech (Rocky Hill, NJ) and the IFN- γ concentration was determined according to the company's instructions using a 96-well Falcon BD Pro-bind plate and ABST (2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid)) liquid substrate (Sigma). Plates were washed with the Biotek ELx405 plate washer (Winooski, VT) and final absorbance data was collected and analyzed with BioRad model 680 scanner (Hercules, CA).

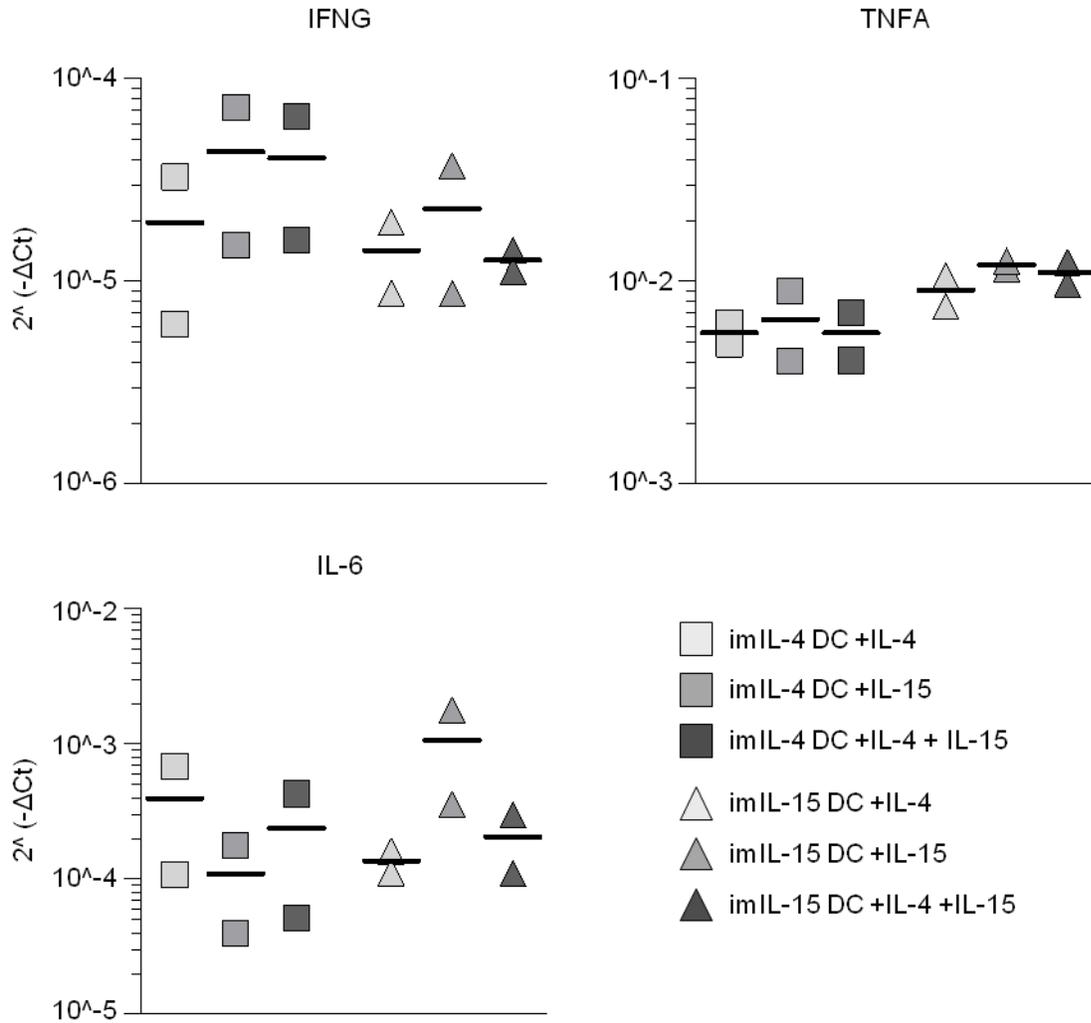


Figure E-1. Presence of IL-15 up-regulates IL-6 transcript levels but does not affect IFN- γ or TNF α transcripts. Relative cytokine mRNA levels were normalized with β -actin gene expression. Bar indicates the mean value. $n=2$

Table E-1. IFN- γ production from DCs matured in presence of IL-4 and/or IL-15

Donor	imIL-4 DC (pg/mL)			imIL-15 DC (pg/mL)		
	+IL-4	+IL-15	+IL-4 +IL-15	+IL-4	+IL-15	+IL-4 +IL-15
1	416.9	309.6	394.3	255.6	174.8	238.4
2	955.0	1050.9	714.0	834.5	999.6	1108.9

imDCs matured with GM-CSF, LPS, TNF α , and indicated cytokine for 24 hours.

APPENDIX F EFFECT OF CYTOKINE ENVIRONMENT ON LEVEL OF CD3⁺ T CELL ACTIVATION

IL-12 and type I IFNs secreted by mDCs is the third essential signal to induce T cell immune responses (56, 169, 187, 240). Since IFN- γ , TNF α , IL-6, and IL-15 are produced in high levels by mIL-15 DCs, we assessed whether the presence of these cytokines would affect T cell stimulation (Table 2-1). PBLs were stimulated with anti-CD3/anti-CD28 beads (DynaLab, Invitrogen, San Diego, CA) in the presence of exogenous cytokines in AIM-V media supplemented with 5% human serum as in our previous DC:PBL co-cultures. We tried to mimic the average secreted cytokine(s) concentration determined by mDC ELISA and were added at the following concentrations: 5 ng/mL IFN- γ (Gentaur), 5 ng/mL TNF α , 10 ng/mL IL-6, 5 ng/mL IL-15, or all four cytokines combined. 3×10^5 PBLs were plated into a 96-well U-bottom plate and then cytokines were added to the appropriate wells. Finally anti-CD3/anti-CD28 beads were added to the wells to stimulate T cells following the manufacturer's instructions overnight. T cell activation was measured via flow cytometry by up-regulation of CD25 and CD69 which are two common markers for activation in CD3⁺-gated T cells. The surface of the stimulated PBLs were stained with anti-CD3, CD25, and CD69 antibodies as described previously prior to collecting the data on BD LSRII and then analysis with Flowjo. Statistical significance was determined by two-tailed, paired t tests due to the small number of donors evaluated. Overall, nearly all the cytokines increased the number of activated T cells in relation to the control. However, IL-6 by itself significantly increased the percentage of CD69⁺ CD3⁺ T cells ($p=0.04$) as well as CD25⁺ CD69⁺ CD3⁺ T cells ($p=0.05$). The presence of TNF α also promoted T cell activation but additional donors would be needed to confirm the slight increase observed. Future experiments

evaluating potential dose-dependence of IL-6 and/or TNF α would necessary to confirm that these inflammatory cytokines help to increase T cell activation. Additionally the potential increase in T cell activation by the cytokines secreted from mIL-15 DCs as well as the effect of this cytokine environment on the T cells' subsequent cytolytic effector activities should also be verified.

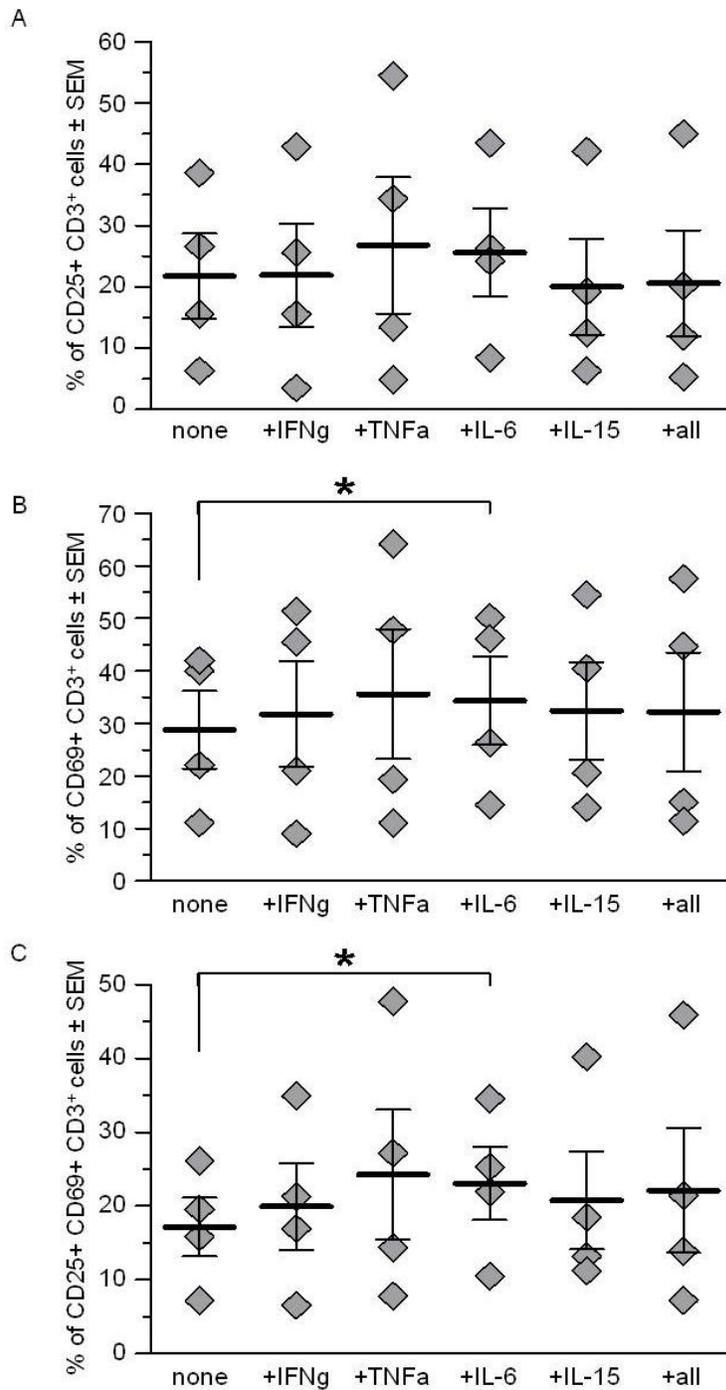


Figure F-1. Effect of IFN- γ , TNF α , IL-15, IL-6, or all four inflammatory cytokines on the activation of CD3⁺ T cells. PBLs were stimulated overnight with anti-CD3/anti-CD28 beads in the presence of the indicated cytokine. T cell activation was evaluated by the up-regulation of CD25 and CD69 expression on the surface of CD3⁺-gated T cells. A) Percentage of CD25⁺ T cells. B) Percentage of CD69⁺ T cells. C) Percentage of CD25⁺ CD69⁺ T cells. Mean values \pm SEM are indicated by the bars and whiskers. Statistical significance determined by two-tailed paired t test with p value ≤ 0.05 was considered significant. $n=4$

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BIOGRAPHICAL SKETCH

Starlyn Leinani Mitsuko Okada was born in 1982 in Honolulu, Hawaii. She lived in Kaneohe, Hawaii until graduation from James B. Castle High School in 2000. That year she moved to Seattle, Washington to attend the University of Washington (UW) as a member of the UW Honors Program.

She became an undergraduate lab assistant for Dr. Fred Farin in 2001. Based on her work on genetic biomarkers for Parkinson's disease, she received a UW Mary Gates Research Scholarship in 2002. In 2003, Starlyn worked as summer research student at the Max Plank Institute of Molecular Cell Biology and Genetics in Dresden, Germany with Dr. David Drechsel. After returning to Seattle, she completed her honors thesis on the foamy virus Gag protein with Dr. Maxine Linial as an UW Early Identification Program Presidential scholar at the Fred Hutchinson Cancer Research Center. She graduated in 2005 with a Bachelor of Science with College Honors in microbiology and a Bachelor of Arts in Japanese language.

She was admitted into the Interdisciplinary Program in Biomedical Sciences at the University of Florida (UF) for a Doctor of Philosophy in 2005 with a UF Alumni Graduate Fellowship. After the first semester, she took a leave of absence to complete research at Keio University School of Medicine in Tokyo, Japan with Dr. Yutaka Kawakami from 2006 to 2007 as a Japanese government (Monbukagakusho)-sponsored research scholar. She returned to UF in 2007 and joined Dr. Lung-Ji Chang's lab to study human monocyte-derived IL-15-induced DCs in the Immunology and Microbiology concentration. Her work in Dr. Chang's lab culminated in a Ph.D. from the University of Florida in the summer of 2012.