THE EFFECTS OF TOLL-LIKE RECEPTOR LIGAND-ACTIVATED DENDRITIC CELLS ON HUMAN $CD4^+$ T CELL RESPONSES

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---

by

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DEDICATION

I dedicate my work to my grandmother, Fumie Murata and my late grandfather, Yoshio Murata.

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LIST OF ABBREVIATIONS

APC, antigen-presenting cell

CTLA, cytotoxic T lymphocyte antigen

DC, dendritic cell

DTH, delayed-type hypersensitivity

dsRNA, double-stranded RNA

EBI3, Epstein-Barr virus induced gene 3

ELISA, enzyme-linked immunosorbent assay

ELISPOT, enzyme-linked immunosorbent spot

FACS, fluorescence-activated cell sorting

FBS, fetal bovine serum

Foxp3, forkhead box P3

GATA3, GATA-binding protein 3

G-CSF, granulocyte colony-stimulating factor

GFP, green fluorescent protein

GITR, glucocorticoid-induced tumor necrosis factor receptor

HIV, human immunodeficiency virus

HLA, human leukocyte antigen

HR, high responder

HSC, hematopoietic stem cell

IBD, inflammatory bowel disease

ICAM-1, inter-cellular adhesion molecule 1

IFN, interferon

Ig, immunoglobulin

IL, interleukin

intDC, interstitial dendritic cell

iTreg, inducible T regulatory cell

IRAK, interleukin 1 receptor-associated kinase

IRF, interferon regulatory factor

LBP, LPS-binding protein

LC, Langerhans cell

LPS, lipopolysaccharide

LR, low responder

LRR, leucine rich repeat

MAL, MyD88 adaptor-like

MDA5, melanoma differentiation-associated gene 5

MDP, muramyl dipeptide

MHC, major histocompatibility complex

moDC, monocyte-derived dendritic cell

MPL-A, monophosphoryl lipid A

MS, multiple sclerosis

Mtb, *Mycobacterium tuberculosis*

MyD88, myeloid differentiation primary response gene 88

NFAT, nuclear factor of activated T cells

NFκB, nuclear factor κ B

nTreg, natural T regulatory cell

NOD2, nucleotide oligomerization domain 2

Pam3CSK, N-Palmitoyl-S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-[R]-Cys-[S]-Serl-[S]-Lys (4) trihydrochloride

PAMP, pathogen-associated molecular pattern

PBS, phosphate saline buffer

pDC, plasmacytoid dendritic cell

PMA, phorbol 12-myristate 13-acetate

Poly I:C, polyinosinic:polycytidylic acid

PRR, pattern recognition receptor

RIG-1, retinoid-inducible gene 1

ROR, retinoid-related orphan receptor

RT-PCR, reverse transcriptase polymerase chain reaction

SARM, Sterile-alpha and Armadillo motif

ssRNA, single-stranded RNA

STAT, signal transducers and activators of transcription

TGFβ, transforming growth factor β

TICAM, Toll-interleukin 1 receptor domain (TIR)-containing adaptor molecule

TIR, Toll-Interleukin 1 receptor

TIRAP, Toll-interleukin 1 receptor (TIR) domain containing adaptor protein

TLR, Toll-like receptor

TNFα, tumor necrosis factor α

TRIF, Toll-interleukin 1 receptor (TIR) domain-containing adapter-inducing interferon β

Treg, T regulatory cell

WNV, West Nile virus

ABSTRACT

Dendritic cells (DCs) play an important role as a link between innate and adaptive immunity through their abilities to detect pathogens and to prime naïve T cells (signal 1 and 2). They not only activate naïve T cells, but also direct differentiation of $CD4^+$ T cells to induce appropriate immune responses against pathogens via cytokine production (signal 3). In this study, human monocyte-derived dendritic cells (moDCs) as a model for myeloid DCs were activated *in vitro* with an array of Toll-like receptor (TLR) ligands derived from or mimicking various pathogens, to determine their cytokine profiles, as well as the ability of these differentially TLR ligand-activated DCs to induce differentiation of naïve $CD4^+$ T cells. moDCs from certain donors activated with a viral TLR ligand, poly I:C (TLR3 ligand), produced interleukin (IL)12p70, whereas moDCs from the other DC donors produced undetectable amounts of IL12p70. Thus, the DC donors were divided into two groups for further investigation: low responders (LR) and high responders (HR). Bacterial TLR ligands failed to induce production of detectable levels of IL12p70 from both LR and HR donor moDCs. Overall, poly I:C-activated moDCs heavily skewed Th1 differentiation in human allogeneic naïve $CD4^+$ T cells relative to that induced by moDCs activated with bacterial TLR ligands (LPS: TLR4 ligand and flagellin: TLR5 ligand) or another viral TLR ligand (ssRNA40: TLR7/8 ligand). Poly I:C-activated HR moDCs induced more heavily skewed Th1 differentiation than that of poly I:C-activated LR moDCs. Conversely, the bacterial TLR ligand-activated moDCs induced relatively balanced Th1/Th2 responses. Neutralization of

IL12p70 in these DC/T cell cocultures demonstrated that IL12p70 plays a predominant role in Th1 differentiation in these cocultures.

We also observed that moDCs activated with two of bacterial TLR ligands tested, LPS and Pam3CSK4 (TLR1/2 ligand), induced their production of IL6 and IL1β. These moDCs also induced the highest levels of Th17 differentiation in the DC/T cell coculture compared to those using moDCs activated with poly I:C, flagellin and ssRNA40. Neutralization of IL1β, IL6 or TGFβ revealed that both TGFβ (presumably T cell-derived) and DC-derived ILβ are absolutely, but IL6 is only partially, required for Th17 differentiation in the cocultures. On the other hand, only TGFβ and IL1β, but not IL6, were required for Th17 differentiation in an APC-free system. This was due to the addition of large amounts of IL1β in the APC-free system, in which case IL6 was dispensable. This differs from the coculture system, in which only small amounts of IL1β were produced by the activated DCs. Taken together, bacterial TLR ligand (Pam3CSK4, LPS and MPL-A, flagellin: TLR1/2, 4, 5 ligands, respectively)- as well as a viral TLR ligand (ssRNA40: a TLR7/8 ligand)-activated moDCs induced relatively more balanced Th1/Th2/Th17 $CD4^+$ T cell differentiation whereas another viral TLR ligand (poly I:C: a TLR3 ligand) induced more heavily skewed Th1 differentiation. These results provide a framework for the use of these TLR ligands in tailoring T cell responses in vaccines and other immunotherapeutic approaches.

CHAPTER I

Literature Review

1. Dendritic Cells

A. Introduction

Dendritic cells (DCs) are a heterogeneous population of antigen-presenting cells (APCs) and are capable of taking up, processing and presenting antigens to induce primary response in T cells. Immature DCs actively phagocytose extracellular materials to be presented as antigens on their surface, whereas mature DCs upregulate their major histocompatibility complex (MHC) class II molecule for activation of $CD4^+$ T cells, which in return help $CD8^+$ T cell and B cell responses. DCs present antigens via MHC class I molecule to $CD8^+$ T cells by conventional or cross-presentation. Although monocytes, macrophages and B cells are also capable of antigen presentation, DCs are the most potent APCs since they prime T cells with a higher efficiency than other APCs (Ueno et al., 2007; Steinman and Cohn, 1973; Steinman and Witmer, 1978; Hart, 1997). The major characteristics of DCs are 1) the ability to prime T cells, 2) the ability to migrate from the periphery or tissues to lymph nodes or lymphoid organs, 3) phagocytic ability, 4) the ability to process antigens into peptides and to present them on MHC molecules, 5) the ability to sense infection or "danger signal" via receptors such as Toll-like receptors (TLRs), 6) the expression of different surface molecules from those expressed on other leukocytes such as macrophages and B cells (Syme and Glnck, 2001; Hart, 1997). DCs arise from either $CD34^+$ bone marrow hematopoietic stem cells (HSCs) or $CD14^+$ monocytes *in vivo* or *in vitro*. They are traditionally categorized into two

groups based on their origin: conventional (myeloid) DCs and plasmacytoid (lymphoid) DCs (pDCs) (Syme and Glnck, 2001; Shortman and Naik, 2007; Wu and Liu, 2007; Hart, 1997).

B. Phenotypes of Dendritic Cells

i. Conventional Dendritic Cells

The conventional DCs are further categorized into two subsets: peripheral non-lymphoid tissue-resident DCs and lymphoid tissue-resident DCs. The peripheral non-lymphoid tissue-resident DC subset includes Langerhans cells (LCs) and interstitial DCs (intDCs). These non-lymphoid DCs sample antigens from peripheral tissues and migrate through lymph to lymph nodes. For example, LCs reside in the skin but can also be found in the intestinal, respiratory and reproductive tracts. Therefore, peripheral non-lymphoid tissue-resident DCs are migratory, and they have mature phenotype upon arriving in lymph nodes since they collect antigens in the periphery (Shortman and Naik, 2007). Human LCs express CD11c, CD1a, E-cadherin, and a C-type lectin (langerin), but lack mannose receptor expression. They also express TLR1, 2, 3, 6, 7 and 10. Human intDCs express CD11c, CD1a, CD14, CD11b, CD36, DC-SIGN and mannose receptors, and TLR 1, 2, 3, 4, 5, 6, 7 and 8. Both LCs and intDCs can be generated *in vitro* from $CD34^+$ hematopoietic cells in the presence of granulocyte monocyte colony-stimulating factor (GM-CSF) and tumor necrosis factor (TNF)α (Hochrein and O'Keeffe, 2008). On the other hand, lymphoid tissue-resident DCs do not migrate into lymphoid organs from lymphatics so they remain immature in phenotype in the lymphoid organs until they encounter antigens. Instead, they sample antigens in the lymphoid organs, in which they reside (e.g. thymus and spleen). (Ueno et al., 2007; Shortman and Naik, 2007). In mice,

lymphoid tissue-resident DCs can be divided into groups based on the phenotypes and functions. For example, murine splenic DCs are separated in to $CD4^-CD8^-$ (25% of splenic cDCs), $CD4^-CD8^+$ (25%) and $CD4^+CD8^-$ (50%) subsets (Hochrein and O'Keeffe, 2008). The $CD4^-CD8^+$ cDC subset has the shortest half-life of three days. They can produce cytokines, such as IL6 and TNFα, upon activation, but only produce low levels of chemokines. They have the ability to cross-present antigens and play an important role in priming $CD8^+$ T cell responses. $CD4^+CD8^-$ cDCs produce the highest levels of chemokines even in the resting state. They inefficiently cross-present antigens unlike $CD4^-CD8^+$ cDCs, but present efficiently to $CD4^+$ and $CD8^+$ T cells in a conventional presentation manner. $CD4^-CD8^-$ cDCs have a similar functions to that of $CD4^+CD8^-$ cDCs, except that the former can produce high levels of interferon (IFN)γ upon stimulation (Hochrein and O'Keeffe, 2008). Although murine splenic DC subsets are defined based on the expression of CD4 and/or CD8, all human DCs express CD4 but not the CD8 surface marker (Hart, 1997). Therefore, studies on human myeloid DCs are often performed using conventional DCs: blood myeloid DCs or monocyte-derived DCs (moDCs) without further subdivision, and it is difficult to correlate results from mouse studies using a subset of murine splenic cDCs with that of human cDCs, since only one of the murine cDC subsets shares the same phenotypes and functions (Hochrein and O'Keeffe, 2008). The most commonly used phenotype of human myeloid DCs for identification is $HLA\text{-}DR^+CD14^{-/low}CD11c^+CD123^-$ (Ueno et al., 2007; Shortman and Naik, 2007; Hart, 1997). The functions of human cDCs are discussed below. Human immature cDCs express DC-SIGN and mannose receptor, as well as chemokine receptors, CCR1, CCR2, CCR4, CCR5, CXCR1 and CXCR4. Upon maturation, these

cells downregulate receptors required for uptake of antigens (i.e., DC-SIGN and mannose receptor) and upregulate MHC class II molecule, the costimulatory molecules, CD80 and CD86, CD40 and the chemokine receptor, CCR7, for more effective antigen presentation (Banchereau et al., 2000).

ii. Plasmacytoid Dendritic Cells

Plasmacytoid DCs differ significantly from cDCs in their phenotype, functions and origin. Traditionally, pDCs are thought to be of lymphoid origin, though this is still controversial. In humans, the key marker on pDCs is IL3Rα chain (CD123), which is required for their differentiation from a resting state. Unlike cDCs, pDCs do not express CD11c. Furthermore, pDCs differ from cDCs in morphology since they lack dendrites unless activated with IL3 and/or CD40L, and in their inability to efficiently prime T cells. Human pDCs are $CD4^{+}CD45RA^{+}CD123^{+}CD11c^{-}$MHC II^{+} and also express C-type lectins, BDCA-2 and BDCA-4, and TLR 1, 6, 7, 9 and 10 (Hochrein and O'Keeffe, 2008). Murine pDCs are $CD11c^{int}$MHC $II^{lo}CD11b^{-}CD123^{-}CD205^{-}$, which is quite distinct from their human counterpart as mentioned above (Hochrein and O'Keeffe, 2008).

iii. Monocyte-derived Dendritic Cells

Human moDCs have been extensively used as a model for human cDCs for the last 20 years. These cells are used almost as equivalent to murine bone marrow-derived or whole splenic DCs, both of which are used as a model for cDCs in mice. Initially, murine blood or bone marrow cells were shown to differentiate into cDCs in culture supplemented with GM-CSF. However, their human counterparts failed to do so in GM-CSF-containing culture. Human monocytes require both granulocyte-macrophage

colony-stimulating factor (GM-CSF) and interleukin (IL)4; GM-CSF promotes DC survival, and IL4 suppresses macrophage growth, allowing differentiation of monocytes into DCs in 5-7 days (Romani et al., 1996; Syme and Glnck, 2001). Fresh monocyte-derived DCs are phenotypically and functionally equivalent to immature myeloid DCs and have a similar TLR expression pattern to myeloid DCs as discussed below (Hochrein and O'Keeffe, 2008).

C. Functions of Dendritic Cells

i. Antigen Uptake, Processing and Presentation

DCs take up antigens by 1) phagocytosis of large particles like bacteria, viruses, parasites and apoptotic host cells and cell fragments, 2) macropinocytosis and 3) receptor-mediated endocytosis (Banchereau et al., 2000). Endocytosis by DCs involves C-type lectin receptors that recognize carbohydrates, such as mannose receptors DEC205 (CD205) and CD206, and DC-SIGN (CD209), or Fcγ receptors types I (CD64) and II (CD32) that recognize antigen-antibody immune complexes or opsonized particles (Banchereau et al., 2000; Steinman and Banchereau, 2007). Antigens from the extracellular environment are presented via MHC class II molecules to prime $CD4^+$ T cells, whereas antigens synthesized intracellularly are presented via MHC class I molecules to prime $CD8^+$ T cells. Briefly, extracellular antigens that have been taken up by DCs are degraded in the endosomes or phagolysosomes and then directed into the MHC class II-rich compartments (MIIC) where MHC class II molecules can be loaded with antigen-derived peptides. Endogenous antigens are ubiquinated and degraded by the proteosomes in the cytoplasm. The antigens are first degraded to ~14 amino acid peptides in length. These peptides are transported via TAP (transporters associated with antigen processing)-

1/TAP-2 into the ER where they are further processed by ERAP (ER-associated peptidase). The resulting peptides of 8 to 10 amino acids in length are then loaded onto MHC class I molecules. DCs are also capable of cross-presentation, i.e., uptake of extracellular particulate antigens and presentation via MHC class I molecules. *In vivo*, peripheral DCs mature upon encountering either pathogens or pathogen products and migrate by CCR7 expression into the T cell area of the draining lymphoid organs where they prime T cells (Banchereau et al., 2000).

ii. TLR Expression and Cytokine Production

DCs express Toll-like receptors that recognize pathogen-associated molecular patterns expressed by pathogens or molecules from damaged host cells. Via TLR signaling, immature DCs can be activated and differentiate into mature DCs. Mature DCs upregulate costimulatory molecule (CD80 and CD86) expression, which is required for efficient T cell priming by DCs. Mature DCs also produce cytokines, such as IL6 and TNFα that further enhance maturation of its DC population, and IL12p70 that directs T cell differentiation. Upon activation, pDCs produce large amounts of type I IFNs, which induce an anti-viral state in innate immunity (Hochrein and O'Keeffe, 2008; Shortman and Naik, 2007). CD40-CD40 ligand interactions between immature DCs and newly activated $CD4^+$ T cells can also induce the maturation of DCs. However, those DCs activated through CD40-CD40 ligand signaling without TLR signaling fail to produce IL12p70 and induce proliferation, but not differentiation of $CD4^+$ T cells (Sporri and Reis e Sousa, 2005). Differential cytokine production by DCs via TLR signaling can influence T cell differentiation resulting in appropriate adaptive immune response against pathogens. Human monocyte-derived DCs have a similar TLR expression pattern to that

of peripheral conventional (myeloid) DCs (Table 1). However, murine TLR expression pattern, at least as measured by mRNA levels, is somewhat different from that of humans (Reis e Sousa, 2004). Most notably, human myeloid DCs do not express TLR9 whereas their murine counterparts do.

iii. Tolerance

The presentation of antigens by immature DCs in the absence of infection or inflammation can lead to T cell anergy (Ardavin et al., 2004). Thymic tolerance is also induced by DC-mediated negative selection (Banchereau et al., 2000). DCs are also capable of inducing tolerance via induction of regulatory T cells, which suppress T cell responses against foreign antigens as well as alloantigens and tumors. In addition, pDCs are a major source of indoleamine 2,3-dioxygenase (IDO), which degrades the essential amino acid, tryptophan, resulting in suppression of T cell proliferation (Rossi and Young, 2005).

Table 1. TLR Expression Pattern in Human Dendritic Cells

	moDC	Myeloid DC	Plasmacytoid DC
TLR1	++	++	+
TLR2	++	++	-
TLR3	++	++	-
TLR4	++	-/+ (Reis e Sousa, 2004)	-
TLR5	+	+	-
TLR6	++	++	++
TLR7	-/+ (Gorden et al., 2005)	+	++
TLR8	++	++	-
TLR9	-	-	++
TLR10	-/+ (Hasan et al., 2005; Kim et al., 2006)	+	+

(Iwasaki and Medzhitov, 2004)

2. T Helper Cells

A. Th1 and Th2 Cells

i. Differentiation of Th1 and Th2 Cells

Naïve $CD4^+$ T cells differentiate into T helper (Th) cells with effector functions. First described by Mosman and Coffman based on the functions and cytokine produced, Th cells were divided into two groups: Th1 cells and Th2 cells (Mosmann and Coffman, 1989). Th1 cells produce the key Th1 effector cytokine, IFNγ, which is crucial for immunity against intracellular pathogens. On the other hand, the key Th2 effector cytokine is IL4, and these cells are responsible for immunity against extracellular pathogens including parasites (Murphy and Reiner, 2002).

Naïve $CD4^+$ T cells are induced to differentiate into Th1 cells by IL12 produced by APCs, primarily DCs, promoting signal transducers and activators of transcription (STAT)4 activation (Moser and Murphy, 2000). Activated T cells via TCR upregulate IL12 receptor β chains to respond to DC-derived IL12 (Szabo et al., 1997). Transcription of *IFNγ* is promoted by activated STAT4 downstream of IL12 stimulation and a transcription factor, nuclear factor of activated T cells (NFAT). Therefore, IL12-mediated STAT4 activation is required for IFNγ production by newly differentiated Th1 cells, and proliferation and survival of these cells, though the effects of IL12 was suppressed in the presence of Th2 effector cytokine, IL4 (Mullen et al., 2001). It was later shown that when naïve $CD4^+$ T cells are activated, T-bet, a T-box transcription factor essential for Th1 development, is induced in STAT1-dependent manner by the autocrine effect of IFNγ, and T-bet promotes remodeling of normally repressed *IFNγ* locus (Szabo et al., 2000; Lighvani et al., 2001). T-bet upregulation was shown to be

STAT4-independent, suggesting initial T-bet induction is upstream of STAT4 activation. However, STAT4 was required for sustained IFNγ production subsequent to initial IFNγ production induced via TCR activation and IL12 receptor β2 chain upregulation (Szabo et al., 1997; Park et al., 2004). In addition, both human and murine DCs have been shown to produce IFNγ, which can promote Th1 differentiation of $CD4^+$ T cells as well (Lugo-Villarino et al., 2003). IFNα/β produced by cDCs or pDCs is also capable of inducing Th1 differentiation by activating STAT4 via STAT2 activation downstream of type I IFN receptor activation in humans, but not in mice (Mullen et al., 2001).

In contrast, Th2 cells differentiate from naïve $CD4^+$ T cells via upregulation of a Th2-specific transcription factor, GATA-binding protein 3 (GATA-3), which is upregulated by IL4 and STAT6 activation (Skapenko et al., 2004). GATA-3 induces remodeling of *IL4* locus, and transcription of IL4 is promoted by another set of Th2-specific transcription factors, b-ZIP (basic-region leucine zipper) protein and c-MAF, and the non-lineage-specific transcription factor, NFAT. Ruslan Medzhitov's group has demonstrated that basophils are directly activated by papain, an allergen with protease activity, to produce IL4 which initiates Th2 differentiation in mice (Sokol et al., 2008). A novel cytokine, IL33, has been shown to induce Th2 cytokines both *in vitro* and *in vivo*, and a recent report demonstrated that IL33 induces antigen-specific IL5-producing Th2 cells in the absence of IL4 (Kurowska-Stolarska et al., 2008; Schmitz et al., 2005). Another recent publication examined the role of IL4 and STAT6 in Th2 differentiation *in vivo* (van Panhuys et al., 2008). In that report, the authors used G4 mice, in which the first exon and intron of *Il4* gene were replaced with green fluorescent protein (GFP) ($IL4^{G4/G4}$), and STAT6-deficient $IL4^{G4/G4}$ mice, and showed that neither IL4 nor STAT6

were required for Th2 differentiation in response to parasite infection. Therefore, it is possible that initial differentiation of Th2 cells is induced by IL33, and these IL4-producing Th2 cells promote further expansion of the Th2 cell population.

ii. Functions of Th1 and Th2 Cells

As mentioned earlier, the Th1 subset plays a central role in immunity against intracellular pathogens (e.g., intracellular bacteria and viruses) and delayed-type hypersensitivity (DTH) (Figure 1). It is also responsible for autoimmunity, though another Th subset, Th17, is also involved in the autoimmunity pathogenesis as discussed below. Th1 cells produce IFNγ, as well as IL2, granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL3. In addition, Th1 cells induce isotype switching to IgG2a production by mouse B cells (Liew, 2002; Moser and Murphy, 2000; Murphy and Reiner, 2002). IFNγ produced by Th1 cells activates macrophages and enhances $CD8^+$ T cell differentiation and proliferation (Whitmire et al., 2005).

Th2 cells produce Th2 effector cytokines, such as IL4, IL5, IL10 and IL13, which play roles in immunity against extracellular pathogens (e.g., helminthes) but also contribute to allergies. Th2 cells also induce isotype switching in B cells to IgE and IgG1 in mice through IL4, which promotes class switching of immunoglobulins and subsequent mast cell activation. IL5 produced by both Th2 cells and mast cells induces eosinophil differentiation and activation (Liew, 2002).

B. Th17 Cells

i. Differentiation and Phenotypes of Th17 Cells

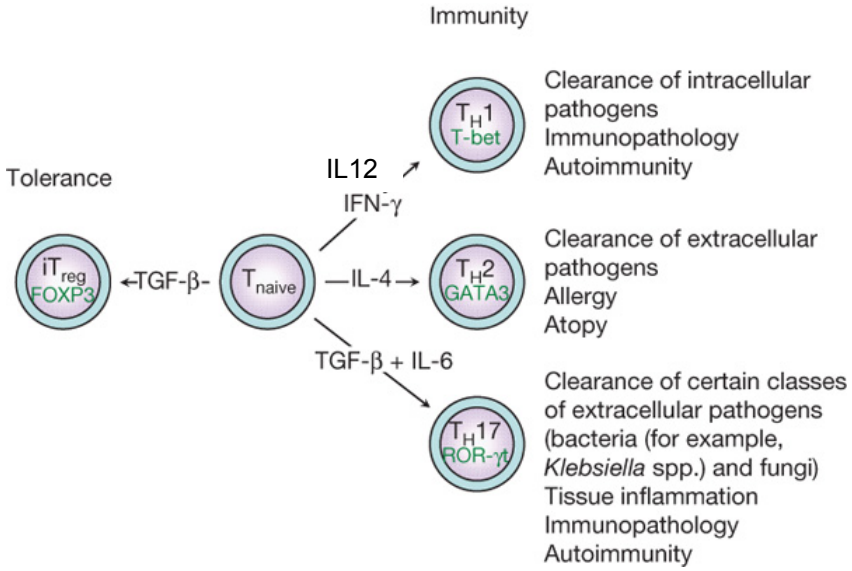
In 2003, Austin Gurney's group first observed that IL23 promotes IL17 production by activated or memory T cells (Aggarwal et al., 2003). Later that year, Daniel Cua's group

reported that mice deficient in the IL23p19 subunit gene were protected against autoimmune inflammation, in this case, collagen-induced arthritis, whereas mice deficient in the IL12p35 subunit gene displayed exacerbated disease (Murphy et al., 2003; Aggarwal et al., 2003). These reports lead to the proposal that IL17-producing T cells are a distinct subset of T helper cells that differ from traditional Th1 or Th2 cells, and are pathogenic in autoimmune diseases. Since the key characteristic of this new T helper subset is production of IL17, a proinflammatory cytokine, these cells were referred to as Th17 cells (Langrish et al., 2005; McKenzie et al., 2006). In 2005, two groups showed that Th17 cells fail to differentiate from Th1 or Th2 cells, but instead, differentiate from naïve $CD4^+$ T cells through their own lineage (Park et al., 2005; Harrington et al., 2005). These two groups also demonstrated that upregulation of Th1- or Th2-specific transcriptional regulators (e.g., STAT1, STAT4 and T-bet, or STAT6 and GATA3, respectively) by the addition of IFNγ or IL4, respectively, suppressed the differentiation of Th17 cells *in vitro* and *in vivo*. Subsequently, three groups demonstrated that TGFβ is the key cytokine required for the differentiation of Th17 cells from naïve $CD4^+$ T cells (Veldhoen et al., 2006; Mangan et al., 2006; Bettelli et al., 2006). In addition, investigators showed that IL6 is also required for Th17 differentiation by suppressing TGFβ-induction of T regulatory cells (Veldhoen et al., 2006; Bettelli et al., 2006). Later, IL2, which is required for functional T regulatory cells, was shown to suppress differentiation of Th17 (Kryczek et al., 2007b). It was also reported that IL1β inhibits IL2-mediated suppression of Th17 differentiation, and that IL6 suppression of T regulatory cell induction is dispensable in the presence of IL1β to allow Th17 differentiation (Kryczek et al., 2007a). Furthermore, TGFβ-producing

$CD4^+CD25^+Foxp3^+$ T regulatory cells were also shown to differentiate into Th17 cells in the presence of IL6 (Xu et al., 2007). In addition, TGFβ as shown to upregulate IL23 receptor (IL23R) expression in Th17 cells, which explains their responsiveness to IL23 (Mangan et al., 2006). However, IL23 is not involved in differentiation of the Th17, but rather acts as an expansion or maintenance factor for Th17 cell population (Veldhoen et al., 2006; Bettelli et al., 2006).

In 2006, Littman's group identified RORγt, a retinoid orphan receptor encoded by *Rorc* as an essential transcription factor for Th17 cell differentiation (Ivanov et al., 2006). RORγt is upregulated in activated T cells in the presence of TGFβ and IL6. This upregulation of RORγt depended on IL6, since T cells isolated from IL6-deficient mice failed to upregulate expression of RORγt, as well as IL17, IL17F and IL23R mRNA. In the same report, the group also demonstrated that RORγt-deficiency resulted in a substantial, but not complete, decrease in Th17 cells and EAE severity in mice. Another retinoid orphan receptor, RORα, was also shown to be induced in Th17 cells (Kryczek et al., 2007a; Yang et al., 2008c). In addition, IRF4 is required for the generation of Th17 cells, since IRF4-deficient mice exhibited less RORγt expression and were completely resistant to the induction of experimental autoimmune encephalomyelitis (EAE) (Brustle et al., 2007)

Figure 1. Differentiation of Naïve CD4$^+$ T Cells into T Helper Cells



"Depending on the cytokine milieu present at the time of the initial engagement of their T-cell receptor and co-stimulatory receptors in the peripheral immune compartment, naive CD4$^+$ T cells can differentiate into various subsets of T helper cells (T_H1, T_H2 and T_H17). However, in the presence of TGF-β, naive T cells convert into FOXP3-expressing induced T_{reg} (iT_{reg}) cells. For each T helper cell differentiation program, specific transcription factors have been identified as master regulators (T-bet, GATA3 and ROR-γt). Terminally differentiated T helper cells are characterized by a specific combination of effector cytokines that orchestrate specific and distinct effector functions of the adaptive immune system." (Bettelli et al., 2006)

In 2006, while using IL27 receptor-deficient mice, Ghilardi and colleagues found that the lack of IL27 signaling exacerbated EAE, leading them to conclude that IL27 suppress the generation of IL17-producing T cells (Batten et al., 2006). In late 2007, four groups reported that IL10-producing suppressive T cells are induced by IL27 (Awasthi et al., 2007; Fitzgerald et al., 2007; McGeachy et al., 2007; Stumhofer et al., 2006). Christopher Hunter's group demonstrated that IL17 but not IL10 production by T cells cultured in Th17-polarizing condition was suppressed by the presence of IL27 (Stumhofer et al., 2006). Rostami and colleagues also showed that IL27 caused suppression of IL17 production by T cells and that IL27-mediated suppression of EAE is due to IL10 production (Fitzgerald et al., 2007). In addition, Awasthi et al. demonstrated that DCs encountering T regulatory cells produce IL27, which subsequently induces T cells to produce IL10 (Awasthi et al., 2007). Finally, Dua's group showed that Th17 cells initially generated in the presence of TGFβ and IL6 are not pathogenic since they produce both IL17 and IL10, but these cells acquire pathogenic function upon restimulation with IL23, which downregulates their expression of IL10 (McGeachy et al., 2007). Taken together, IL27 suppresses IL17 production by Th17 cells, and the IL10 produced by Th17 cells or other Th subsets, stimulated by IL27, suppresses the pathogenic effector functions of Th17 cells (Jankovic and Trinchieri, 2007).

In addition, IL21 was shown to be produced by Th17 cells and acts in an autocrine manner in the presence of TGFβ to enhance differentiation of naïve $CD4^+$ T cells into Th17 cells (Wei et al., 2007; Nurieva et al., 2007; Zhou et al., 2007; Korn et al., 2007). Vijay Kuchroo's group demonstrated that Th17 cells can be generated in IL6-deficient mice, and IL21 in the presence of TGFβ can alternatively induce differentiation

of Th17 cells (Korn et al., 2007). IL21 is produced by Th17 cells as well as RORγ-deficient T cells activated in the presence of IL6 or IL21 itself. IL21 induces IL23 receptor and RORγt upregulation more efficiently than IL6. Therefore, IL21 produced by IL6-stimulated T cells can act in an autocrine matter to induce IL23 receptor and RORγt expression to further enhance differentiation of Th17. How IL21 is initially produced in IL6-deficient mice is unknown, especially since IL21 production is normally dependent on IL6. However, it is also possible that small amounts of IL21 are produced by Th1 or Th2 cells. Despite many *in vitro* studies demonstrating the involvement of IL21 in Th17 differentiation, a recent report suggests that IL21 or IL21 receptor is not required for Th17 differentiation and development of autoimmune diseases *in vivo* (Sonderegger et al., 2008).

STAT3 has also been demonstrated to be an important transcription factor involved in Th17 differentiation, which is induced by IL6, IL21 or IL23 (Bettelli et al., 2006; Chen et al., 2007a; Zhou et al., 2007). Activated STAT3 binds to *IL17a/f* and *IL21* promoters to induce IL17, IL17F and IL21 production. IL23 receptor, RORγt and RORα upregulation are also STAT3-dependent as their expression is decreased in STAT3-deficient cells. Although the precise mechanisms are unknown, STAT3 induction by IL6, IL21 or IL23 is suppressed by other STATs that are activated by other cytokines that are known to suppress Th17 differentiation (i.e., STAT1 by IFNγ, STAT6 by IL4, STAT5 by IL2, STAT1 by IL27). Hoeve et al. have demonstrated that IL12 is capable of suppressing Th17 differentiation directly, in addition to skewing T cell response toward Th1 to suppress Th17 differentiation via IFNγ (Hoeve et al., 2006). It is unknown

whether this is due to suppression of STAT3 by STAT4 or terminal production of IFNγ and STAT1 induction.

In 2007, three groups reported that TGFβ, which was shown to be a key cytokine required for mouse Th17 differentiation in the presence of IL6 was not required for human Th17 differentiation (Chen et al., 2007b; Wilson et al., 2007; Acosta-Rodriguez et al., 2007a). This claim was disproved later by three other groups as well as our study described here (Gerosa et al., 2008; Yang et al., 2008a; Volpe et al., 2008; Manel et al., 2008). In addition, human Th17 cells were shown to be $CCR2^{+}CCR5^{-}$ (Sato et al., 2007) and $CCR4^{+}CCR6^{+}$ (Acosta-Rodriguez et al., 2007b), while another report showed that they are $CCR4^{+}CCR5^{+}CCR6^{+}$ (Annunziato et al., 2007). Upregulated CCR6 expression in human Th17 cells correlates with their murine counterparts (McGeachy et al., 2007).

ii. Functions of Th17 Cells

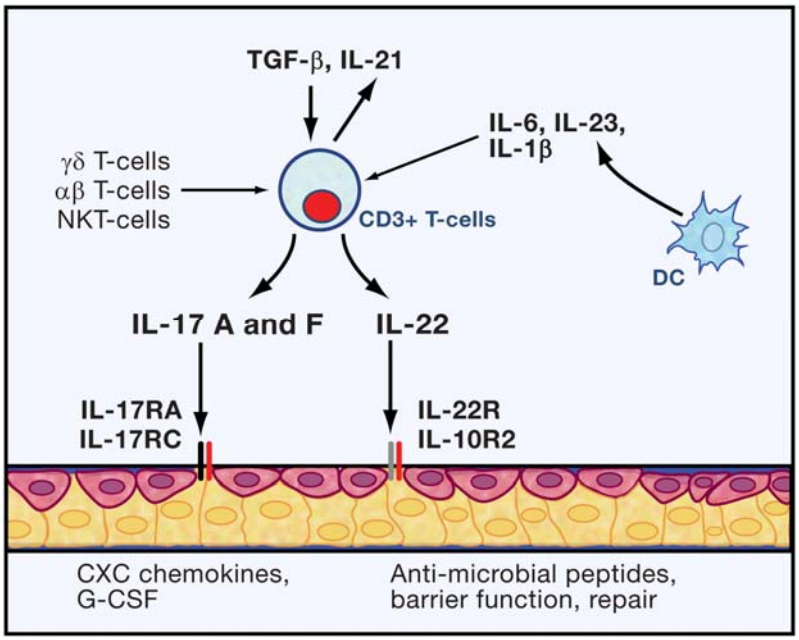
Evidence from clinical observations in humans and *in vivo* experiments in mice has shown the involvement of Th17 in pathogenesis of autoimmune diseases including arthritis, psoriasis, multiple sclerosis (MS), inflammatory bowel diseases (IBD) in both mouse models and humans (Aggarwal et al., 2003; Batten et al., 2006; Bettelli et al., 2008; Murphy et al., 2003). Both IL17 and IL22 are found in synovial fluid as well as in inflamed endothelium in MS lesions and psoriatic lesional skin (Ikeuchi et al., 2005). IL17 induces ICAM-1, IL6 and IL8 expression in human keratinocytes, and IL22 acts in synergy with IL17 to induce β defensin-2, calcium binding proteins, calgranulin A and B, and psoriasin in psoriatic skin (Ouyang et al., 2008). In patients with ulcerative colitis or Crohn's disease, IL17 mRNA is upregulated in gut tissue (Annunziato et al., 2007), and

IL17 protein is detectable in the serum from patients with active IBD (Ouyang et al., 2008).

Th17 cells are also part of natural immunity against pathogens, such as bacteria (e.g., *Mycobacteria tuberclosis, Klebsiella pneumoniae, Bacteroides fragilis*), fungi (e.g., *Candida albicans*) and certain viruses (e.g., HIV and hepatitis C virus) (Matsuzaki and Umemura, 2007; Zelante et al., 2007; Scriba et al., 2008; Khader and Cooper, 2008; Yue et al., 2008; Rowan et al., 2008). The production of IL17 recruits neutrophils to initiate inflammation by inducing upregulation of chemokines by many types of cells such as epithelial cells (Figure 2), endothelial cells, fibroblasts, osteoblasts and monocytes/macrophages (Weaver et al., 2007; Ouyang et al., 2008; Kolls and Linden, 2004). IL17A and IL17F induce granulocyte colony-stimulating factor (G-CSF), CXCL1, CXCL2 and CXCL5 upregulation in murine fibroblasts and epithelial cells, and G-CSF, CXCL1, CXCL2, CXCL5 and CXCL8 in human epithelial cells. IL17A can also induce β defensin-2 and CCL20 in human lung epithelial cells and CXCL8 in synoviocytes (Ouyang et al., 2008). In addition, IL17 induces protective Th1 recruitment to granulomas by upregulating CXCR3 ligands, CXCL9, CXCL10 and CXCL11 in mice vaccinated against *Mycobacterium tuberculosis* (Khader et al., 2007).

Although IL22 can be produced by Th1 and Th2 cells as well as NK cells and newly activated naïve $CD4^+$ T cells, Th17 cells are the dominant IL22-producers. IL22 not only acts in synergy with IL17 to initiate inflammation as mentioned above, but also induces production of antimicrobial peptides, chemokines and acute-phase proteins, and upregulates genes involved in wound healing and tissue-repair in keratinocytes (Ouyang et al., 2008).

Figure 2. Functions of IL-17 and IL-22 during *K. pneumonia* Infection in the Lung



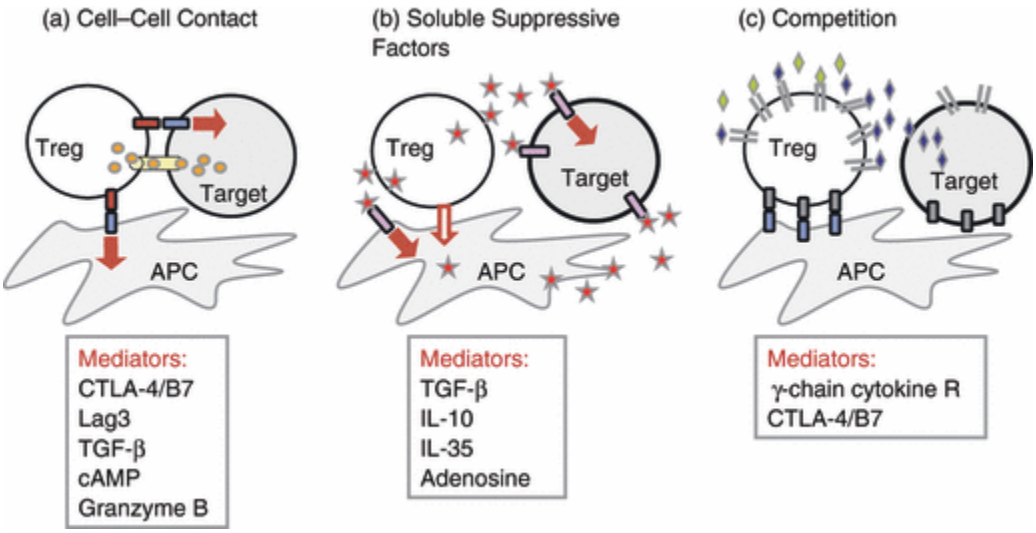
"IL-17 and IL-22 are induced rapidly in experimental bacterial pneumonia and are produced by several T cell populations in the lung, including γδ-T cells and NKT cells as well as effector memory αβ $CD4^+$ T cells. IL-17 signaling regulates granulopoiesis, through the regulation of G-CSF, as well as neutrophil recruitment, via the regulation of CXC chemokines by epithelial cells. IL-22 and IL-17 induce antimicrobial peptides from the same target cells, and IL-22 can augment epithelial repair. This cooperative induction of neutrophil recruitment and this antimicrobial-peptide production augment epithelial-barrier function and are critical for mucosal host defense against Gram-negative bacterial pneumonia." (Ouyang et al., 2008)

C. T Regulatory Cells

T regulatory cells (Tregs) are a subset of Th cells with suppressive functions. Sakaguchi et al. first demonstrated in 1995 that CD25 (IL2 receptor α chain) is a phenotypic marker for Tregs. Naturally occurring Tregs are generated in the thymus, and $CD4^+CD25^+$ "natural Tregs (nTregs)" comprise 5-10% of peripheral $CD4^+$ T cell population. nTregs also express other markers, including glucocorticoid-induced tumor necrosis factor receptor-related protein (GITR), OX40 (CD134), L-selectin (CD62L) and cytotoxic T lymphocyte-associated antigen 4 (CTLA-4/CD152). Tregs also express a transcription factor, forkhead box P3 (Foxp3), identified in Scurfy mice, in which a single mutation in the *Foxp3* gene causes deficiency in Tregs resulting in spontaneous autoimmune diseases (Sakaguchi et al., 2007). The most notable limitation to studying Tregs is to obtain a pure population of Tregs to work with since none of these markers are exclusively expressed on Tregs. However, it is widely accepted that majority of Treg population is $CD4^+$ $CD25^+$ $Foxp3^+$. In humans, the majority of the $CD4^+$ $CD25^+$ cells were also thought to be $CD45RA^+$ and $CD45RO^+$ in neonates and adults, respectively. However, a recent publication has shown that $CD4^+CD25^+CD45RA^+$ Tregs exist in the periphery of adults (Seddiki et al., 2006). Moreover, another report demonstrated that $CD4^+CD25^+CD45RA^+$ Tregs maintained Foxp3 expression during *in vitro* expansion whereas $CD4^+CD25^+CD45RO^+$ Tregs failed to so do (Hoffmann et al., 2004). Tregs can also be generated in the periphery, and these cells are called induced Tregs (iTregs). Naïve $CD4^+CD25^-$ T cells can differentiate into Tregs in the presence of TGFβ or by induction with antigen-presenting semi-mature DCs (Mills, 2004; Neufert et al., 2007).

Tregs function to suppress self-reactive effector T cells via cell-cell contact as well as by production of immunosuppressive cytokines, TGFβ and IL10 in a contact-independent way (Figure 3). Tregs suppress effector T cell activity or proliferation and function by cell-cell contact-dependent stimulation between Tregs and effector T cells through CTLA-4 and CD80/CD86 ligation, respectively. B7 (CD80/CD86)-deficient responder T cells were resistant to suppression by Tregs *in vitro* and the B7-deficient $CD4^+$ T cells induced a lethal wasting disease in lymphopenic mice even in the presence of Tregs (Paust et al., 2004). CTLA-4 expressed on Tregs can also bind to CD80/CD86 on APCs inducing IDO, which subsequently suppresses effector T cell activation and survival. TGFβ and IL10 produced by Tregs suppress DC maturation, causing the DCs to become tolerogenic and induce anergic T cells or suppress effector T cells (Cools et al., 2007). Tregs can also compete for IL2 with effector T cells since Tregs constitutively express CD25 whereas naïve $CD4^+$ T cells do not upregulate CD25 until they are activated (Sojka et al., 2008). Recently, it became clear that Tregs regulate Th17 at the level of differentiation. TGFβ induces simultaneous upregulation of both Foxp3 and RORγ required for Treg and Th17 functions, respectively. However, Foxp3 directly interacts with RORγ to suppress RORγ-mediated IL17 expression (Zhou et al., 2008; Ichiyama et al., 2008; Zhou et al., 2008). In the presence of proinflammatory cytokines such as IL6 and/or IL1, Foxp3 expression is downregulated and RORγ-expressing cells complete their differentiation into Th17 cells by expressing IL17 (Zhou et al., 2008).

Figure 3. Possible Mechanisms of Suppression by T Regulatory Cells (Tregs)



"(a) Cell–cell contact. Tregs may suppress target cells via direct interaction of receptor–ligand pairs on Tregs and target cells; delivery of suppressive factors via gap junctions including cyclic adenosine monophosphate (cAMP); direct cytolysis; membrane-bound suppressive cytokines such as transforming growth factor-β (TGF-β); and/or indirectly via modulating the antigen-presenting cell (APC) through cell–cell contact, possibly through reverse signaling via Treg–cytotoxic T-lymphocyte antigen-4 (CTLA-4) engagement of B7 on dendritic cells. (b) Soluble suppressive factors. Tregs can directly secrete interleukin-10 (IL-10), TGF-β and IL-35 or induce APCs to secrete such factors. Expression of CD73/CD39 by Tregs facilitates the local generation of adenosine that can down-modulate immune function. (c) Competition. Tregs may compete for some cytokines that signal via receptors that contain the common γ-chain (IL-2, IL-4 and IL-7). Additionally they may compete for APC costimulation via constitutive expression of CTLA-4. Red arrow indicates an inhibitory signal**."** (Sojka et al., 2008)

3. Toll-like Receptors and Their Ligands

A. Introduction on Toll-like Receptors

TLRs were first identified as mammalian homologues of the *Drosophila melanogaster* Toll receptor through sequence comparison by database searches. *Drosophila* Toll receptor was originally identified as a transmembrane protein that is required for induction of antimicrobial peptides, and the mammalian TLRs were also shown to be involved in anti-microbial immunity. The first TLR to be identified was TLR4. Mouse substrains C3H/HeJ and C57BL/10ScCr were known to be hyporesponsive to LPS, and the locus responsible for that was named *Lps*. In 1998, *Lps* locus was identified to be identical to *Tlr4* locus (Beutler and Poltorak, 2001). There have been 13 TLRs (1-10 in humans and 1-9, 11-13 in mice) identified to date. TLR1/2 and TLR2/6 heterodimers, TLR4, TLR5 and TLR10 homodimers are expressed on cell surface, whereas TLR3, TLR7/8 and TLR9 homodimers are usually expressed intracellularly. TLRs can also be divided into groups based on their ligands. TLR1, TLR2, TLR4 and TLR6 recognize lipids and TLR3, TLR7, TLR8 and TLR9 nucleic acids. TLR5 is the only receptor that recognizes a protein (Brikos and O'Neill, 2008). TLR10 is the only orphan receptor in humans, and TLR12 and TLR13 in mice (Brikos and O'Neill, 2008).

TLRs are members of pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs), which are microbial components. Like all the PRR members, TLRs are germline-encoded, nonclonal molecules that are constitutively expressed in the host (Uematsu and Akira, 2008; Kawai and Akira, 2007). TLRs are type I integral member glycoproteins that consist of a cytoplasmic signaling domain, transmembrane domain and extracellular domain. The cytoplasmic domain of

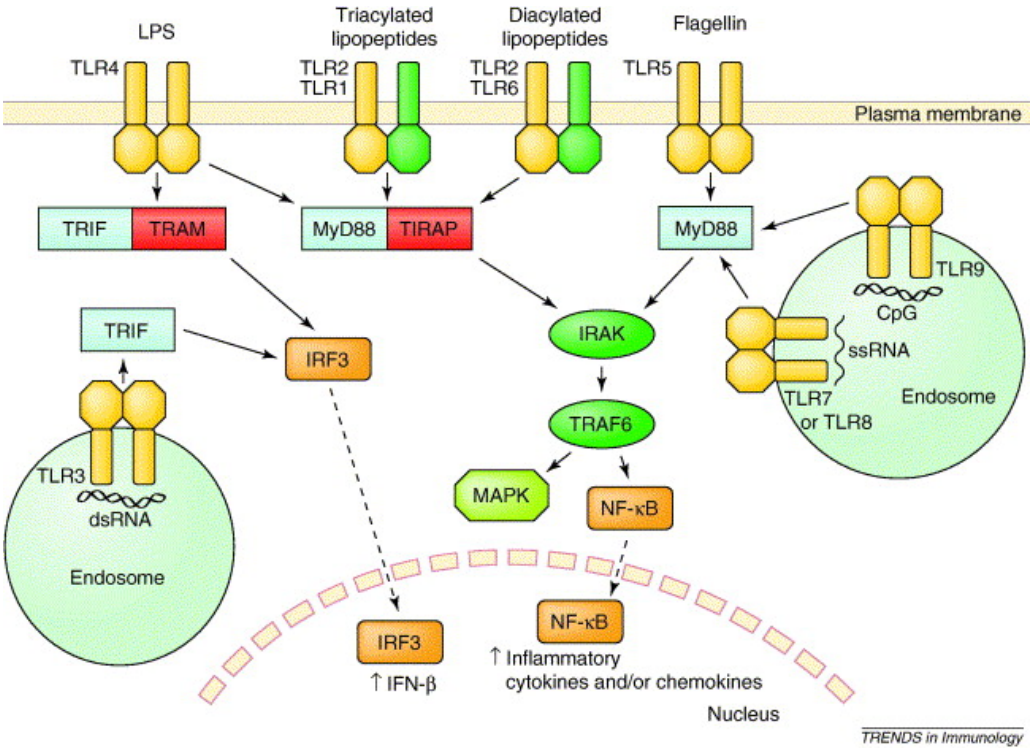
TLRs is homologous to that of IL1 receptors, and hence called the Toll/IL1 receptor (TIR) domain. The TIR and extracellular domains are connected by a single transmembrane helix. The extracellular pathogen-binding ectodomain of TLRs contains 19-25 tandem leucine-rich repeats (LRR), each of which consists of 24-29 amino acid residues. Each LRR consists of β-strands and α-helices connected by loops forming a horseshoe structure. These horseshoe-shaped molecules oligomerize to form homodimers or heterodimers to compose functional TLRs upon binding of ligands (Bell et al., 2005). Upon binding of a ligand to a TLR, it forms a dimer inducing conformational change of itself to recruit adaptor molecules via TIR-TIR domain interaction. Four adaptor molecules involved in TLR signaling have been identified to date: MyD88, TIR-associated protein (TIRAP)/MyD88-adaptor-like (MAL), TIR-domain-containing adaptor protein-inducing IFNβ (TRIF)/TIR-domain-containing molecule 1 (TICAM1) and TRIF-related adaptor molecule (TRAM). There is a fifth adaptor molecule, sterile α- and armadillo-motif containing protein (SARM), but this is a negative regulator of TLR signaling (Watters et al., 2007). In general, there are two distinct pathways of TLR signaling: MyD88-dependent and TRIF-dependent (Figure 4).

The MyD88-dependent pathway downstream of TLR4 or TLR2 activation is analogous to IL1 signaling. When TLR4 is activated upon ligation and dimerization, TIRAP is recruited to the TIR domain of TLR4, which subsequently recruits MyD88. The N-terminal death domain (DD) of MyD88 then recruits IRAK1 and IRAK4, ultimately leading to the activation of NFκB, which results in proinflammatory cytokine production (Watters et al., 2007). TLR5, TLR7, TLR8 and TLR9 activation also utilize MyD88-dependent pathway. In humans, TLR7 and TLR9 expression is limited to pDCs.

Activation of these receptors in pDCs does not result in NFκB activation, but instead, leads to IRF-7 activation and type I IFN production without IRF-3 activation and the positive feedback loop of IFNβ (Hornung et al., 2008; Solis et al., 2006). TLR5 and TLR8 activation in cDCs leads to canonical MyD88 pathway and NFκB activation. The MyD88-dependant pathway via TLR5, TLR7, TLR8 and TLR9 activation does not require recruitment of TIRAP, since macrophages from TIRAP knockout mice function similarly to that of wild type mice (Kawai and Akira, 2007).

MyD88-independent or TRIF-dependent pathway is activated upon TLR3 stimulation. TRIF is recruited to the TIR domain of TLR3, which leads to activation of IRF-3 resulting in type I IFN production as well as recruitment of RIP-1 activating NFκB (Watters et al., 2007; Kawai and Akira, 2007). TLR4 can also signal via a MyD88-independent pathway, since MyD88-deficient mice are capable of activating NFκB in response to LPS. Unlike the TRIF-dependent pathway via TLR3, recruitment of TRIF to activated TLR4 requires TRAM. The TRIF-dependent pathway initiated via TLR4 activation occur subsequently, rather than simultaneously, to MyD88-dependent pathway via TLR4 activation (Kagan et al., 2008). When induction through the MyD88-pathway is complete, TLR4 is endocytosed, and the TRIF-dependent pathway is initiated in the endosome.

Figure 4. Major TLR Signaling Pathways



"All TLRs signal through MyD88, with the exception of TLR3. TLR4 and the TLR2 subfamily (TLR1, TLR2, TLR6) also engage TIRAP. TLR3 signals through TRIF. TRIF is also used in conjunction with TRAM in the TLR4–MyD88-independent pathway. Dashed arrows indicate translocation into the nucleus. LPS, lipopolysaccharide; dsRNA, double-stranded RNA; ssRNA, single-stranded RNA; MAPK, mitogen-activated protein kinases; NF-κB, nuclear factor-κB; IRF3, interferon regulatory factor-3." (van Duin et al., 2006)

B. Bacterial Toll-like Receptor Ligands and Their Receptors

TLR4 recognizes lipopolysaccharide (LPS; a cell wall component of Gram-negative bacteria), lipid A (a component of LPS, endotoxin), core oligosaccharide and O-antigen (Uematsu and Akira, 2008). Although there is no evidence that TLR4 directly binds to its ligands, the recognition of the ligands requires a complex consisting of TLR4, MD2 and CD14 (Uematsu and Akira, 2008; Fitzgerald et al., 2004). First, LPS complexed with host-derived LPS-binding protein (LBP) in serum associates with CD14. CD14 was originally identified as a receptor for LBP-bound LPS. There are two forms of CD14, a GPI-linked membrane-bound form and a soluble form. Soluble CD14 enhances TLR4 recognition of LPS by acting on cells that do not express CD14. CD14 enhances the response to LPS by 1000-fold, and CD14-deficient mice are resistant to LPS stimulation. β2 integrins can also function in place of CD14 in macrophages but only when LPS is aggregated. MD2 is a protein that lacks a transmembrane domain and is either bound to TLR4 in the Golgi before being expressed on the cell surface or secreted as a soluble form by MD2-expressing cells (Fitzgerald et al., 2004; Visintin et al., 2001). The CD14-LBP-LPS complex is brought into close proximity to TLR4-MD2 complex where MD2 can directly bind to LPS via lipid A to initiate TLR4 signaling. Monophosphoryl lipid A (MPL-A) has also shown to bind to MD2 to induce TLR4 signaling. Mata-Haro et al. demonstrated that lipid A induces TLR4 signaling by activating the Mal-MyD88-dependent and TRAM-TRIF pathways, whereas MPL-A only activates the TRAM-TRIF pathway (Mata-Haro et al., 2007). Ohto et al. resolved the crystal structure of MD2 bound to lipid IVa, which is a lipid A precursor with four acyl groups. It clearly shows that lipid IVa binds directly to an oval-shaped MD2 in its hydrophobic cavity. The

authors proposed that lipid A with six to eight acyl chains binds to MD2 to induce a conformational change that results in more efficient TLR4 signaling by providing a platform for adaptor molecules such as Mal, MyD88, TRAM and TRIF. They also demonstrated that, in contrast to the previous predictions, phosphate groups on lipid IVa do not bind to MD2. Therefore, it is proposed that phosphates on lipid A somehow interact with TLR4, and the number of the phosphates interacting with TLR4 determines which pathway(s) will be activated (Ohto et al., 2007) (Figure 5).

TLR2 forms heterodimers with either TLR1 or TLR6, each of which is structurally related to TLR2 (Takeda and Akira, 2005). TLR2 homodimers are not functional in humans, though it is unknown if this applies in mice as well (Farhat et al., 2008). TLR1/TLR2 heterodimers recognize bacterial triacyl lipopeptides, whereas TLR2/TLR6 heterodimers sense diacyl lipopeptides, since macrophages from TLR1-deficient mice failed to produce inflammatory cytokines upon stimulation with triacyl lipopeptides but showed normal responses with diacyl lipopeptides, and macrophages from TLR6-deficient mice exhibited the opposite responses (i.e., production of cytokines upon stimulation with diacyl lipopeptides, but an impaired response with triacyl lipopeptides) (Uematsu and Akira, 2008). TLR2 heterodimers also recognize many other bacterial components including lipoteichoic acid from Gram-positive bacteria, lipoarabinomannan from mycobacteria and a phenol-soluble modulin from *Staphylococcous epidermis* (Takeda and Akira, 2005). Although TLR1/TLR2 and TLR2/TLR6 heterodimers recognize slightly different bacterial components, activation of these receptors reportedly does not result in differential signaling (Farhat et al., 2008).

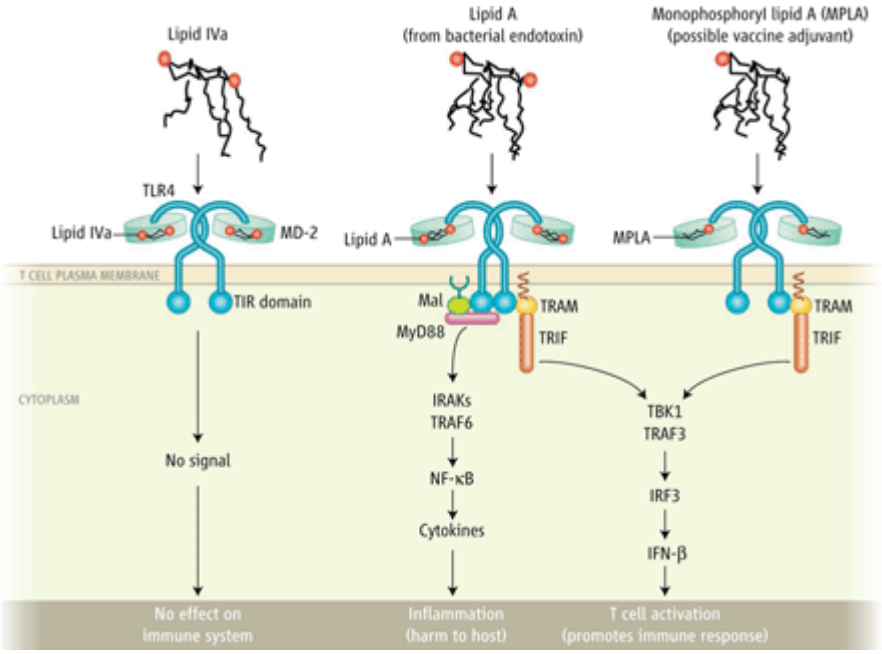
Fungal components can also bind to TLRs. Zymosan, a cell wall component of *Saccharomyces cerevisiae* composed of β-glucan, mannans, proteins and lipids, is recognized by TLR2/TLR6 heterodimers and Dectin-1, another C-type lectin PRR (Dostert and Tschopp, 2007). Mannans from *Cryptococcus neoformans*, *Saccharomyces cerevisiae, Candida albicans* and conidia from *Aspergillus fumigatus* and *C. albicans* have been shown to be recognized by TLR4 (Roeder et al., 2004).

Bacterial flagellin is a single protein that forms the majority of flagella, and it is a ligand for TLR5. Many human pathogenic bacteria, such as *Salmonella, Eshcerichia coli, Campylobactor, Legionella pneumophila, Pseudomonas*, are flagellated (Steiner, 2007). TLR5-expressing lamina propria DCs in mice produce proinflammatory cytokines upon detecting pathogenic bacteria in TLR5-dependent manner. In addition, TLR5 polymorphism is associated with susceptibility to Legionnaires' disease caused by *Legionella pheumophila* (Uematsu and Akira, 2008).

Unmethylated CpG DNA is another bacterial TLR ligand and is recognized by TLR9. CpG DNA motifs are found in bacteria and vertebrates. However, bacterial CpG DNA is found more frequently and in unmethylated form, whereas the frequency of CpG DNA in vertebrates is reduced and cytosines of the vertebrate CpG motifs are highly methylated (Uematsu and Akira, 2008). Under a normal condition, mammalian CpG DNA is not available for TLR9 signaling. However, defective removal of apoptotic cells may lead to the release of host CpG DNA potentially causing autoimmune responses (Pasare and Medzhitov, 2003).

Uropathogenic bacteria probably possess a ligand for TLR11 since TLR11-deficient mice are susceptible to this infection (Uematsu and Akira, 2008). Functional TLR11 is not expressed in humans, since the human *Tlr11* gene contains a stop codon.

Figure 5. Distinct TLR Signaling Pathways Activated by Lipid A and Its Analogs



"The TLR4-MD-2 complex is expressed as dimers on the surface of immune cells (T and B cells, phagocytes). Lipid A and two of its analogs, lipid IVa and monophosphoryl lipid A, bind to MD-2. There is no direct evidence that these lipids bind TLR4. The specific engagement of each lipid may cause structural changes in MD-2 and TLR4, resulting in new protein binding sites in the cytoplasmic domain of TLR4. The resulting recruitment of specific adapter proteins activates distinct signaling pathways and associated cellular responses." (Fitzgerald and Golenbock, 2007).

C. Viral Toll-like Receptor Ligands and Their Receptors

TLR3 senses double-stranded (ds)RNA, which is a marker for viral infection since dsRNA is generated only during replication of ssRNA and DNA viruses. The prototypical TLR3 ligand used is synthetic dsRNA, polyinosine-polycytidylic acid (poly I:C) (Ohto et al., 2007). TLR3 is unique among all the TLRs, since its signaling does not utilize the adaptive molecule MyD88. Despite the prediction that TLR3-deficient mice are more susceptible to viral infections, Michael Oldstone and colleagues. demonstrated that these mice responded to infections with murine cytomegalovirus, vesicular stomatitis virus, lymphochorinomeningitis virus and reovirus similarly to the wild type (Edelmann et al., 2004). In conventional DCs, additional receptors for dsRNA, retinoic acid-inducible gene 1 (RIG-1) and melanoma differentiation-associated gene-5 (Mda-5), both of which are cytosolic receptors, were identified (Takeda and Akira, 2005). Recognition of dsRNA by either RIG-1 or Mda-5 leads to activation of both IRF-3 and IRF-7, inducing proinflammatory cytokine and type I IFN production (Kawai and Akira, 2007).

TLR9 also recognizes viral CpG DNA. In humans, TLR9 is predominantly expressed in pDCs. TLR9 stimulation of pDCs induce anti-viral type I IFN production.

Single-stranded (ss)RNA derived from RNA viruses, such as human immunodeficiency virus and influenza virus, is recognized by TLR7 and TLR8. Both TLR7 and TLR8 are expressed from genes in close proximity on the X chromosome and share 60% homology to each other at the amino acid level in humans (Du et al., 2000). Therefore, they are structurally related and can recognize the same compound independently. However, each can also recognize TLR7- or TLR8-specific agonists (Gorden et al., 2005). It was originally postulated that murine TLR8 is nonfunctional,

since murine TLR8-expressing cells failed to respond to stimulation with ssRNA. However, a more recent study demonstrated that murine TLR8 was activated with a combination of imidazoquinoline and poly T oligodeoxynucleotides (Hornung et al., 2008). Although ssRNA can also be found in host cells, TLR7 and TLR8 do not respond to the host ssRNA under a normal circumstances due to the fact that TLR7 and TLR8 are expressed in the endosomes.

4. Cytokines

A. Interleukin 6 Family Cytokines

i. Interleukin 6

Interleukin 6 (IL6) is a monomeric cytokine, originally identified as a B cell differentiation factor, but it is now known to act on many cells and has pleiotropic functions, including T cell differentiation, maturation of macrophages and acute-phase protein synthesis by hepatocytes (Kishimoto et al., 1995). IL6 binds to its cognate receptor, the IL6 receptor α chain (IL6Rα), and this leads to dimerization of two signal-transducing glycoprotein gp130 (Figure 6). The fully assembled IL6-IL6R complex consists of two IL6 molecules, two IL6Rα chains and two gp130 molecules (Paonessa et al., 1995). IL6 also binds to soluble IL6 receptor (sIL6R), and this IL6-sIL6R complex subsequently binds to membrane-bound gp130 to trigger cellular responses through activation of STAT1 and STAT3 via "IL6-*trans*-signaling". This sIL6R is generated as a result of alternative splicing of IL6Rα mRNA. Likewise, soluble gp130 is also produced, and it binds to the IL6-sIL6Rα complex to suppress the IL6-*trans*-signaling by

sequestering it from the membrane-bound gp130. Therefore, there are multiple ways to control IL6 signaling (Jones, 2005).

ii. Interleukin 12

Interleukin 12 (IL12) is the prototype of heterodimeric members of IL6 family, and it is a heterodimer consisting of IL12p35 subunit, a homolog of IL6, and IL12p40 subunit, a homolog of sIL6Rα (Figure 6). Therefore, IL12 is also referred to as IL12p70, and it is not secreted unless IL12p35 is expressed simultaneously with IL12p40 to form the disulfide-linked heterodimer, whereas IL12p40 is secreted as either a homodimer or a monomer (Kastelein et al., 2007). However, IL12p40 homodimer production has been observed in mice but not in humans, and the human IL12p40 homodimer produced by transfected cells binds to the human IL12 receptor with lower affinity. Therefore, IL12p40 homodimer is not a physiological/natural IL12 receptor antagonist in humans (Trinchieri, 2003). IL12p35 mRNA is found in many cell types, including T cells that do not produce IL12p70, whereas IL12p40 is produced by cells that also produce bioactive IL12p70 (Kastelein et al., 2007; Trinchieri, 2003). IL12 is produced by monocytes, macrophages, DCs, neutrophils and B cells (Paunovic et al., 2008). IL12 produced by these cells acts on T cells to induce their differentiation into Th1 cells and IFNγ production. IL12p70 can be produced by APCs upon stimulation with TLR ligands, and subsequent CD40L-CD40 interaction between the APCs and T cells. IL12p70 signals via IL12 receptor (IL12R) that consists of IL12 receptor β1 chain (IL12Rβ1) and β2 chain (IL12Rβ2), which are both related to gp130. IL12R is primarily expressed on T cells and natural killer (NK) cells (Kastelein et al., 2007). IL12Rβ1 and IL12Rβ2 expression is maintained by T cell activation and IFNγ stimulation, and downregulated by IL4

stimulation. IL12Rβ1 has no signaling functions, but is required for optimal binding of IL12p70 to the high affinity IL12R, whereas IL12Rβ2 functions as the signal transducer of IL12 signaling, through which STAT4 is activated resulting in Th1 differentiation (Trinchieri, 2003; Paunovic et al., 2008).

iii. Interleukin 23

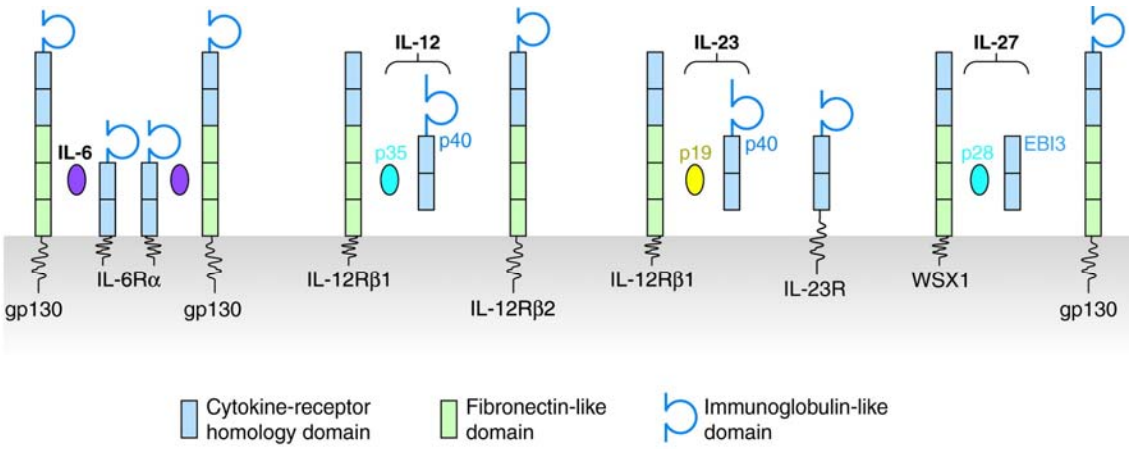
Interleukin 23 (IL23) is also a heterodimer member of the IL6 family, and it shares IL12p40, which is covalently linked to the IL23p19 subunit, a homolog of IL12p35 and IL6 (Figure 6). Similar to IL12, IL23 is secreted as disulfide-linked heterodimer by activated DCs and other APCs. IL23 receptor complex also shares IL12Rβ1 with IL12R, but uses a unique IL23R subunit, which is a homolog of gp130. IL23R is predominantly expressed on activated/memory T cells, which correlates with the fact that IL23 is not involved in T cell differentiation as initially thought, but instead acts on Th17 cells as a maintenance/expansion factor for this population. IL23R is also expressed on T cell clones, NK cell clones, as well as on monocytes, macrophages and DCs at low levels (Kastelein et al., 2007). IL23 signaling activates STAT3 and STAT4 (Kastelein et al., 2007; Paunovic et al., 2008).

iv. Interleukin 27

Another heterodimeric member of IL6 family is IL27. IL27 consists of IL27p28 subunit, a homolog of IL6, and Epstein-Barr virus-induced molecule 3 (EBI3), a homolog of sIL6R and IL12p40 (Figure 6). IL27 is secreted upon coexpression of both subunits similar to the other heterodimeric members of the family, and the receptor consists of gp130 and a homolog of IL12Rβ1, WSX-1/TCCR, an orphan type I cytokine receptor. This receptor is mainly expressed on lymphocytes and NK cells with high expression

levels on effector and memory T cells (Kastelein et al., 2007; Paunovic et al., 2008). IL27 signaling leads to the activation of STAT1, STAT2, STAT3, STAT4 and STAT5. As described above, IL27 was shown to suppress IL17 production by TGFβ plus IL6-induced Th17 cells mediated by IL10 production by these cells (Batten et al., 2006). In addition, IL27 induces Th1 differentiation in naïve T cells, since WSX-1-deficient mice exhibit decreased level of IFNγ production resulting in increased susceptibility to *Listeria monocytogenes* and increased IL4 production (Paunovic et al., 2008).

Figure 6. IL6 Family Cytokines and Their Receptors



Kastelein RA, et al. 2007.
Annu. Rev. Immunol. 25:221–42

IL6 binds to IL6 receptor complex composed of IL6Rα chains and gp130. IL12p35 subunit, IL23p19 subunit and IL27p28 subunit are homologous to IL6, whereas IL12p40 subunit and EBI3 are homologous to soluble IL6Rα.

B. Interleukin 1 Family Cytokines

i. Interleukin 1

IL1 family consists of 11 members (IL1α, ILβ, IL18/IL1γ, IL33, IL1Ra, IL1F5/IL1δ, IL1F6/IL1ε, IL1F7/IL1ζ, IL1F8/IL1η, IL1F9, and IL1F10). The last six members have no known functions to date (Blanco et al., 2008). Two forms of the prototypic member of the family, IL1α and IL1β, are derived from separate genes, but their functions are indistinguishable (Dinarello, 1997). Interleukin 1α (IL1α) is a pro-inflammatory cytokine that is primarily expressed by many cell types including monocytes, macrophages, keratinocytes. IL1α is not usually found in circulation or body fluids of healthy individuals. ProIL1α is bioactive and bound to the cell membrane. Upon cell death, proIL1α is cleaved by an extracellular protease, calpain, and released as soluble mature IL1α. ProIL1β is found in the cytoplasm and not bioactive until cleaved by caspase-1. Mature, bioactive IL1β is secreted by many cell types, but predominantly by monocytes and macrophages (Bird et al., 2002; Blanco et al., 2008; Dinarello, 1997; Dinarello, 1996). IL1α and IL1β share homology of less than 26%, but share structural similarities (Dinarello, 1996). The activities of IL1 are regulated by soluble IL1 receptor (Type II IL1R, IL1RII) and IL1 receptor antagonist (IL1Ra). The biological effects of IL1 include fever, upregulation of cytokines such as IL2, IL4, IL10 and IL12, and increased expression of ICAM-1 and VCAM-1. IL1 binds to the receptor complex consisting of type I IL1 receptor (IL1RI) and IL1 receptor accessory protein (IL1RAcP). Upon ligation with IL1, IL1R signals to activate STAT3, JNK, ERK1/2, MAPKp38 and NF-κB (Schmitz et al., 2005).

ii. IL1 Receptor Antagonist

IL1Ra also shares structural similarities with IL1α and IL1β, but it is a naturally-occurring antagonist of IL1 signaling. IL1Ra is produced by the same cells that produce IL1. For example, monocytes can produce IL1Ra upon stimulation with LPS, and keratinocytes and epithelial cells produce it to prevent intracellular proIL1α from binding to nuclear DNA. It has no agonist function even when it was injected into humans at a very high dosage. IL1Ra binds to IL1RI at one binding site, whereas agonists such as IL1α and IL1β bind to the receptor at two binding sites allowing the conformational change in the receptor, which is important for the formation of heterodimer of IL1RI and IL1RAcP. Since IL1Ra binds to IL1RI at one site, the receptor does not undergo a conformational change and fails to form the heterodimer receptor complex, which starts the IL1-signaling cascade (Dinarello, 1997; Dinarello, 1996).

C. IL17 Family Cytokines

IL17 was originally named as CTLA8 as it was isolated from murine cytotoxic T lymphocyte (CTL) cDNA library. Now the IL17 family consists of six members (IL17A-F), and they do not share any sequence homology with other known mammalian proteins. IL17A (IL17) and IL17F are predominantly produced by $CD4^+$ activated/memory cells, but also by CD8+ T cells, γδ T cells, NK cells and neutrophils (Weaver et al., 2007; Kolls and Linden, 2004). Their genes are located in close proximity (46,050 base pairs away) on the same chromosome, and IL17A and IL17F share the highest homology in the family (50%) (Kolls and Linden, 2004). IL17B is expressed in the pancreas, small intestine, stomach and spinal cord. IL17C is expressed in testes, thymus, spleen and prostate. IL17D is expressed in the skeletal muscle, neuronal cells, prostate and resting

$CD4^+$ T cells. IL17E, also named IL25, is the least related to IL17A (16%) and is expressed in the brains, lung, testis and prostate. It is produced by eosinophils and mast cells, as well as Th2 cells at least in mice. Relatively little is known about functions of IL17 family cytokines. IL17A and IL17F induce IL6 and IL8 production by fibroblasts, and recruitment and activation of neutrophils. They also act on many types of cells and induce GM-CSF, G-CSF, and chemokines such as CXCL8 and CXCL1 as mentioned above (Weaver et al., 2007; Ouyang et al., 2008; Kolls and Linden, 2004). Although IL17A and IL17F seem to share many functions, they were also shown to have distinct effects in disease models. IL17A-deficient mice were resistant to EAE, exhibited a reduced Th2 response in an asthma model, and developed severe dextran sulfate sodium-mediated colitis, whereas IL17F-deficient mice developed EAE, exhibited more severe asthma, but reduced colitis induced by dextran sulfate sodium (Yang et al., 2008b). IL17B and IL17C are potent activators of TNFα and IL1β. The function of IL17D is unknown. IL17E induces CCL5 and CCL11 expression and Th2 cytokines such as IL5, IL13 and IL4, implicating its involvement in allergy. In addition, IL17E-deficient mice develop more severe EAE indicating IL17E may have opposing functions from IL17A (Kleinschek et al., 2007).

IL17 receptors have yet to be studied in detail. There are five known members in IL17 receptor family. IL17R (IL17RA) binds to both IL17A and IL17F, with higher affinity to IL17A, whereas IL17RB binds to IL17B and IL17E. IL17RC has been shown to be essential for response to IL17F homodimers (Ouyang et al., 2008). The functions of IL17RD and IL17RE are not yet known (Weaver et al., 2007; Kolls and Linden, 2004). IL17RA is expressed ubiquitously and binds to IL17A and IL17F with a low affinity

(Toy et al., 2006). Therefore, it is likely that IL17RA requires a coreceptor. One report has demonstrated that IL17RA forms a multimer in the plasma membrane (Kramer et al., 2006), but another report has shown that IL17RC participates in IL17 receptor signaling with IL17RA and possibly forms a heterodimeric IL17 receptor complex (Toy et al., 2006), which may determine the tissue specificity of functional IL17 receptor complex expression. Therefore, it appears that both IL17RA and IL17RC are required to form functional receptors for IL17A and IL17F homodimers and heterodimers (Ouyang et al., 2008).

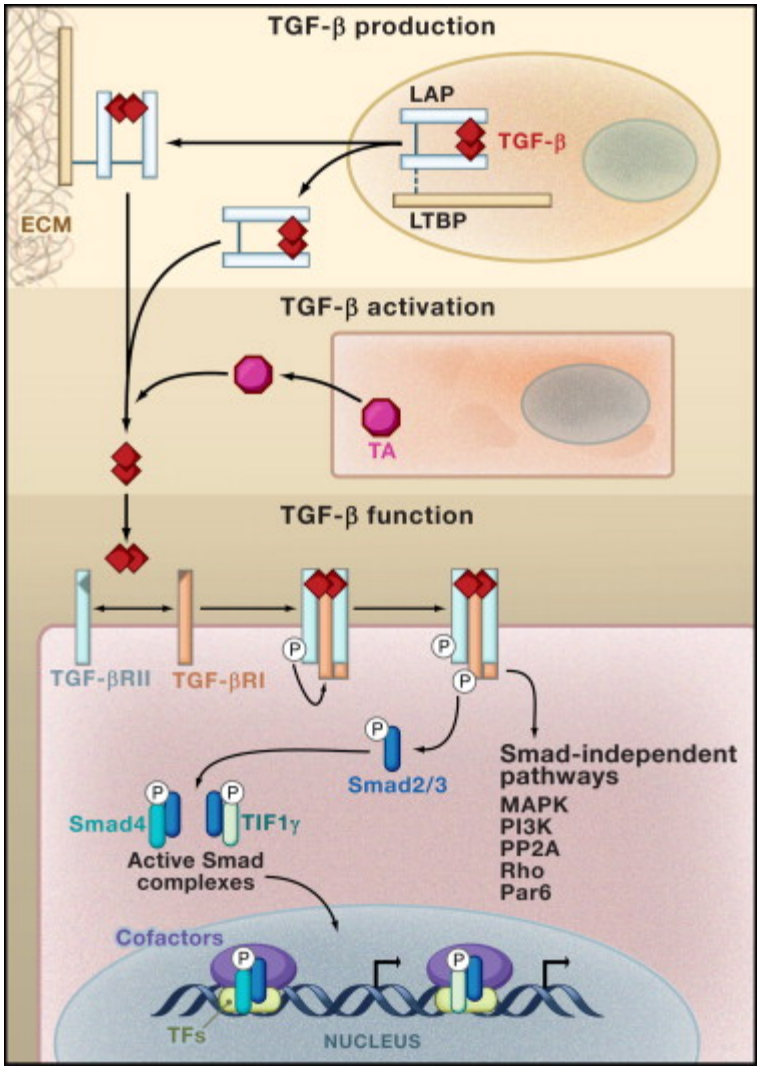
D. Transforming Growth Factor β

There are three isoforms of transforming growth factor β (TGFβ1, TGFβ2 and TGFβ3) encoded by different genes in mammals (Li et al., 2006b). TGFβ is produced by many tissue cells including epithelial, endothelial, hematopoietic, neuronal and connective tissue cells (Blobe et al., 2000). Although all three isoforms have similar properties *in vitro*, TGFβ1 is expressed predominantly in immune cells and plays the major role in TGFβ signaling (Li et al., 2006a; Li et al., 2006b). TGFβ is first synthesized as pre-proTGFβ precursor (Figure 7). Pre-proTGFβ contains a signal peptide, and proTGFβ is processed in the Golgi, cleaving the N-terminus of the propeptide to generate a homodimer of protein called latency-associated protein (LAP) noncovalently bound to a homodimer of mature TGFβ. LAP-bound TGFβ may be secreted in the latent form, or it can also be associated with latent-TGFβ-binding protein (LTBP) to be targeted to the extracellular matrix. Latent TGFβ needs to be activated by LAP proteolysis or a conformational change cleaving the LAP-LTBP complex (Li et al., 2006a; Li et al., 2006b). TGFβ signals through a tetrameric receptor complex consisting of two TGFβ

receptor type I molecules (TGFβRI) and two TGFβ receptor type II molecules (TGFβRII) (Li et al., 2006a; Li et al., 2006b). TGFβRII homodimer binds to the activated TGFβ and associates with the TGFβRI homodimer, which is necessary for subsequent signaling in Smad-dependent pathway to regulate target genes and Smad-independent pathway with no known functions in immunity (Li et al., 2006b). TGFβ is involved in CD8+ T cell, natural T regulatory and NKT cell development in the thymus, survival of low affinity T cells in the periphery, inhibition of high-affinity self-reactive T cells via peripheral tolerance, and maintenance of natural T regulatory cells. It is also involved in differentiation of induced T regulatory cells and Th17 cells (Li et al., 2006b). TGFβ induces expression of both transcription factors Foxp3 and RORγt (Ivanov et al., 2006). With expression of Foxp3, RORγt-expressing cells retain immunoregulatory function since Foxp3 suppresses RORγt activities (Lochner et al., 2008; Ivanov et al., 2006). However, in the presence of IL6, Foxp3 is suppressed allowing RORγt to function as Th17-sepcific transcription factor and induce Th17 differentiation (Ivanov et al., 2006).

Figure 7. TGFβ Signaling

"TGF-β is synthesized in an inactive form composed of a TGF-β dimer in association with the latency-associated protein (LAP). This latent TGF-β molecule can be secreted as such, or can form a complex with latent-TGF-β-binding protein (LTBP) that mediates its deposition to the extracellular matrix (ECM). TGF-β becomes activated after the engagement of a TGF-β activator (TA) that triggers LAP degradation or alters LAP's conformation in response to environmental cues. Active TGF-β binds to a tetrameric complex composed of TGF-β receptor II (TGF-βRII) and TGF-β receptor I (TGF-βRI) and initiates signaling pathways that are dependent on the kinase activity of the receptors. Activated TGF-βRI phosphorylates the transcription factors Smad2 and Smad3, triggering their translocation into the nucleus in complex with the proteins Smad4 or TIF1γ. Smad complexes in association with additional transcription factors (TFs) bind to the regulatory sequences in target genes and regulate gene expression by recruiting transcription cofactors. In addition, TGF-β activates Smad-independent pathways such as those mediated by mitogen-activated protein kinase (MAPK), PI3K kinase, PP2A phosphatase, Rho family proteins, and the epithelial polarity protein Par6, which trigger different cell type-specific responses." (Li and Flavell, 2008)


TGF-β production
LAP
TGF-β
LTBP
ECM
TGF-β activation
TA
TGF-β function
TGF-βRII
TGF-βRI
P
Smad2/3
Smad-independent pathways
MAPK
PI3K
PP2A
Rho
Par6
Smad4
TIF1γ
Active Smad complexes
Cofactors
TFs
NUCLEUS

CHAPTER II

Materials and Methods

1. Preparation of Human Primary Cells

A. Generation of Immature Monocyte-derived Dendritic Cells

Peripheral blood obtained from human donors was used in compliance with a protocol approved by the University of Missouri Health Sciences Institutional Review Board. HLA class II alleles of the human donors are listed in Table 2. Peripheral blood mononuclear cells (PBMCs) were isolated from 90 ml of peripheral blood of healthy human donors by density centrifugation on Histopaque-1077 (polysucrose and sodium diatrizoate; Sigma, St. Louis, MO). Untouched monocytes were isolated from the PBMCs by depleting non-monocytes using magnetic beads (Monocyte Isolation Kit II, Miltenyi Biotec, Aurora, CA) according to the manufacturer's protocol. Magnetic bead-treated cell suspensions were separated using an AutoMACS cell separator (Miltenyi Biotec). Resulting monocytes were cultured at 6 $x10^6$ cells/3 ml/well in 6-well plates in complete culture medium [RPMI 1640 (Gibco, Grand Island, NY), 10% fetal bovine serum (FBS, US Bio-Technologies, Parkerford, PA), 25mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), 2 mM L-glutamine, 100 U/ml penicillin/streptomycin, and 0.5 mM sodium pyruvate (Gibco)] at 37°C in 5% CO_2. 1000 U/ml recombinant human (rh)GM-CSF (Leukine sargramostim; Berlex Laboratories, Richmond, CA) and 500 U/ml rhIL4 (PeproTech, Rocky Hill, NJ) were also added to the culture to allow the monocytes to differentiate into immature monocyte-derived dendritic cells (moDCs), similar to previously developed protocols (Romani et al., 1994; Bender et

al., 1996). On day 3, the cells were refed by adding 1 ml/well of fresh complete medium containing 1000 U/ml rhGM-CSF and 500 U/ml rhIL4, and the resulting immature moDCs were harvested on day 5. The purity of the DCs was approximately 95% based on flow cytometric analysis (data not shown).

B. Isolation of Naive $CD4^+$ T Cells

PBMCs were isolated from 120 ml of peripheral blood of healthy human donors by density centrifugation on Histopaque-1077. Naïve $CD4^+$ T cells were isolated from the PBMCs by magnetic beads (Naïve $CD4^+$ T cell Isolation Kit, Miltenyi Biotec), which depleted non-$CD4^+$ T cells (i.e., $CD8^+$ T cells, B cells, NK cells, γ/δ T cells, monocytes, DCs, granulocytes, platelets, and erythroid cells) as well as $CD4^+CD45RO^+$ (activated/memory) T cells according to the manufacturer's protocol using an AutoMACS cell separator. The purity of naïve $CD4^+CD45RA^+$ cells was approximately 90% as determined by surface staining (data not shown).

C. Depletion of $CD25^+$ T Cells

In some experiments, $CD25^+$ cells were depleted subsequently to naïve $CD4^+$ T cell isolation using a $CD25^+$ cell isolation kit (CD25 MicroBeads II, Miltenyi Biotec). The size of $CD25^+$ T cell population after the depletion was reduced by approximately 90% as determined by surface staining (data not shown).

Table 2. List of Human Donors and HLA Class II Alleles

Donor	HLA Class II Alleles (DR and DQ only)
6	DR7, 13, 52, 53; DQ2, 6
90	DR11, 13, 52; DQ6, 7
99	DR9, 15, 51, 52; DQ3, 6
101	DR1, 15, 51; DQ5, 6
103	NT
104	DR4, 14, 52, 53; DQ3, 5
108	DR11, 15, 51, 52; DQ6, 7
109	DR4, 8, 53; DQ3, 6
110	DR13, 52; DQ6
112	DR1, 8; DQ5
116	DR4, 7, 53; DQ8, 9
118	DR11, 52; DQ7
119	DR1, 7, 53; DQ2, 5
121	DR13, 52; DQ6
122	DR1, 13, 52; DQ5, 6

2. Cell Activation

A. Activation of Monocyte-derived Dendritic Cells

Immature moDCs were plated at 3 x 10^5 cells/ml/well in 24-well plates and left unstimulated or activated with different TLR ligands: 100 ng/ml LPS (*S. minnesota* Re 595; Calbiochem, San Diego, CA or Ultra pure *S. Minnesota* LPS; Invivogen, San Diego, CA), 100 μg/ml poly I:C (Amersham Biosciences, Piscataway, NJ), 2.5 μg/ml ssRNA40 packaged in liposomes (Invivogen), 0.5 μg/ml flagellin (*S. typhimurium*; Invivogen), 1 μg/ml Pam3CSK4 (Invivogen), or 1 μg/ml MPL-A (Invivogen) at 37°C in 5% CO_2 for 24 hours in either complete culture medium or X-VIVO15 serum-free medium (Lonza, Walkersville, MD) for experiments described in chapters III and IV, respectively, to generate activated/mature moDCs. Supernatants from these cultures were collected and analyzed by enzyme-linked immunosorbent assay (ELISA) for cytokine production by the activated moDCs as described below. For DC/T cell cocultures, moDCs were activated at 5-7.5 x 10^4 cells/ 0.5 ml/well so that the cultures were set up at a 1:10 (DC:T cell) ratio when naïve $CD4^+$ T cells were added at 5-7.5 x 10^5 cells/ 0.5 ml/well after 24 hours as described below.

B. Activation of Naïve $CD4^+$ T Cells in Dendritic Cell/T Cell Cocultures

Immature moDCs were left unstimulated or activated with different TLR ligands at 37°C in 5% CO_2 for 24 hours at 5 x 10^4 cells/0.5 ml/well and 7.5 x 10^4 cells/0.5 ml/well for cocultures set up for IL17 and IL22 ELISA, and IFNγ, IL5 and IL17 ELISPOT, respectively, as described above. Naïve $CD4^+$ T cells were added at a 1:10 (DC:T cell) ratio (i.e., 5 x 10^5 cells/0.5 ml/well and 7.5 x 10^5 cells/0.5 ml/well, respectively) and cultured at 37°C in 5% CO_2 for 5 + 7 days for IFNγ and IL5 ELISPOT and 7 days for

IL17 enzyme-linked immunosorbent spot (ELISPOT), IL17 and IL22 ELISA. Resulting primed $CD4^+$ T cells were restimulated, and supernatants from these cultures were collected to be analyzed by ELISA for cytokine production by the differentiated $CD4^+$ T cells. The restimulated $CD4^+$ T cells were also harvested and analyzed by ELISPOT for the frequency of IFNγ-, IL5- or IL17-produing cells as described in details below.

C. Activation of Naïve $CD4^+$ T Cells with Plate-bound Anti-CD3 and Soluble Anti-CD28

CD25-depleted naïve $CD4^+$ T cells were plated at 2.5 x 10^5 cells/ml/well and activated with 5 μg/ml plate-bound anti-CD3 (OKT3, eBioscience, San Diego, CA; 24-well plates were coated with anti-CD3 antibody in PBS at 37°C in 5% CO_2 for 2 hours and washed twice with PBS) and 2.5 μg/ml soluble anti-CD28 (CD28.2, eBioscience) in X-VIVO15 serum-free medium at 37°C in 5% CO_2 for 7 days. The resulting activated $CD4^+$ T cells were restimulated and analyzed by IL17 ELISPOT for the frequency of IL17-producing cells as described in detailed in below.

3. Measurement of mRNA Upregulation

A. Isolation of Total RNA from Monocyte-derived Dendritic Cells

Total RNA from moDCs was isolated using RNAqueous-4PCR RNA isolation kit (Ambion, Austin, TX) according to manufacturer's protocol. Briefly, moDCs were left unstimulated or activated with different TLR ligands at 37°C in 5% CO_2 for 6 hours before being harvested, washed once in PBS and pelleted in RNase-free 1.5 ml eppendorf tubes (Fisher Scientific, Waltham, MA). The cells were mixed with 500 μl lysis/binding buffer (guanidinium thiocyanate) followed by addition of 500 μl 64% ethanol. The

mixtures were put through filters assembled onto collection tubes by centrifugation and the flow-throughs were discarded. The filters were washed once with 700 µl wash buffer I and twice with 500 µl wash buffer II/III. They were then transferred to fresh collection tubes and RNA was eluted from the filters by applying total of 90 µl pre-heated elution buffer (0.1 mM EDTA) directly onto the filter followed by centrifugation. Finally, the eluates were treated with DNase (TURBO DNA-free DNase treatment kit; Ambion) to remove contaminating residual DNA. Briefly, 20 µl 10X TURBO DNase buffer and 2 µl TURBO DNase were added to the eluates and incubated for 30 minutes at 37˚C in a water bath. 22 µl DNase inactivation reagent was then added to the mixture, which was incubated for 2 minutes at room temperature and centrifuged for 1 minute. Supernatants containing RNA were recovered and stored at -80˚C until use.

B. Quantification of Total RNA

Total RNA isolated from moDCs was quantified using RiboGreen RNA quantification kit (Molecular Probes, Eugene, OR). Briefly, 4.7 µl RNA samples were diluted in 345.3 µl 1X TE buffer (10 mM Tris-HCl and1 mM EDTA). Either 100 µl of the diluted RNA samples or ribosomal RNA standard, and 100 µl of 200-fold diluted RiboGreen reagent were added to each well (100 µl total/well) in triplicates on 96-well black polystyrene flat-bottom plates (Corning, Lowell, MA) achieving 1:150 final dilution of the RNA samples. Fluorescence was measured with a 485-nm excitation filter and a 535-nm emission filter on a Fusion Universal Microplate Reader (Perkin-Elmer, Wellesley, MA). The original RNA concentration was determined by the standard curve generated with the standard and multiplying the measured RNA concentration by 150.

C. cDNA Synthesis

cDNA samples were generated using 1st Strand cDNA Synthesis kit (Roche, Indianapolis, IN). Briefly, RNA samples were denatured by heating them at 65˚C for 15 minutes on a heat block (Fisher Scientific) and then cooling on ice for 5 minutes. Then 100 ng denatured RNA sample, 2 µl 10X reaction buffer, 4 µl of 25 mM $MgCl_2$, 2 µl deoxynucleotide mix, 2 µl random hexamers, 1 µl RNase inhibitor, 0.8 µl AMV reverse transcriptase were mixed in thin-walled PCR tubes (Fisher Scientific). Negative control reactions were prepared the same way without the reverse transcriptase. The total amount of the mixtures was then adjusted to be 20 µl with nuclease-free water. The reactions were incubated at 25˚C for 10 minutes (primer annealing), 42˚C for 60 minutes (cDNA synthesis), 99˚C for 5 minutes (denaturing RT) and 4˚C for 5 minutes (cooling) using a GeneAmp 2400 thermal cycler (Perkin-Elmer).

D. Real-time RT-PCR

Real-time RT-PCR was performed using the cDNA synthesized as described above. Briefly, 10.25 µl nuclease-free water, 12.5 µl TaqMan Universal PCR Master Mix), 1.25 µl Assays-on-Demand primer/probe sets (IFNβ: Hs00277188-s1, IL12p35: Hs00168405-m1, IL12p40: Hs00233688-m1, IL23p19: Hs00372324-m1, IL27p28: Hs00377366-m1, EBI3: Hs00194957-m1; Applied Biosystems, Foster City, CA) and 1 µl cDNA were mixed in each well on MicroAmp optical 96-well reaction plates, which were sealed with optical adhesive covers (Applied Biosystems). Real-time assays were run on an ABI Prism 7000 Sequence Detection System (Applied Biosystems) using default PCR thermal cycling conditions. Endogenous control was assayed by using primer/probe set for human β-glucoronidase (GUSB: Hs99999908-m1; Applied Biosystems). Relative

expression was calculated using a formula RE = $2^{-\Delta\Delta CT}$ where ΔCT is CT (target) – mean of CT (GUSB) and ΔΔCT is ΔCT – mean of CT (unstimulated).

4. Flow Cytometry

A. Surface Staining of Monocyte-derived Dendritic Cells

TLR ligand-activated moDCs were harvested and plated at 2-5 x 10^5 cells/well in 96-well plates and blocked in 100 µl/well 50% human serum (Cambrex, Walkersville, MD) in PBS at 4˚C. After 15 minutes, the cells were washed twice with staining buffer (PBS, 1% FBS, 0.1% sodium azide). The cells were then stained with fluorochrome-conjugated mAb (1 µg/1 x 10^6 cells) at 4˚C for 30 minutes. After being washed twice in the staining buffer, the cells were fixed with 100 µl Cytofix (4% paraformaldehyde; BD Biosciences, Franklin Lakes, NJ) at 4˚C for 30 minutes, washed twice, resuspended in 300 µl staining buffer and analyzed by flow cytometry using a FACScan analyzer (Becton Dickinson, Franklin Lakes, NJ). Antibodies used were mouse FITC-α human CD40 (5C3, eBiosciences), mouse FITC-α human CD80 (2D10.4, eBioscience), mouse FITC-α human CD86 (2331/FUN-1, BD Biosciences), mouse FITC-α HLA-DR (G46-6, BD Biosciences), mouse FITC-IgG1 (eBioscience) and mouse FITC-IgG2a (BD Biosciences).

B. Intracellular Staining of Monocyte-derived Dendritic Cells

Immature moDCs were plated, blocked and washed as described in the surface staining protocol above. The cells were first permeablized with 100 µl Cytofix/Cytoperm (saponin buffer with paraformaldehyde) for 20 minutes at 4˚C and washed twice in 1X Perm Wash buffer (FCS and saponin; BD Biosciences). Permeablized cells were then

resuspended in 50 µl Perm Wash buffer, stained with mouse PE-α human TLR3 (TLR3.7, eBioscience) or an isotype control (mouse PE-IgG1; eBioscience) (1 µg/1 x 10^6 cells) at 4˚C for 30 minutes, washed twice in 1X Perm Wash buffer, resuspended in 300 µl staining buffer and analyzed by flow cytometry.

5. Measurement of Cytokine Production

A. Cytokine Production by Monocyte-derived Dendritic Cells

Supernatants from the cultures of unstimulated or TLR ligand-activated moDCs were collected at 24 hours post-activation, and cytokine concentrations for TNFα, IL6, IL10, IL12p40 and IL12p70 were determined by ELISA using paired capture and detection Ab sets (human TNFα, IL6, IL10, IL12p40 and IL12p70 ELISA DuoSets; R&D Systems, Minneapolis, MN) according to manufacturer's protocols. Briefly, 96-well EIA/RIA polystyrene flat-bottom high-binding plates (Corning Inc., Lowell, MA) were coated with 100 µl of diluted capture Ab at recommended concentration in PBS, sealed with adhesive plate sealers (R&D Systems) and incubated overnight at room temperature. The plates were washed three times with wash buffer (PBS, 0.05% Tween20; Fisher Biotech, Fair Lawn, NJ), blocked with 200 µl /well of dilution buffer (PBS, 1% BSA) and incubated at room temperature for 2 hours. After washing the plates three times, either standards diluted in the complete medium or the culture supernatants were added at 100 µl/well in duplicates or triplicates, and the plates were incubated at room temperature for 2 hours. After the plates were washed three times, diluted detection (biotinylated) Ab at the recommended concentration in dilution buffer was added at 100 µl/well and incubated at room temperature for 2 hours. The plates were then washed again three times, and

streptavidin-horseradish peroxidase (SA-HRP) diluted at 1:200 in the dilution buffer was added at 100 µl/well and incubated at room temperature for 20 minutes. After the plates were washed three times, TMB Blue substrate (hydrogen peroxide and 3,3',5,5'-tetramethyl-benzidine in an organic solvent/buffer solution; DAKO, Carpinteria, CA) was added at 100 µl/well and incubated at room temperature for 20 minutes. The reaction was stopped with 50 µl/well of 2 N H_2SO_4 (5.5 ml 36N H_2SO_4 + 94.5 ml water). Absorbance at 450 nm was read with the SPECTRAMax 190 microplate spectrophotometer, and the results were analyzed by SOFTMax Pro software (Molecular Devices, Sunnyvale, CA).

IL1β ELISA was also performed using a paired capture and detection Ab set (human IL1β ELISA Ready-SET-Go!, eBioscience) according to manufacturer's protocol, similar to that described above.

IL23 ELISA was performed as previously described (Vanden Eijnden et al., 2006). Briefly, 50 µl/well of capture Ab (goat anti-human IL-23p19 polyclonal Ab, AF1716; R&D Systems) diluted to 5 µg/ml in PBS was added, and the plates were sealed and incubated overnight at room temperature. Plates were blocked with PBS + 1% BSA at room temperature for 1 hour. Standard was prepared by serially diluting rhIL23 (1290-IL; R&D Systems) by 2-fold starting at 4 ng/ml. Detection Ab (biotinylated mouse anti-human IL12p40, AHC7129; Biosource, Camarillo, CA) was diluted to 0.5 µg/ml in dilution buffer (PBS, 0.5% BSA), added at 50 µl/well and incubated at room temperature for 2 hours. The rest of the procedure is the same as described above.

B. Cytokine Production by $CD4^+$ T Cells

Immature moDCs were left unstimulated or activated with different TLR ligands at 5 x 10^4 cells/0.5 ml/well in the complete medium or X-VIVO15 at 37°C in 5% CO_2 for 24 hours as described above. Allogeneic naïve $CD4^+$ T cells were added to the moDCs at 5 x 10^5 cells/0.5 ml/well. These cocultures were incubated at 37°C in 5% CO_2 for 7 days. The differentiated $CD4^+$ T cells from the cocultures were then washed in PBS, resuspended in 1 ml X-VIVO15 and restimulated with 50 ng/ml phorbol myristate acetate (PMA) and 1 μg/ml ionomycin (Calbiochem) at 37°C for 24 hours. Supernatants were collected and assayed by IL17 and IL22 ELISA using capture and detection Ab sets (ELISA DuoSet; R&D Systems) according to manufacturer's protocol.

6. $CD4^+$ T Cell Proliferation Analysis

Allogeneic naïve $CD4^+$ T cells were stained with 1μM carboxyfluorescein succinimidyl ester (CFSE; Molecular Probes, Eugene, OR) as previously described (Parish, 1999). Briefly, allogeneic naïve $CD4^+$ T cells were resuspended at 2 x 10^7 cells/ml in PBS with 0.1% BSA. An equal volume of 2 μM CFSE in PBS with 0.1% BSA was added to the cell suspension and incubated at 37°C for 10 minutes. The reaction was quenched with an equal amount of sterile FBS. The CFSE-stained cells were then washed in serum-free RPMI 1640 three times and resuspended in either the RPMI complete medium or X-VIVO15, added to the culture of moDCs that had been previously activated or left unstimulated with TLR ligands for 24 hours as described above at a 1:10 DC:T ratio, and incubated for 48, 72, 96 or 120 hours. Occasionally, coincubation times were extended to 6, 7 or 12 days. Cell division of the T cells was analyzed by flow cytometry.

7. $CD4^+$ T Cell Differentiation Analysis

A. IFNγ and IL5 ELISPOT

Allogeneic naïve $CD4^+$ T cells were added to the culture of activated DCs as described above at a 1:10 DC:T ratio (7.5×10^4 DCs/0.5 ml/well + 7.5×10^5 T cells/0.5 ml/well) and incubated for 5 days. The primed $CD4^+$ T cells were collected and washed in PBS, resuspended in the complete medium supplemented with 5 U/ml rhIL-2 (R&D Systems), and cultured for an additional 7 days. The rested $CD4^+$ T cells were plated and restimulated with PMA and ionomycin for 6 hours, and assayed for the frequency of both IFN-γ- and IL-5-producing cells by ELISPOT using paired capture and detection Abs ELISPOT sets (Cytokine ELISPOT Pair, BD Biosciences) according to the manufacturer's protocol. Briefly, wells in 96-well filtration plates (MultiScreen high protein binding Immobilon-P membrane plate, 0.45 μm, MAIPS4510; Millipore, Billerica, MA) were first coated with 100 μl of capture Ab diluted to 5 μg/ml in PBS at 4°C overnight. After the capture Ab was discarded, the plates were blocked with the RPMI complete medium for 2 hours at room temperature. Blocking solution was then discarded, and the rested $CD4^+$ T cells were added to each well at 50 μl/well (200 cells/well for IFN-γ and 1×10^5 cells/well for IL-5). The T cells were then restimulated in the wells by adding 50 μl/well of 50 ng/ml PMA and 1 μg/ml ionomycin (Calbiochem) and incubating at 37°C in 5% CO_2 for 6 hours. After the plates were washed twice with deionized water and three times with wash buffer (PBS, 0.05% Tween20), detection Ab diluted in dilution buffer (PBS, 10% FCS) to 2 μg/ml was added to each well at 100 μl/well, and the plates were incubated for 2 hours at room temperature. The plates were washed again three times with wash buffer, and 100 μl/well of 100-fold diluted SA-HRP

was added to each well followed by incubation of the plates at room temperature for 1 hour. Finally, the plates were washed four times with wash buffer and twice with PBS, and 100 μl/well of AEC substrate (1 drop AEC chromogen + 1 ml AEC substrate, 3-amino-9-ethylcarbozole; BD Biosciences) was added to each well. The plates were incubated at room temperature for 10 to 20 minutes until spots developed, washed with deionized water and dried overnight. They were analyzed on an ImmunoSpot ELISPOT plate reader (Cellular Technology Ltd., Cleveland, OH). IL12 neutralization experiments were performed by adding either affinity purified polyclonal goat anti-IL12 IgG (catalog number AF-219-NA, R&D Systems) or normal goat IgG (R&D Systems) at 1 μg/ml on days 1 and 3 to the DC/$CD4^+$ T cell cocultures, followed by ELISPOT analysis on day 12 as above.

B. IL-17 ELISPOT

For cocultures, allogeneic naïve $CD4^+$ T cells were added to the cultures of TLR ligand-activated DCs as described above at a 10:1 (T cell:DC) ratio and incubated for 7 days. For APC-free cultures, CD25-depleted naïve $CD4^+$ T cells (2.5×10^5 cells/ml) were activated with 5 μg/ml plate-bound anti-CD3 (eBioscience) and 2.5 μg/ml soluble anti-CD28 (eBioscience) in the absence or presence of 30 ng/ml rhIL1β, 30 ng/ml rhIL6, 20 ng/ml rhIL23, or 5 ng/ml rhTGFβ (PeproTech, concentrations used unless otherwise indicated) for 7 days. The primed $CD4^+$ T cells were collected, washed in PBS, plated in duplicate wells and restimulated with 50 ng/ml phorbol myristate acetate (PMA) and 1 μg/ml ionomycin (Calbiochem) for 10 hours. The restimulated cells were assayed for the frequency of IL17-producing cells by enzyme-linked immunosorbent spot (ELISPOT, human IL17 ELISpot development module and ELISpot Blue Color Module, R&D

Systems) according to the manufacturer's protocol. The plates were analyzed on an ImmunoSpot ELISPOT plate reader (Cellular Technology Ltd., Cleveland, OH). In the neutralization experiments, cytokines in the DC/T cocultures were neutralized using 1 μg/ml recombinant human IL1 receptor antagonist (IL1Ra, R&D Systems), 10 μg/ml anti-IL6R (17506, R&D Systems), or 10 μg/ml mouse monoclonal anti-TGFβ (1D11, R&D Systems), which were added to the activated moDC culture 10 minutes prior to addition of the naïve $CD4^+$ T cells. Mouse IgG_1 was used as an isotype control at 10 μg/ml.

8. Statistical Analyses

Data were analyzed by one-way ANOVA with Dunnett's or Tukey post-test using Prism software (GraphPad Software, La Jolla, CA). Where indicated, some data were analyzed with Student's t test.

9. List of Reagents

Recombinant human IL4 (PeproTech, catalog number 200-04)

Recombinant human IL4 was reconstituted at 100 μg/ml in sterile water. This working solution was kept at -20°C and used at 100 ng/ml (500 U/ml).

Recombinant GM-CSF (Leukine sargamostim; Berlex Laboratories, product number NDC 50419-002-33)

Recombinant human GM-CSF (Leukine®) was reconstituted at 500 μg/ml (=2.8×106 U/ml) in sterile water. This working solution was kept at 4°C and used at 1000 U/ml.

Recombinant human IFNγ (PeproTech, catalog number 300-02)

Recombinant human IFNγ was reconstituted at 1 mg/ml. This stock solution was kept at -20°C. Working solution was made in PBS and used at 50 ng/ml.

Recombinant human IL2 (PeproTech, catalog number 200-02)

Recombinant human IL12 was reconstituted at 20 μg/ml. This stock solution was kept at -20°C. Working solution was made in PBS and used at 2 ng/ml.

Recombinant human IL1α (PeproTech, catalog number 200-01A)

Recombinant human IL1α was reconstituted at 100 μg/ml in sterile water. This working solution was kept at -20°C. Working solution was diluted in culture medium and used at 30 ng/ml.

Recombinant human IL1β (PeproTech, catalog number 200-01B)

Recombinant human IL1β was reconstituted at 100 μg/ml in sterile water. This working solution was kept at -20°C. Working solution was diluted in culture medium and used at 30 ng/ml.

Recombinant human IL6 (PeproTech, catalog number 200-06)

Recombinant human IL6 was reconstituted at 100 μg/ml in sterile water containing 10 mM acetic acid. This working solution was kept at -20°C. Working solution was diluted in culture medium and used at 30 ng/ml.

Recombinant human TGFβ1 (PeproTech, catalog number 100-21)

Recombinant human TGFβ1 was reconstituted at 50 μg/ml in sterile water containing 1 mM sodium citrate. This stock solution was diluted to 500 ng/ml in PBS with 2% BSA and kept at -20°C. The working solution was used at 5 ng/ml.

Recombinant human IL23 (R&D Systems, catalog number 1290-IL)

Recombinant human IL23 was reconstituted at 20 μg/ml in PBS with 0.1% BSA. This working solution was kept at -20°C. Working solution was made in culture medium and used at 20 ng/ml.

Lipopolysaccharide (LPS), *Salmonella Minnesota* Re 595 (Calbiochem, catalog number 437629)

LPS was reconstituted at 1 mg/ml in 0.5% triethylaminein in sterile water. This stock solution was kept at 4°C and was diluted at 1:10 in sterile water to make 10 μg/ml working solution. The working solution was kept at 4°C and used at 100 ng/ml.

Lipopolysaccharide (LPS), Ultra pure *Salmonella Minnesota* (Invivogen, catalog number tlrl-smlps)

LPS was first dissolved in ethanol and sterile water was added to make stock solution at 5 mg/ml. This stock solution was kept at -20°C and was diluted at 1:10 in sterile water to make 10 μg/ml working solution. The working solution was kept at 4°C and used at 100 ng/ml.

Poly I:C (Amersham, product number 27-4729)

Poly I:C was reconstituted at 1 mg/ml in PBS at 50°C for 20 minutes and cooled slowly to room temperature. This working solution was kept at -20°C and used at 100 μg/ml.

Single-stranded RNA40/LyoVec (Invivogen, catalog number tlrl-lrna40)

ssRNA40 was reconstituted at 100 μg/ml in sterile water. This working solution was kept at -20°C and used at 2.5 μg/ml.

Flagellin, *Salmonella typhimurium* (Invivogen, catalog number tlrl-stfla)

Flagellin was reconstituted 100 μg/ml in of sterile water. This working solution was kept at -20°C and used at 0.5 μg/ml.

Monophosphoryl Lipid A (MPL-A), *Salmonella Minnesota* Re 595 (Invivogen, catalog number trl-mpl)

MPL-A was reconstituted at 1 mg/ml in 10% ethanol in sterile water. This working solution was kept at -20°C and used at 1 μg/ml.

Pam3CSK4 (Invivogen, catalog number tlrl-pms)

Pam3CSK4 was reconstituted at 100 μg/ml in sterile water. This working solution was kept at -20°C and used at 1 μg/ml.

Phorbol-12-myristate-13-acetate (PMA; Calbiochem, catalog number 524400)

PMA was reconstituted at 10 mg/ml in DMSO. This stock solution was kept at -20°C. Working solution was made by dilution in culture medium and used at 50 ng/ml.

Ionomycin, *Streptomyces conglobatus* (Calbiochem, catalog number 407950)

Ionomycin was reconstituted at 1 mg/ml in DMSO. This working solution was kept at 4°C and used at 1 μg/ml.

CHAPTER III

The Effects of Toll-like Receptor Ligand-activated Monocyte-derived Dendritic Cells on Human $CD4^{\pm}$ T Cell Responses and Th1/Th2 Differentiation

1. Introduction

A number of TLRs are expressed by antigen presenting cells including both mouse and human DCs. Myeloid DCs in both species express TLR1/2 and TLR2/6 heterodimers, TLR3, TLR4, TLR5, TLR7, and TLR8, whereas plasmacytoid cells express TLR1/6 heterodimers, TLR7, TLR8, and TLR9 (Mazzoni and Segal, 2004; Iwasaki and Medzhitov, 2004). Human moDCs express basically the same pattern of TLRs as human myeloid DCs and thus serve as a good model system for TLR ligand activation of human myeloid DCs and their subsequent ability to direct T cell differentiation (Mazzoni and Segal, 2004; Iwasaki and Medzhitov, 2004). Three distinct nucleic acid-based TLR ligands have been identified: double stranded (ds) ribonucleic acid (RNA), a TLR3 ligand; single stranded (ss) RNA, a TLR7 and TLR8 ligand; and cytosine-guanine dinucleotide repeat motifs (CpG) deoxyribonucleic acid (DNA), a TLR9 ligand (Heil et al., 2004; Bauer and Wagner, 2002; Alexopoulou et al., 2001). dsRNA and ssRNA species are generated by viruses, and CpG motifs are generated by both bacteria and viruses (Hochrein et al., 2004; Bauer and Wagner, 2002). Several bacterial products also serve as TLR ligands. Some species of lipopolysaccharide (LPS), lipotechoic acid, lipoglycans, and lipoproteins derived from bacterial cell walls can activate through TLR2, while LPS from many other bacterial species and flagellin from several bacterial species activate through TLR4 and 5, respectively (Kapsenberg, 2003; Didierlaurent et

al., 2004; Aggrawal et al., 2003). Although investigators have generally shown that TLR ligand-activated DCs produce IL12p70 and skew $CD4^+$ T cell differentiation toward a Th1 pathway (Mazzoni and Segal, 2004; Kapsenberg, 2003; Iwasaki and Medzhitov, 2004), others have suggested that LPS- or flagellin-activated DCs can induce $CD4^+$ Th2 differentiation (Didierlaurent et al., 2004; Amsen et al., 2004).

Thus, our hypothesis was that activation of DCs with different TLR ligands results in differential DC production of cytokines and subsequent differentiation of human naïve $CD4^+$ T cells. To test this hypothesis, we compared the differentiation of human naïve $CD4^+$ T cells induced by different bacterial and viral TLR ligand-activated human moDCs and identified cytokine factors expressed by the differentially TLR ligand-activated DCs that play major roles in the T cell outcomes. We also examined how DCs from different human donors responded to bacterial and viral TLR ligands, and how those affected T cell differentiation. To this end, we utilized four different TLR ligands in these studies, two bacterial TLR ligands and two viral ligands, to activate moDCs from eight different human donors and examined their production of selected cytokines including IL12p70. Furthermore, differentially TLR ligand-activated moDCs from four of the donors were examined for their effects on naïve $CD4^+$ T cell proliferation and differentiation. Thus, these studies help provide a basis for the potential use of these TLR ligands or analogues thereof to customize human immune responses during vaccination against pathogens and cancer, and in other immunotherapeutic interventions.

2. Results

A. TLR Ligands Differentially Activate moDCs from Different Human Donors

moDCs from eight different human donors (Table 3) were examined for their production of four cytokines, IL12p40, IL12p70, TNFα, and IL10, in response to activation with two bacterial and two viral TLR ligands. The two bacterial TLR ligands used in this study are LPS (TLR4 ligand) and flagellin (TLR5 ligand). The two viral TLR ligands used are poly I:C (synthetic dsRNA, TLR3 ligand) and ssRNA40 (synthetic ssRNA, TLR7/8 ligand). Despite considerable donor to donor variation and experiment to experiment variation using the same donor, several trends emerged. As shown in Figure 8 and Table 3, the eight donors from whom we generated moDCs were divided into two groups based on the ability of their moDCs to produce IL12p70 in response to poly I:C; low responders (LR) whose moDCs produced barely detectable or undetectable levels of IL12p70, and high responders (HR) whose moDCs produced significantly higher levels of IL12p70 in response to poly I:C. The production of TNFα and IL12p40 in HR moDCs in response to poly I:C was significantly higher than that induced in LR moDCs. In contrast, the responses of LR and HR moDCs to LPS with regard to production of IL12p40, TNFα, and IL10 were similarly high. Furthermore, in the absence of exogenous IFNγ, LPS failed to consistently induce detectable levels of IL12p70 in all moDCs. Finally, flagellin induced minimal levels of all four cytokines examined here in moDCs relative to those induced by the other TLR ligands.

Since we were interested in examining human $CD4^+$ T lymphocyte Th1-Th2 responses to the TLR ligand-activated moDCs and since IL12 plays an important role in Th1 cell differentiation, we further analyzed the production of both IL12p70 and IL12p40

in moDCs derived from HR and LR donors in response to poly I:C (Figure 9). Statistical analyses based on the individual IL12p70 experiments (Figure 9A) indicated that the responses of the moDCs from two of the four HR donors differed significantly from at least one of the LR donors. In addition, the moDC responses of all four LR donors differed significantly from at least one HR donor. Finally, the mean of the mean IL12p70 responses from each of the four HR donors differed significantly from that of the four LR donors (see inset, Figure 9A).

Interestingly, the IL12p40 responses of the moDCs derived from the HR and LR donors also displayed significant differences between individual donors in each group (Figure 9B). IL12p40 production by donor 112 (HR) moDCs differed significantly from the responses from each of the four LR donor moDCs. Furthermore, the mean of the mean IL12p40 responses from each donor differed significantly between the moDCs derived from HR and LR donors (Figure 9B inset). Thus, the results presented in Figures 8 and 9 together indicate that the moDCs derived from donors in the two groups differ in their IL12 responses to poly I:C.

To examine whether TLR ligands induce different levels of maturation in LR or HR donor moDCs, TLR ligand-activated moDCs from a HR and a LR were stained for costimulatory molecules CD40 and CD86, and a MHC class II molecule, HLA-DR (Figure 10). All TLR ligand-activated moDCs exhibited similar levels of CD40, CD86 and HLA-DR upregulation. In addition, there was no significant difference in levels of upregulation of these molecules in both HR and LR TLR ligand-activated moDCs.

Table 3. List of Human Donors, Responder Status and HLA Class II Alleles

Donor	Status[1]	HLA Class II Alleles (DR and DQ only)
6	T cells	DR7, 13, 52, 53; DQ2, 6
99[3]	HR	DR9, 15, 51, 52; DQ3, 6
101	HR	DR1, 15, 51; DQ5, 6
103	LR	NT[2]
104	LR	DR4, 14, 52, 53; DQ3, 5
108[3]	LR	DR11, 15, 51, 52; DQ6, 7
109[3]	HR	DR4, 8, 53; DQ3, 6
110[3]	LR	DR13, 52; DQ6
112[3]	HR	DR1, 8; DQ5
116	T cells	DR4, 7, 53; DQ8, 9

[1]Responder status was based on the moDC level of IL12p70 induced by poly I:C within a 24h timeframe: low responder (LR), ≤100 pg/ml, high responder (HR), >100pg/mL. Donors 6 and 116 were used a source of allogeneic naïve $CD4^+$ $CD45RO^-$ T cells for co-culture experiments.

[2]NT, not typed.

[3]moDCs from these donors were used in the DC:T cell co-culture experiments shown in Figures 11-13.

Figure 8. Cytokine Production by TLR-activated moDCs from LR and HR Donors

moDCs from the four low responder (LR, open symbols) and four high responder (HR, closed symbols) human donors were activated for 24h with the indicated TLR ligand or left unstimulated. Subsequently, the production of cytokines was assessed in the culture supernatants from the cells by ELISA. LR and HR donors were classified on the basis of the production of IL12p70 by poly I:C-activated moDCs. The LR and HR experiments were compared by one-way ANOVA with a Dunnett's post-test comparing all TLR ligand-activated LR responses to unstimulated LR responses and all TLR ligand activated HR responses to unstimulated HR responses. Significant differences from unstimulated moDCS are indicated with the symbol,★, underneath the x-axis labeling. In addition, Student's t (two-tailed) test was used to compare LR and HR responses to a particular TLR ligand for a particular cytokine. Significant differences between LR and HR responses are indicated with brackets and the indicated *p* values.

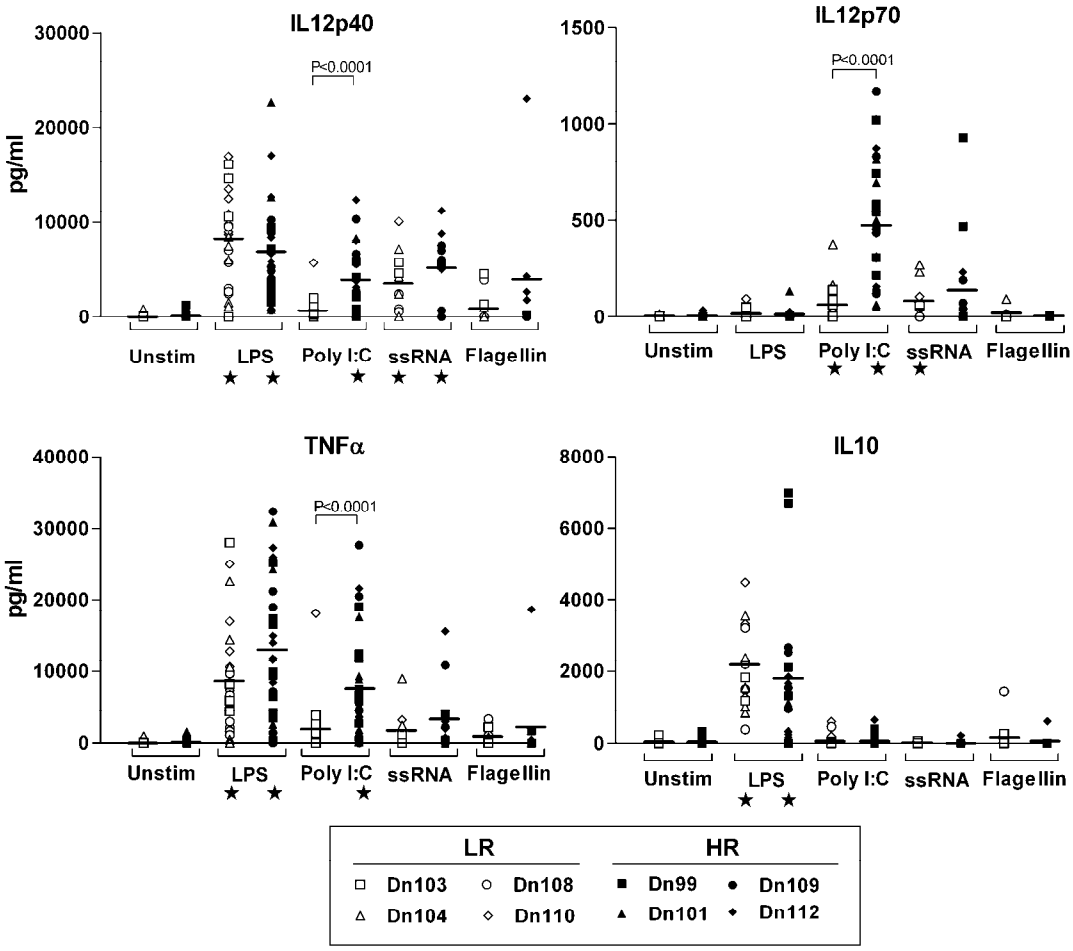

IL12p40
IL12p70
TNFα
IL10
pg/ml
P<0.0001
Unstim
LPS
Poly I:C
ssRNA
Flagellin
LR
HR
Dn103
Dn104
Dn108
Dn110
Dn99
Dn101
Dn109
Dn112

Figure 9. HR moDCs Produce Statistically Significant Levels of IL12p70 and IL12p40 in Response to Poly I:C Relative to LR moDCs

A, Comparison of IL12p70 levels produced by poly I:C-activated moDCs from HR and LR donors in individual experiments. * denotes that the indicated responses demonstrate statistically different responses from the responses of at least one donor from the other donor type, based on paired one-way ANOVA with a Tukey post-test: donor 99 versus 103 and 104, $p < 0.05$; donor 99 versus 108 and 110, $p < 0.01$; donor 101 versus 108 and 110, $p < 0.05$. Inset show the mean of the means for each HR donor versus the mean of the means of each LR donor. These means were compared using a two-tailed Student's t test. **B**, Comparison of IL12p40 levels produced by poly I:C-activated moDCs from HR and LR donors in individual experiments. * denotes that the indicated responses demonstrate statistically different responses from the responses of at least one donor from the other donor type: donor 112 versus 103 and 110, $p < 0.05$; donor 112 versus 104 and 108, $p < 0.01$. Inset show the mean of the means for each HR donor vs. the mean of the means of each LR donor. These means were compared using a two-tailed Student's t test.

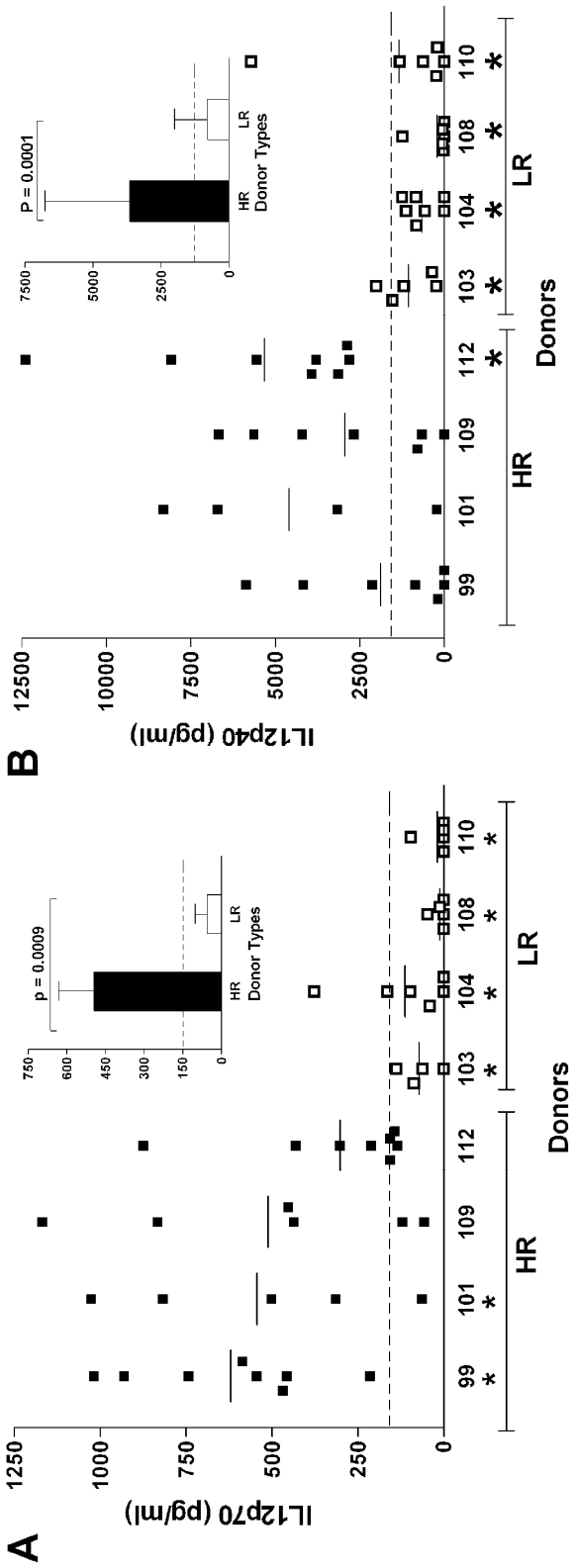
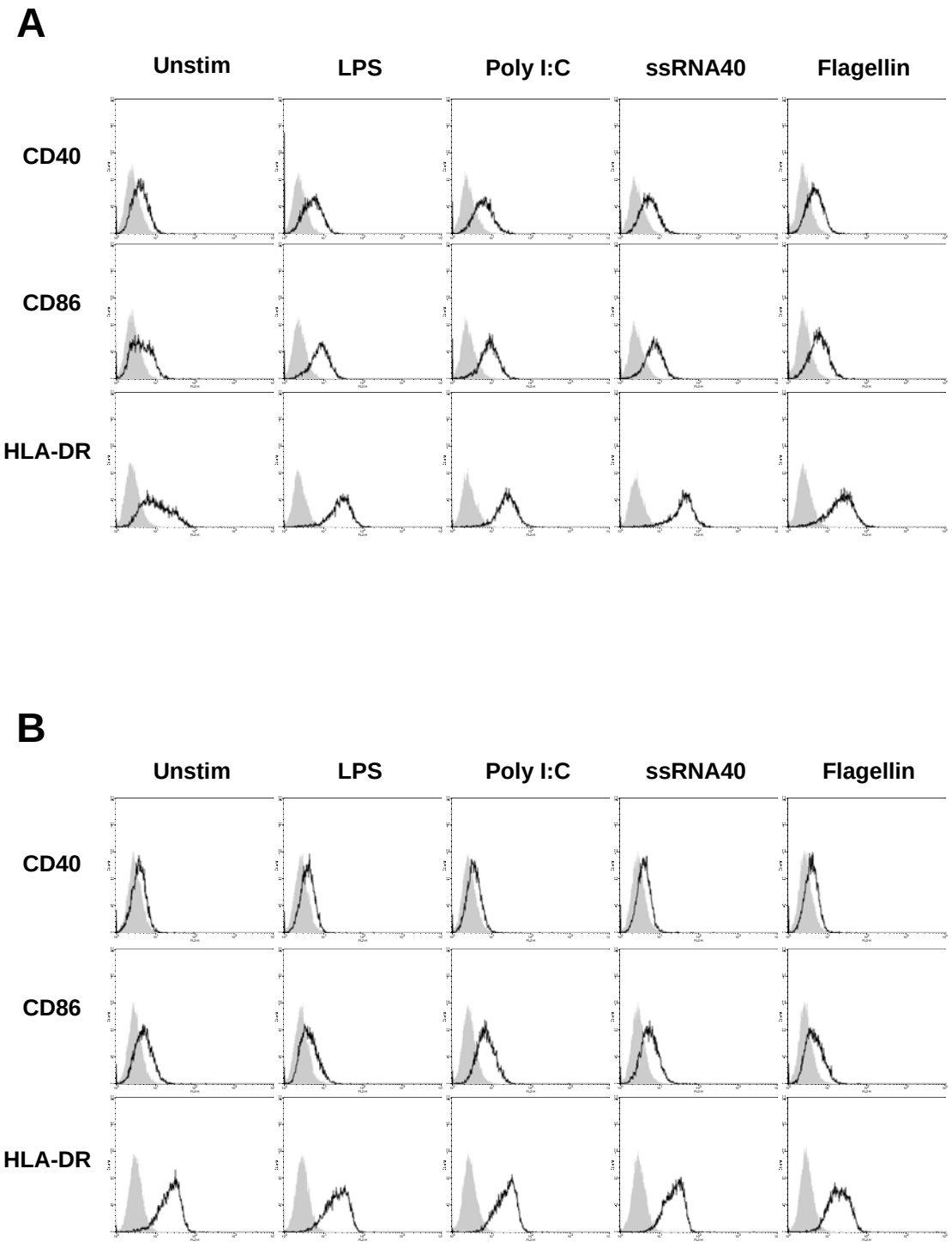

A
IL12p70 (pg/ml)
1250
1000
750
500
250
0
p = 0.0009
750
600
450
300
150
0
HR
LR
Donor Types
99
101
109
112
103
104
108
110
HR
LR
Donors
B
IL12p40 (pg/ml)
12500
10000
7500
5000
2500
0
P = 0.0001
7500
5000
2500
0
HR
LR
Donor Types
99
101
109
112
103
104
108
110
HR
LR
Donors

Figure 10. CD40, CD86 and HLA-DR Upregulation on TLR Ligand-activated moDCs

moDCs from Dn 112 (HR, Figure 10A) and Dn 108 (LR, Figure 10B) were left unstimulated or activated with TLR ligands for 24 hours. These cells were then stained for surface expression of CD40, CD86 and HLA-DR. Staining with isotype control is shown in gray.


A
Unstim
LPS
Poly I:C
ssRNA40
Flagellin
CD40
CD86
HLA-DR
B
Unstim
LPS
Poly I:C
ssRNA40
Flagellin
CD40
CD86
HLA-DR

B. TLR Ligand-activated moDCs Induce Distinct Patterns of $CD4^+$ T Cell Proliferation, Independent of Human Donor Status

To examine the effect of TLR ligand-activated moDCs from LR and HR donors on T cell activation and proliferation, naïve ($CD45RO^-$) $CD4^+$ T cells, isolated from allogeneic (HLA-DR- and -DQ-mismatched) donors using negative depletion-magnetic bead technology, were labeled with CFSE, followed by coculture with unstimulated or TLR ligand-activated allogeneic LR and HR moDCs (DC:T cell ratio of 1:10). At 48 hours, 72 hours, 96 hours, and 120 hours of coculture, the cells were analyzed by flow cytometry to compare the effect of TLR-activation of the moDCs on T cell proliferation (and dilution of CFSE in the T cells). At 96 hours, small peaks of dividing cells could be observed (data not shown) with larger peaks of cells that had undergone 5-7 cell divisions visible at 120 hours (Figure 11). Stimulation of the CFSE-labeled $CD4^+$ T cells with plate-bound anti-CD3 and soluble anti-CD28 were used as a positive control in the absence of DCs to stimulate a larger proportion of the naïve $CD4^+$ T cells, thereby allowing us to distinguish how many cell divisions the T cells had undergone in the cocultures with activated DCs (data not shown). At 120 hours, the bacterial TLR ligand-activated moDCs from both LR and HR donors induced increased $CD4^+$ T cell proliferation relative to that induced by poly I:C-activated moDCs from the same donors, based on the percentage of $CD4^+$ T cells that have undergone at least one cell division (given in each histogram in Figure 11, including those activated with unstimulated moDCs shown in gray shading). After 7 days of coculture, T cell proliferation induced by poly I:C-activated moDCs from the same donors still lagged behind $CD4^+$ T cell proliferation induced by the bacterial TLR ligand-activated moDCs (data not shown). It

is noteworthy that, although flagellin-activated moDCs produced either barely detectable or undetectable levels of the cytokines measured here (Figure 8), they induced significantly increased $CD4^+$ T cell proliferation (Figure 11) as compared to unstimulated moDCs, verifying that flagellin was indeed activating the moDCs.

These results demonstrate that, despite the differences in cytokine production after TLR-activation of LR and HR moDCs, LR and HR moDCs were similar in their ability to induce $CD4^+$ T cell proliferation. Furthermore, bacterial TLR ligand-activated moDCs induced statistically significant increases in $CD4^+$ T cell proliferation relative to that induced by unstimulated and poly I:C-activated moDCs (repeated measures one-way ANOVA with Tukey post-test).

Figure 11. Proliferative Responses of Naïve $CD4^+$ T Cells Stimulated with Allogeneic Unstimulated or TLR Ligand-activated moDCs from LR or HR Donors

CFSE-labeled naïve $CD4^+$ T cells isolated from human donors were stimulated with allogeneic unstimulated (gray-filled histograms) or LPS-, flagellin-, poly I:C-, or ssRNA-activated moDCs as indicated. moDCs were generated from two LR donors (108 and 110) and two HR donors (99 and 109). The cells were harvested at 48, 72, 96, and 120h of co-culture, and the cells were analyzed by flow cytometry to determine the relative loss of CFSE staining resulting from cell division. Only the results from 120h are shown. moDCs and dead cells were excluded from the analysis by forward and side light scatter gating. Results from each row of histograms for each moDC donor are from the same experiment.

LPS
Flagellin
Poly I:C
ssRNA
Dn 108
Dn 110
Dn 99
Dn 109
Cell Count
CFSE
13.07%
36.17%
40.63%
6.51%
14.57%
22.05%
65.10%
45.41%
25.08%
52.25%
12.58%
34.89%
42.47%
11.96%
18.68%
13.62%
22.61%
28.72%
7.22%
19.57%

C. Poly I:C-activated moDCs Induce Increased Skewing of $CD4^+$ T Cell Differentiation toward the Th1 Pathway

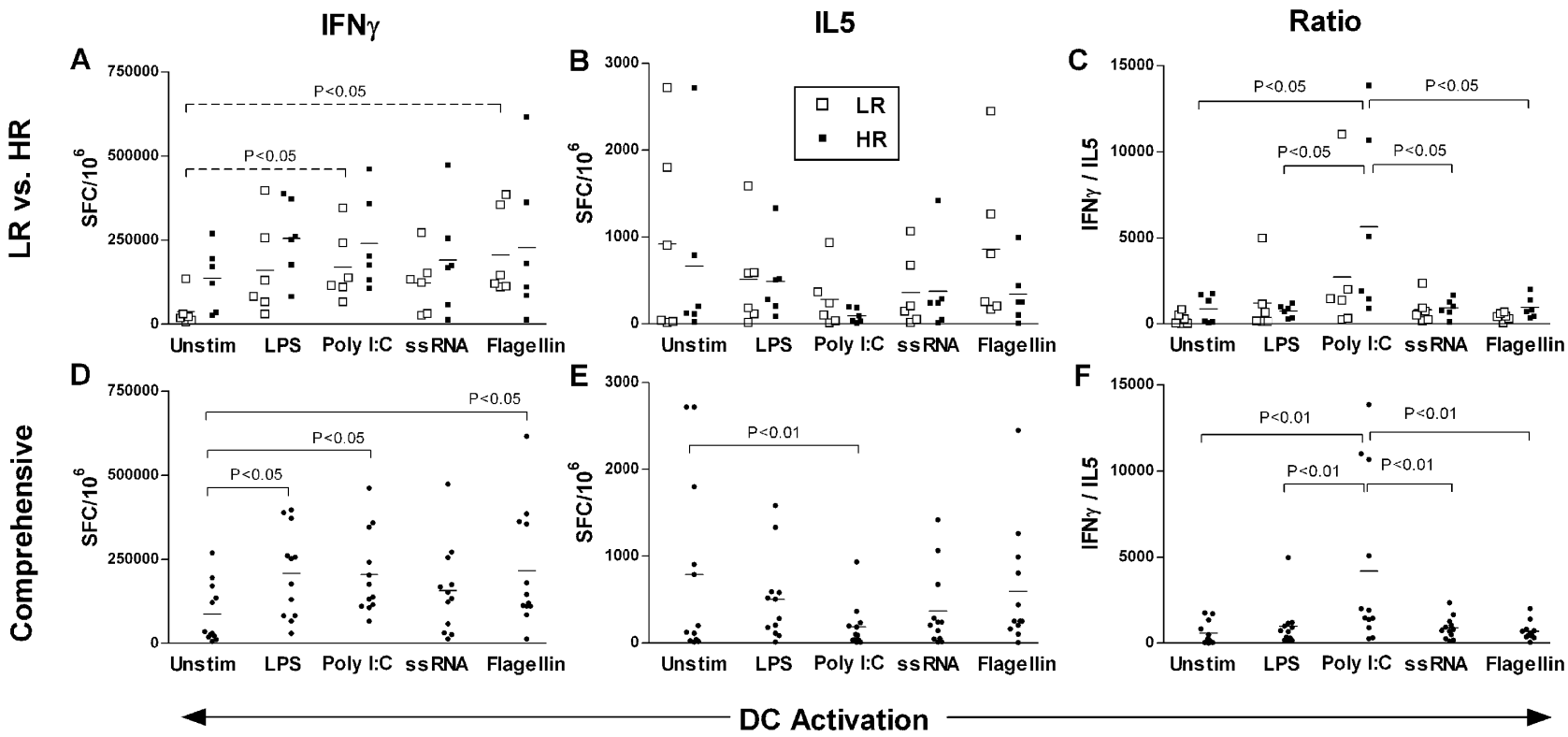
To examine the effect of bacterial and viral TLR ligand-activated moDCs on $CD4^+$ T cell differentiation and whether TLR ligand-activated moDCs from LR and HR donors differed in this capacity, we examined the ability of unstimulated and TLR ligand-activated HR and LR moDCs to induce differentiation of naïve $CD4^+$ T cells into Th1 and Th2 cells by assessing the frequency of IFNγ- and IL5-producing cells in the co-cultures. moDCs from HR and LR donors were either left unstimulated or activated with TLR ligands for 24 hours prior to the addition of naïve ($CD45RO^-$) $CD4^+$ T cells isolated from one of two different allogeneic donors (donors 6 and 116). After 12 days of coculture, the frequency of cells that produce IFNγ (Th1) or IL5 (Th2) was determined using ELISPOT.

As shown in Figure 12A-C, there is a trend for TLR ligand-activated moDCs from HR donors to induce greater frequencies of IFNγ responses than those induced by TLR ligand-activated moDCs from LR donors, though these differences are not statistically significant. However, the ratios of the frequencies of IFNγ to IL5 responses induced by poly I:C-activated HR moDCs differ significantly from ratios induced by either unstimulated HR moDCs or HR moDCs stimulated with other TLR ligands, whereas the ratios of IFNγ to IL5 responses induced by poly I:C-activated LR moDCs did not differ significantly from the ratios induced by either unstimulated LR moDCs or LR moDCs stimulated by other TLR ligands. These results correlate the higher production of IL12p70 by poly I:C-activated HR moDCs with induction of increased Th1/Th2 skewing relative to that induced by poly I:C-activated LR moDCs.

When all of the experiments using LR and HR moDCs were grouped together (Figure 12D-F), LPS-, poly I:C-, and flagellin-activated moDCs were found to induce increased frequencies of IFNγ-producing $CD4^+$ T lymphocytes relative to unstimulated moDCs (Figure 12D). However, only poly I:C-activated moDCs induced significantly lower frequencies of IL5-producing cells (Figure 12E). The combination of these effects resulted in significantly higher ratios of IFNγ- to IL5-producing cells using poly I:C-activated moDCs relative to the ratios induced by either unstimulated moDCs or moDCs activated with other TLR ligands (Figure 12F). While other TLR ligand (LPS, ssRNA40, and flagellin)-activated moDCs induced skewing toward IFNγ-producing $CD4^+$ T lymphocytes relative to unstimulated moDCs (Figure 12D), they failed to induce significantly higher ratios of IFNγ- (Th1) to IL5- (Th2) producing $CD4^+$ T lymphocytes similar to poly I:C-activated moDCs (Figure 12F).

Figure 12. Differentiation of Naïve $CD4^+$ T Cells after Stimulation with Unstimulated or TLR-activated Allogeneic moDCs from LR and HR Donors

ELISPOT was used to quantitate the frequency of IFNγ- (A, D) and IL5- (B, E) producing T cells, and the ratios of those frequencies are presented (C, F), induced by unstimulated and TLR-activated LR versus HR moDCs (A-C, open squares denote LR moDCs; closed squares denote HR moDCs) or all donors (D-F). Paired one-way ANOVA comparisons with a Tukey post-test were performed to determine statistical differences, which are noted on the figure. In A-C, solid lines indicate differences among the frequencies or ratios induced by HR moDCs, and dotted lines indicate differences among the frequencies or ratios induced by LR moDCs. Two-tailed Student's t tests were performed to compare the responses induced by LR versus HR moDCs for every moDC activation condition. None of the differences induced by LR versus HR moDCs were statistically significant.


IFNγ
IL5
Ratio
LR vs. HR
Comprehensive
A
B
C
D
E
F
SFC/10^6
IFNγ / IL5
LR
HR
P<0.05
P<0.01
Unstim
LPS
Poly I:C
ssRNA
Flagellin
DC Activation

E. IL12p70 Produced by Poly I:C-activated moDCs is Responsible for Th1 Skewing

From the above results, poly I:C induced increased production of IL12p70 from HR donor moDCs, and these activated moDCs induced increased skewing toward Th1 and away from Th2 $CD4^+$ T effectors cells. To determine if the increased production of IL12p70 directly resulted in the observed increased skewing, we examined the effect of IL12p70 neutralization in the cocultures. A representative experiment is shown for each of type of donors in Figure 13 (LR donor 108: Figure 13A-C; HR donor 109: Figure 13D-F). IL12 neutralization decreased the frequency of IFNγ-producing (Th1) $CD4^+$ T lymphocytes induced by all TLR ligand-activated LR and HR donors except for flagellin-activated moDCs. Thus, despite the lack of detectable IL12p70 produced by both LR and HR moDCs activated with LPS or ssRNA40 and by LR moDCs activated with poly I:C, IL12 neutralization decreased the ability of these activated moDCs to induce differentiation into Th1 cells (Figure 13A and D). Importantly, IL12 neutralization also decreased the induction of T cell differentiation into Th1 cells by poly I:C-activated HR moDCs (Figure 13D). When T cell differentiation into IFNγ-producing cells induced by TLR ligand-activated moDCs from all four donors was analyzed together, neutralization of IL12 significantly decreased the frequency of Th1 cells induced by LPS- and poly I:C-activated moDCs (Figure 13G). In addition, neutralization of IL12 generally enhanced differentiation into IL5-producing Th2 cells (Figure 13B and E); when examined comprehensively for all four donors, the effects of IL12 neutralization on IL5 responses induced by all TLR ligand-activated moDCs were statistically significant (Figure 13H).

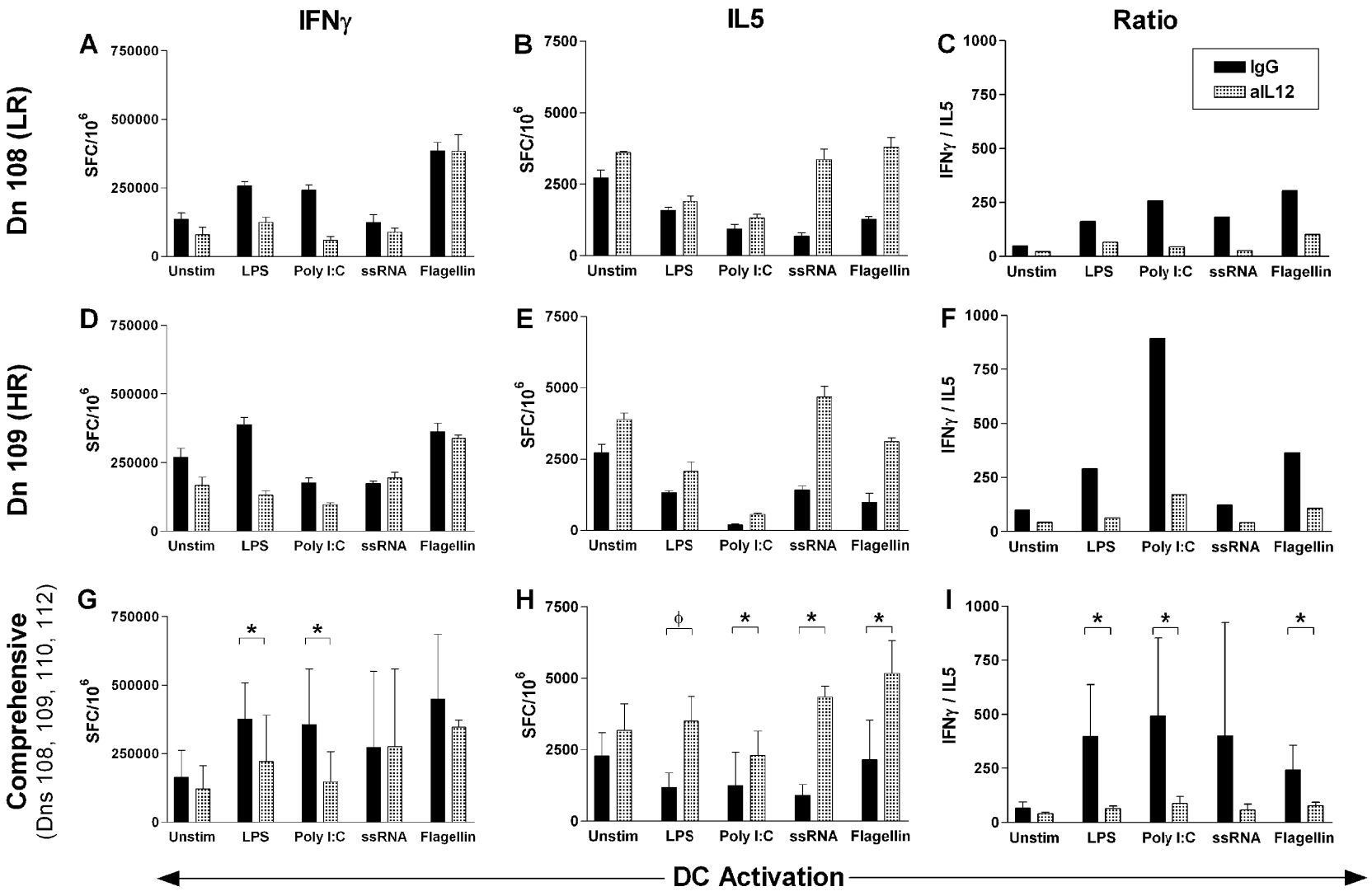
The greatest effect of IL12 neutralization was observed in the ratios of frequencies of IFNγ- to IL5-producing cells (Figure 13C and F). This effect is observed

in the T cell responses induced by all TLR ligand-activated moDCs, regardless of TLR ligand or donor type (LR vs. HR) of moDC employed. When examined comprehensively for all four donors, these effects were statistically significant for responses induced by all TLR ligand-activated moDCs examined except ssRNA40-activated moDCs (Figure 13I).

Thus, these results indicate that IL12p70 played a critical role in the induction of naïve human $CD4^+$ T cell differentiation to Th1 cells by TLR ligand-activated moDCs. Furthermore, these results indicate that the synthetic viral TLR3 ligand, poly I:C, induced higher levels of IL12p70 production by moDCs than the other TLR ligands tested here. The increased IL12p70 production by poly I:C-activated moDCs resulted in more highly skewed Th1 populations. Conversely, the other TLR ligand-activated moDCs produced decreased IL12p70 levels and subsequently induced more balanced Th1/Th2 $CD4^+$ T cell responses.

Figure 13. IL12 Plays a Critical Role in Th1 Skewing of Naïve $CD4^+$ T Cells by Allogeneic TLR-activated moDCs

Unstimulated or TLR-activated moDCs from two LR and two HR donors were used to stimulate naive T cell differentiation in the presence of normal goat IgG or affinity purified goat anti-IL12 IgG (1 μg/ml) added simultaneous to the initiation of co-culture and again on day 3. ELISPOT was used to measure the frequencies of IFNγ- (A, D, G) and IL5- (B, E, H) producing T cells and their ratios (C, F, I) induced by unstimulated or TLR ligand-activated moDCs. A representative experiment for the two LR (108, A-C) and two HR (109, D-F) donors along with a comprehensive analysis of all four donors (G-I) are shown. A two-tailed Student's t test was used to statistically compare differences observed between normal goat IgG and goat anti-IL12 IgG treatments in G-I. * denotes $p<0.05$, whereas Φ denotes a near statistically significant difference of $p=0.0578$.


IFNγ
IL5
Ratio
Dn 108 (LR)
Dn 109 (HR)
Comprehensive
(Dns 108, 109, 110, 112)
A
B
C
D
E
F
G
H
I
SFC/10^6
IFNγ / IL5
IgG
aIL12
Unstim
LPS
Poly I:C
ssRNA
Flagellin
DC Activation

3. Discussion

This is one of the first studies to comprehensively compare the effects of several different bacterial and viral TLR ligands on DC activation and the subsequent effect of the activated DCs on the differentiation of naïve $CD4^+$ T cells in humans. These results suggest that the poly I:C and potentially other dsRNA species could shunt immune responses more toward $CD4^+$ Th1 responses producing IFNγ. Perhaps more surprisingly, the two bacterial TLR ligands tested in these studies activated DCs to induce relatively more balanced Th1/Th2 responses. Teleologically, the more skewed Th1 response induced by poly I:C-activated DCs would produce more effective cellular responses against viral and other intracellular pathogens, whereas more balanced responses induced by the two bacterial TLR ligand-activated DCs would allow the production of both cellular and Ab responses, which would be more protective against extracellular pathogens.

The polyclonal Ab that was used to neutralize IL12p70 in the experiments shown in Figure 5 may also bind and potentially neutralize IL23, since these two cytokines share a common subunit (p40). Thus, it is possible that IL23 could be the cytokine produced by activated DCs that is skewing Th1 differentiation unless neutralized in the cocultures. However, two results argue against this. First, all four TLR ligands used here have been shown to induce moDC production of IL23 (R.K. Benwell and D.R. Lee, manuscript in preparation), and this fails to correlate with the Th1/Th2 differentiation patterns observed here. Second, while early studies suggested a role for IL23 in Th1 skewing, more recent studies demonstrate that this cytokine is not involved in Th1 skewing of naïve T cells but rather serves as a survival/proliferation factor for Th17 cells (Ghilardi et al., 2004;

Langrish et al., 2005; Murphy and Reiner, 2002; Cua et al., 2003; Murphy et al., 2003; Hunter, 2005). Thus, our results and those of others suggest that IL23 does not play a role in skewing of naïve $CD4^+$ T cells toward a Th1 pathway in this system, and that the polyclonal Ab used in Figure 5 indeed functions in reducing Th1 differentiation by neutralizing IL12p70 present in these co-cultures.

The expression of IFNβ was examined by quantitative real time RT-PCR as a potential contributor to Th1 skewing induced by poly I:C-activated HR DCs, since IFNβ is one of the first type I IFNs produced and acts to increase its own expression as well as those of many IFNα subtypes through the induction of IRF7 (Honda et al., 2005). In humans, type I IFNs can activate Stat4, much like IL12p70 does in both mice and humans, thereby inducing differentiation along the Th1 pathway (Rogge et al., 1998; Davis et al., 2005). While LPS was found to induce expression of modest levels of IFNβ transcripts in moDCs, poly I:C induced much higher expression (data not shown). Thus, type I IFNs could contribute to the increased Th1 skewing induced by poly I:C-activated HR DCs, but probably play a more minor role in this system, since IL12p70 neutralization abrogates much of the Th1 skewing by these activated moDCs (Figure 13).

As mentioned above, all four TLR ligands examined here increase moDC expression of IL23 heterodimer (R.K. Benwell and D.R. Lee, manuscript in preparation). Furthermore, LPS- and flagellin-activated moDCs induce more balanced Th1/Th2 responses (Figure 12) and increased Th17 differentiation (Benwell RK and Lee DR, manuscript in preparation) relative to that induced by poly I:C-activated moDCs. Thus, the lower levels of IL12p70 produced by LPS- and flagellin-activated moDCs relative to poly I:C-activated moDCs may result in comparatively more balanced Th1/Th2

responses, thereby resulting in relatively decreased levels of IFNγ in the cultures. Decreased levels of IFNγ would theoretically result in enhanced Th17 differentiation, if positive factors were present for that, since IFNγ has been shown to inhibit Th17 differentiation (Park et al., 2005; Hunter, 2005; Harrington et al., 2005). More recently, studies have also shown that IL12p70 can directly inhibit Th17 differentiation (Hoeve et al., 2006).

In these studies, poly I:C-activated DCs uniquely promoted extremely high skewing of Th1 compared to Th2 responses. The synthetic viral dsRNA analogue, poly I:C, can act through TLR3 (Alexopoulou et al., 2001), which is expressed in the endosomes of myeloid and monocyte-derived DCs (Matsumoto et al., 2003), resulting in signaling through the adaptor TRIF. In addition, poly I:C can also act through the cytoplasmic sensor, MDA5, a retinoid acid-inducible gene I-like receptor (Meylan and Tschopp, 2006; Yoneyama and Fujita, 2008). When poly I:C accesses the cytoplasm, it can interact with MDA5 when expressed, and ultimately results in NF-κB, IRF3 and IRF7 activation, similar to that observed downstream of TLR3 activation (Meylan and Tschopp, 2006; Kumar et al., 2008). Thus, poly I:C could generate signals through MDA5 in addition to those generated through TLR3 in the endosomes, which ultimately could result in qualitatively or quantitatively different signals in DCs than those generated through the other TLRs engaged in this study. *In vivo* studies in which poly I:C was used as an adjuvant in mice deficient in the MDA5 adaptor (IPS-1) or the TLR3 adaptor (TRIF) provide support for this concept (Kumar et al., 2008) . Alternatively, the unique ability of poly I:C-activated DCs to highly skew Th1 responses may be due to the unique signaling of TLR3 through TRIF alone (Akira and Takeda, 2004). In contrast,

TLR4 signals through either a heterodimer of TRIF and TRAM or a heterodimer of MyD88 and TIRAP (van Duin et al., 2006; Akira and Takeda, 2004). TLR5 and TLR7/8 reportedly signal through MyD88 only. Nevertheless, of the TLR ligands engaged in this study, poly I:C, through TLR3, the cytoplasmic sensor MDA5, or both, uniquely results in higher levels of IL12p70 production and induces significantly greater Th1 skewing. The levels of IL12p70 produced in response to poly I:C and the resulting skewing of Th1 responses are more elevated with moDCs derived from certain human donors (HR), but this is still evident with moDCs derived from other donors as well based on IL12 neutralization experiments (Figure 13).

T cells are also known to express TLRs, and it is possible that TLR ligands are directly acting on T cells to modulate their differentiation. However, we speculate that the effects of TLR ligands in the cocultures predominantly influence the maturation and functions of moDCs, which subsequently affect T cell differentiation rather than acting directly on T cells for several reasons. First, TLR signaling does not have any effects on T cells that have not been activated via TCR and CD28 (data not shown). T cells first must be activated, but this is done by APCs, or TLR ligand-activated moDCs in our cocultures, and the difference in levels of T cell activation may result from differentially TLR ligand-activated moDCs. Second, TLR ligands may directly act on αCD3/αCD28-activated T cells, but αCD3/αCD28 treatment induces a very strong and artificial T cell activation, and it is difficult to correlate the results from such experiments with that of our cocultures which emulates more physiological T cell activation by APCs. Third, it is also very difficult to selectively stimulate moDCs, but not T cells, in the cocultures without washing away moDC-derived cytokines that are important for T cell

differentiation. Furthermore, moDCs are stimulated with TLR ligands 24 hours prior to addition of the naïve $CD4^+$ T cells, after which TLR ligands in the cultures may be internalized by moDCs or degraded, though the half-lives of TLR ligands in such conditions are not known. In addition, if TLR ligands were to be administered as adjuvants *in* vivo, they will act not only DCs but also many other types of cells, though adjuvants are not likely to be directly administered to where naïve T cells encounter antigen-bearing DCs (i.e., lymphoid organs) and the concentrations of TLR ligands in the T cell zone of lymphoid organs may be diluted to minimum *in vivo*. Therefore, we concluded that distinct T cell differentiation patterns induced in the DC/T cell are mainly due to the differential activation of moDCs by TLR ligand stimulation.

Collectively, these studies indicate that TLR ligands or nontoxic analogues can be utilized to customize immune responses generated in vaccines and other immunotherapeutic approaches. The viral TLR ligand, poly I:C, activated DCs to induce increased $CD4^+$ Th1 differentiation which would afford enhanced protection against viral and other intracellular pathogens. In contrast, the bacterial TLR ligands, LPS and flagellin, activated DCs to induce relatively more balanced Th1/Th2 responses, which may be more favorable to the development of cellular responses including all three $CD4^+$ T cell effector types, Th1, Th2, and Th17, as well as providing help for Ab responses, all of which would be beneficial in fighting bacterial pathogens (Kleinschek et al., 2006; Happel et al., 2005; Ghilardi et al., 2004; Murphy and Reiner, 2002; Murphy et al., 2003; Hunter, 2005; Cua et al., 2003).

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CHAPTER IV

The Effects of Toll-like Receptor Ligand-activated Monocyte-derived Dendritic Cells on Human Th17 Differentiation

1. Introduction

The recently discovered subset of T helper cells, Th17, has received much attention for its involvement in the pathogenesis of autoimmune diseases such as rheumatoid arthritis, psoriasis, inflammatory bowel diseases, diabetes and multiple sclerosis in both mice and humans (Bettelli et al., 2008; Romagnani, 2008; Wilson et al., 2007; Bettelli et al., 2006). However, the Th17 response also exists as a part of normal immunity against extracellular as well as some intracellular pathogens such as *Mycobacterium tuberculosis, Mycoplasma pneumoniae, Klebsiella pneumoniae, Escherichia coli, Aspergillus fumigatus, Cryptococcus neoformans, Candida albicans* and *Toxoplasma gondii* (Matsuzaki and Umemura, 2007; Zelante et al., 2007; Scriba et al., 2008; Khader and Cooper, 2008). Furthermore, recent reports suggest Th17 responses also play protective roles against hepatitis C virus and human immunodeficiency virus type 1 infections (Yue et al., 2008; Rowan et al., 2008; Ndhlovu et al., 2008).

It has been shown extensively that Th17 differentiation requires TGFβ as well as antigen presenting cell (APC)-derived cytokines such as IL1β, IL6, IL23 and TNFα (Sutton et al., 2006; Mangan et al., 2006; Veldhoen et al., 2006; Ivanov et al., 2006; Li et al., 2007b; Kimura et al., 2007; Bettelli et al., 2008; Bettelli et al., 2006; Kryczek et al., 2007a; Murphy et al., 2003; Zhou et al., 2007). Especially in mice, it is widely accepted that the minimal requirement for Th17 induction is TGFβ and IL6 by inducing RORγt

and RORα, transcription factors responsible for Th17 differentiation and suppressing Foxp3, a regulator of T regulatory cell differentiation and functions, respectively (Yang et al., 2008c; Bettelli et al., 2006; Zhou et al., 2008). In mice, IL23 is not involved in initiation of Th17 differentiation but maintains the Th17 population (Veldhoen et al., 2006; Bettelli et al., 2006; Zhou et al., 2007). Moreover, IL1β and TNFα work synergistically with IL6 and TGFβ to enhance more Th17 differentiation (Veldhoen et al., 2006). IL21 produced by Th17 cells act in an autocrine manner to enhance Th17 differentiation (Zhou et al., 2007; Wei et al., 2007; Nurieva et al., 2007; Korn et al., 2007). In contrast, the cytokine requirements for human Th17 differentiation are still not well defined. Several publications originally suggested that there is a distinct difference in cytokine requirements for Th17 differentiation between mice and humans, since TGFβ was not shown to be required for human Th17 differentiation (Acosta-Rodriguez et al., 2007a; Wilson et al., 2007; Chen et al., 2007b; Evans et al., 2007). More recently, other publications showed that TGFβ is in fact required for Th17 differentiation in humans (Manel et al., 2008; Volpe et al., 2008; Yang et al., 2008a; Gerosa et al., 2008). Although these reports have shown a strong involvement of TGFβ, IL1β, IL6 and IL23 in human Th17 differentiation, the minimum cytokine requirements reported in these studies were inconsistent. Furthermore, the cytokine requirements for Th17 differentiation in a more physiological setting (i.e., APC/T cell cocultures) have not yet been determined.

Toll-like receptors (TLRs) are members of pattern recognition receptors. These receptors exist to recognize pathogen-associated molecular patterns that are conserved throughout many pathogens (e.g., bacteria, viruses and parasites). The TLRs are expressed on many cell types but are highly expressed on APCs, such as dendritic cells

(DCs) and macrophages that can respond to infection as part of innate immunity (Kawai and Akira, 2007; Akira and Hemmi, 2003). Since TLR ligands have great potential as immune modulators for their ability to directly induce innate immunity, as well as to indirectly manipulate adaptive immunity, we were interested in exploring the use of TLR ligands as vaccine adjuvants to either induce or limit Th17 responses against pathogens or in autoimmune diseases, respectively.

In our current study, we hypothesize that different TLR ligand-activated DCs induce disparate levels of human Th17 differentiation. Moreover, we propose that bacterial TLR ligand-activated DCs are capable of inducing the highest levels of Th17 differentiation, since Th17 responses play more essential roles in anti-bacterial immunity. We determined the cytokine profile (IL1β, IL6 and IL23) produced by monocyte-derived dendritic cells (moDCs) activated with various TLR (TLR1/2, 3, 4, 5, 7/8) ligands, as well as the ability of those TLR ligand-activated DCs to induce Th17 differentiation of human naïve $CD4^+$ T cells. Our results suggest that lipopolysaccharide (LPS)- and tripalmitoylated lipopeptide (Pam3CSK4)-activated moDCs induce the highest levels of Th17 differentiation in human $CD4^+$ T cells. In addition, DC-derived IL1β and T cell-derived TGFβ were absolutely required for Th17 differentiation, but IL6 was only partially required in our DC/T cell cocultures. Furthermore, we confirmed the requirement of IL1β as well as TGFβ in Th17 differentiation in an APC-free culture system. Surprisingly, IL6 was dispensable in the presence of high concentrations of IL1β. Overall, this is the first study to provide a comprehensive analysis of TLR ligand-activated DCs with regard to their cytokine profile in relation to their ability to induce Th17 differentiation in human naïve $CD4^+$ T cells. We also describe the previously

underappreciated ability of IL1β in the presence of TGFβ as minimal requirements for human Th17 differentiation.

2. Results

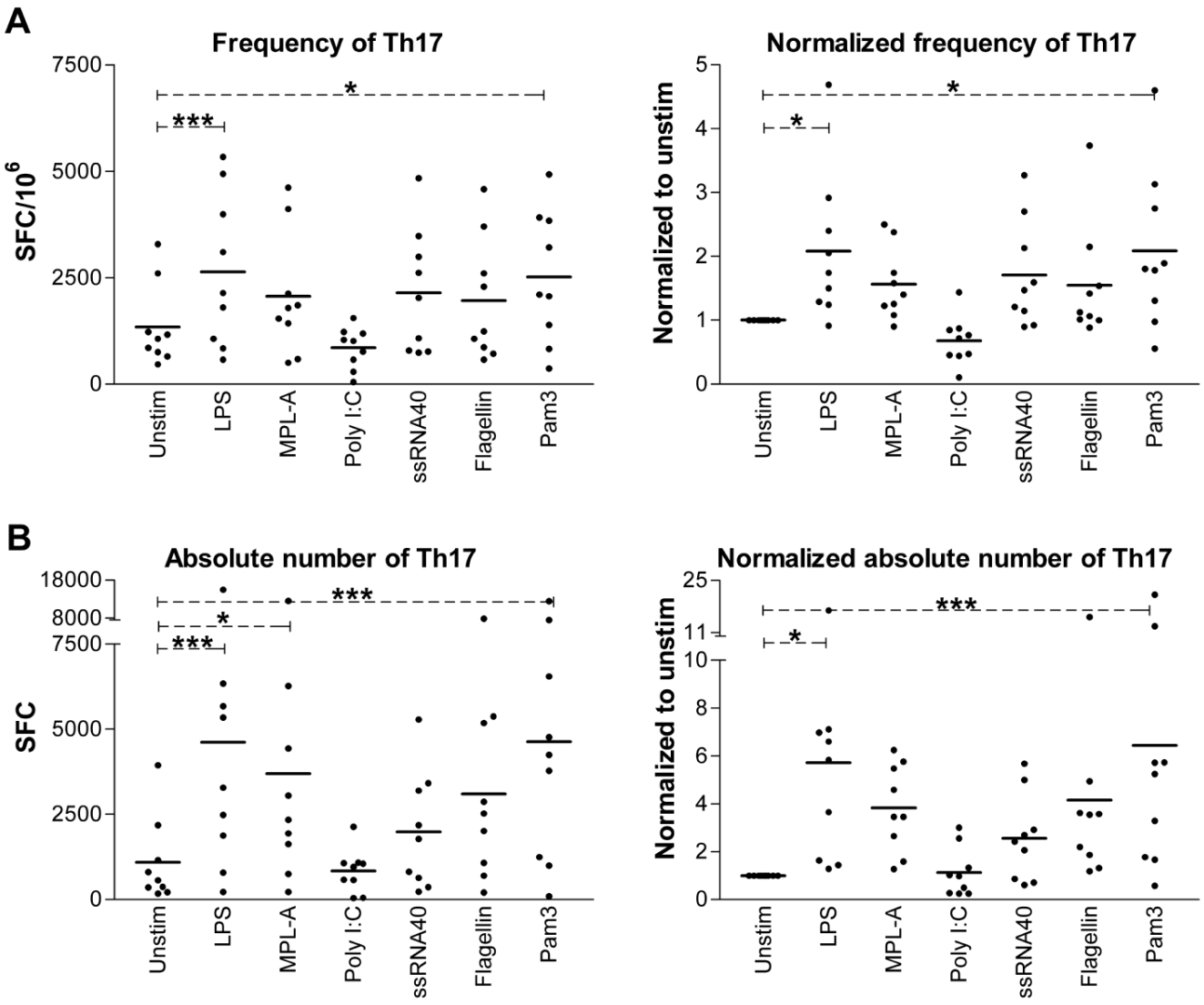
A. Th17 Differentiation in TLR Ligand-activated DC/Naïve $CD4^+$ T Cell Cocultures

To better understand how to induce beneficial Th17 cells in immunity against pathogens in humans, we determined if TLR ligand-activated moDCs were capable of inducing Th17 differentiation of human naïve $CD4^+$ T cells. moDCs were first activated with different TLR ligands for 24 hours and cocultured with freshly isolated allogeneic naïve ($CD45RO^-$) $CD4^+$ T cells in serum-free medium for 7 days. Serum-free medium was used for all of the experiments done in this study to avoid the presence of exogenous TGFβ in the cocultures, since fetal bovine serum contains bovine TGFβ (identical to human TGFβ (Van Obberghen-Schilling et al., 1987). We tested four bacterial TLR ligands (LPS, TLR4 ligand; MPL-A, TLR4 ligand; flagellin, TLR5 ligand and Pam3CSK4, TLR1/2 ligand) and two viral TLR ligands (poly I:C, double-stranded RNA, TLR3 ligand and ssRNA40, single-stranded RNA, TLR7/8 ligand). The primed T cells were then assayed for the frequency of Th17 cells using IL17 ELISPOT (Figure 14A). The frequencies of Th17 cells induced with TLR ligand-activated DCs were normalized to that with unstimulated DCs to control for the magnitude of the allogeneic responses (number of class II MHC mismatches between the moDC donors and T cell donors) in the cocultures. We previously showed that different TLR ligand-activated moDCs induce disparate patterns of Th1/Th2 differentiation in this coculture system (Chapter III). To consider the effects of TLR ligand-activated DCs on both T cell differentiation and

proliferation, we estimated the absolute number of Th17 cells resulting from the 7 day-cocultures based on the frequencies of Th17 cells and the total cell counts (Figure 14B). DCs activated with the two bacterial TLR ligands, LPS and Pam3CSK4, induced significant levels of Th17 differentiation of human naïve $CD4^+$ T cells as measured by both the frequencies and the absolute numbers, correlating with previous reports by others, which indicated that the Th17 response is vital to effective immunity against many bacteria and some fungi. DCs activated with other bacterial TLR ligands, MPL-A and flagellin, as well as one of the viral TLR ligands, ssRNA40, also induced higher levels of Th17 differentiation than unstimulated DCs (though these differences were not statistically significant). Although the role of Th17 cells in anti-viral immunity is still being determined, this observation suggests that Th17 responses may be involved in immunity against certain viruses. In contrast, DCs activated with the other viral TLR ligand tested here, poly I:C, failed to induce any observable increase in Th17 differentiation over unstimulated DCs. These results indicate that DCs activated with bacterial TLR ligands are effective inducers of human Th17 differentiation. αIFNγ and αIL4 antibodies were also added to the APC-free cultures, since it was shown that Th1 and/or Th2 cells suppress Th17 differentiation *in vitro* (Harrington et al., 2005; Park et al., 2005). The αIFNγ/αIL4 treatment of the APC-free cultures did result in a slight increase (~5%) in frequencies of Th17 cells (data not shown). However, to demonstrate more physiologically relevant effects of TLR ligand-activated moDCs in the cocultures, further coculture experiments were performed in the absence of these neutralizing antibodies.

Figure 14. Th17 Differentiation Induced by TLR Ligand Activated DCs

Naïve $CD4^+$ T cells were cocultured with moDCs activated with TLR ligands for 7 days at a 1:10 DC:T ratio in X-VIVO15 medium. The resulting differentiated T cells were restimulated with PMA/ionomycin for 10 hours and assayed for the frequency of IL17-producing cells by ELISPOT in duplicate wells. Data are shown from nine experiments (all different DC donor/T cell donor combinations) and analyzed using repeated measurement one-way ANOVA with Dunnett's post-test (unstimulated as control). A, Frequency of IL17-producing cells per one million cells is shown as spot-forming cells (SFC)/10^6. The frequency was also normalized to the frequency induced by unstimulated DCs to control for the number of HLA mismatch between DC donors and T cell donors in each experiment (normalized frequency). B, Absolute number of IL17-producing cells per coculture is shown. The absolute number was also normalized to that induced by unstimulated DCs. *$p<0.05$, ***$p<0.001$.


A
Frequency of Th17
SFC/10^6
7500
5000
2500
0
***
*
Unstim
LPS
MPL-A
Poly I:C
ssRNA40
Flagellin
Pam3
Normalized frequency of Th17
Normalized to unstim
5
4
3
2
1
0
*
*
B
Absolute number of Th17
SFC
18000
8000
7500
5000
2500
0
***
*
***
Normalized absolute number of Th17
Normalized to unstim
25
11
10
8
6
4
2
0
***
*

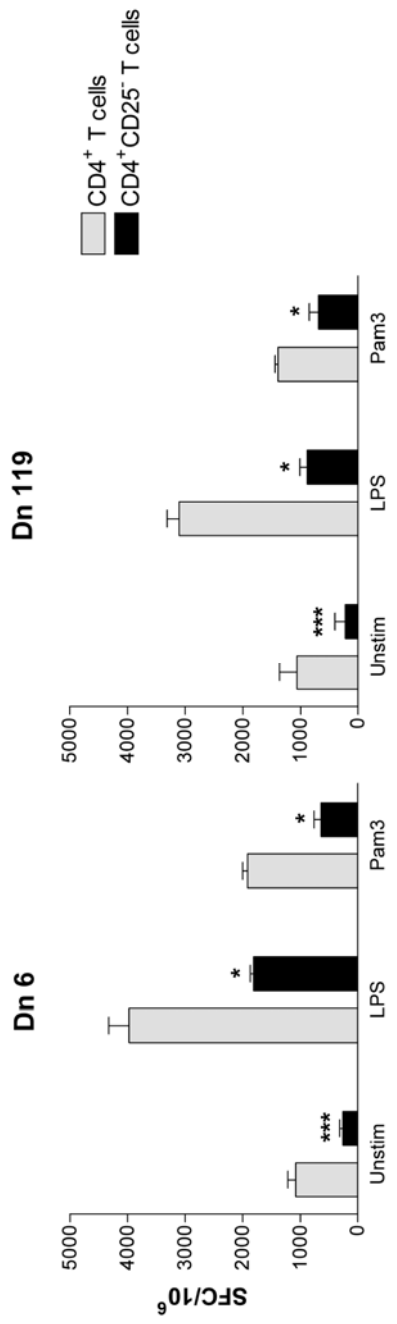
B. $CD4^+CD25^+$ T Cells Play a Major Role in Th17 Differentiation in the TLR Ligand-activated DC/Naïve $CD4^+$ T Cell Coculture System

The majority of human T regulatory cells that produce TGFβ are thought to be contained in the $CD4^+CD45RO^+$ T cell population (Baecher-Allan et al., 2001; Yi et al., 2006; Cools et al., 2007). CD45 can be found as two different isoforms in humans, and they are expressed in a mutually exclusive matter: the CD45RO isoform is expressed primarily on activated and memory T cells, and the CD45RA isoform primarily on naïve T cells. CD25 is also expressed on activated T cells along with CD45RO. Interestingly, recent publications have suggested that the $CD4^+CD45RA^+$ T cell population contains some $CD25^+$ cells, and these cells are indeed T regulatory cells with suppressive functions (Seddiki et al., 2006). Earlier reports on human Th17 cells suggested that the process of human Th17 differentiation differed from that of the murine counterpart, since the latter requires both IL6 and TGFβ whereas the former was suggested to be independent of TGFβ (Acosta-Rodriguez et al., 2007a; Wilson et al., 2007; Chen et al., 2007b). Instead it was reported that TGFβ actually inhibits human Th17 differentiation, which further conflicted with the findings in mice mentioned earlier. More recent reports on human Th17 differentiation indicated that TGFβ is indeed required for human Th17 differentiation (Manel et al., 2008; Volpe et al., 2008; Yang et al., 2008a; Gerosa et al., 2008). Therefore, to confirm that TGFβ is indeed required for human Th17 differentiation and to identify the putative primary cellular source of TGFβ, we examined whether the population of $CD4^+CD45RA^+CD25^+$ T cells play a major role in human Th17 differentiation in our DC/T cocultures. To test this, we cocultured moDCs activated with either LPS or Pam3CSK4 with either naïve $CD4^+$ T cells

($CD4^+CD45RA^+CD45RO^-$) or CD25-depleted naïve $CD4^+$ T cells ($CD4^+CD25^-CD45RA^+CD45RO^-$), and determined the frequency of Th17 differentiation. LPS and Pam3CSK4 were selected to activate DCs, because these TLR ligand-activated DCs induced the highest levels of Th17 differentiation in the coculture system as described above. 3-5% of $CD4^+CD45RA^+$ T cell population was $CD25^+$ (determined by surface staining), and $CD25^+$ cell-depletion decreased the frequency to 0.3-0.5%, achieving 90% reduction in $CD25^+$ cell numbers (data not shown). The frequency of Th17 differentiation decreased significantly with CD25-depletion in cocultures using both LPS- and Pam3CSK4-activated DCs as well as unstimulated DCs (Figure 15). Since $CD4^+CD25^+CD45RO^-$ T cells in our coculture system are critical for efficient Th17 differentiation and since human $CD4^+CD25^+$ T regulatory cells can produce TGFβ, our results support the model that $CD4^+CD25^+CD45RO^-$ T cells act as a major source of TGFβ in our coculture system for human Th17 differentiation.

Figure 15. $CD4^+CD25^+$ T Cells Play a Major Role in Th17 Differentiation in DC/T Cell Cocultures

TLR ligand-activated DCs were cocultured with naïve $CD4^+$ T cells from which $CD25^+$ cells had been depleted or not ($CD4^+$ T cells vs. $CD4^+CD25^-$T cells). After 7 days, the resulting differentiated T cells were restimulated with PMA/ionomycin for 10 hours and assayed for the frequency of IL17-producing cells by ELISPOT in duplicate wells. The results are shown as frequency of IL17-producing cells per one million cells ($SFC/10^6$). Statistical analysis was done on the two experiments combined using unpaired one-tailed Student's t test. *$p<0.05$, ***$p<0.001$.


CD4+ T cells
CD4+CD25- T cells
Dn 6
Dn 119
SFC/10^6
5000
4000
3000
2000
1000
0
Unstim
LPS
Pam3
***
*

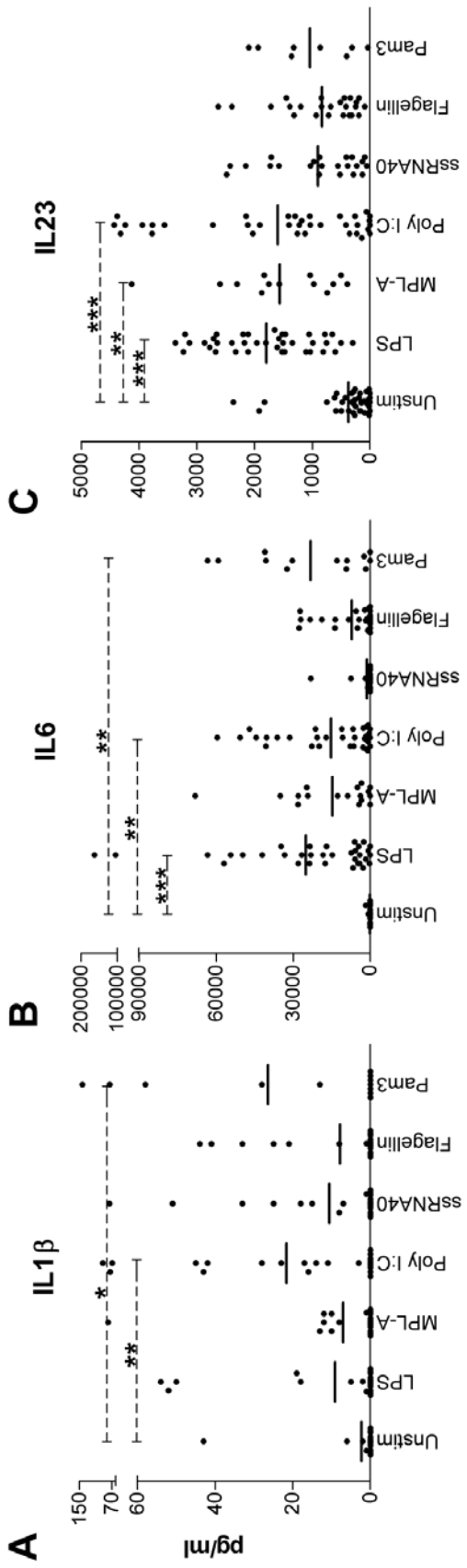
C. Cytokines Produced by Human moDCs upon Stimulation with TLR Ligands

To better understand how TLR ligand-activated moDCs regulate human Th17 responses in our DC/T cell cocultures, we determined the cytokine profile of moDCs upon activation with the selected TLR ligands to correlate the level of Th17 differentiation in the cocultures with the levels of DC-derived cytokines. We measured the production of IL1β, IL6, and IL23, all of which have been implicated involved in Th17 differentiation in mice and/or humans (Manel et al., 2008; Volpe et al., 2008; Yang et al., 2008a; Gerosa et al., 2008; Sutton et al., 2006; Mangan et al., 2006; Veldhoen et al., 2006; Ivanov et al., 2006; Li et al., 2007b; Kimura et al., 2007; Bettelli et al., 2008; Romagnani, 2008; Kryczek et al., 2007a; Zhou et al., 2007). Poly I:C- and Pam3CSK4-activated DCs produced low levels of IL1β but significantly more than unstimulated DCs; the other TLR ligands used here also induced low levels of IL1β (Figure 16A). Monocytes were used as a positive control for IL1β production, and they produced up to 2 ng/ml IL1β when stimulated with LPS (data not shown). These data agree with previous report that TLR ligand-activated DCs (myeloid or monocyte-derived) produce significantly decreased amounts of IL1β than monocytes (Acosta-Rodriguez et al., 2007a). LPS- and Pam3CSK4-, as well as poly I:C-activated DCs produced 20-25 ng/ml IL6. Notably, DCs activated with ssRNA40 consistently produced significantly decreased amounts of IL6 compared to other TLR ligand-activated DCs. In addition, LPS-, MPL-A-, and poly I:C-activated DCs produced significant amounts of IL23 (Figure 16C). DCs stimulated with the other TLR ligands tested (ssRNA40, flagellin and Pam3CSK4) also produced ~1 ng/ml IL23. Overall, poly I:C activation of DCs induced production of all three cytokines determined (IL1β, IL6 and IL23), whereas LPS induced IL6 and IL23 (and

lower levels of IL1β), and Pam3CSK4 induced IL1β and IL6. The cytokine profile of TLR ligand-activated DCs determined here did not seem to correlate with the levels of human Th17 differentiation observed in the coculture experiments (Figure 14), suggesting that other cytokines or factors may be involved in either promoting or inhibiting Th17 differentiation in the cocultures.

Figure 16. Cytokine Profile of TLR Ligand-activated moDCs

moDCs were left unstimulated or stimulated with LPS, MPL-A, poly I:C, ssRNA40, flagellin or Pam3CSK4 for 24 hours. Supernatants were collected and assayed for IL1β, IL6 or IL23 production by ELISA in duplicate wells. Statistical analysis was done using one-way ANOVA with Dunnett's post-test (unstimulated as control). **A**, IL1β ELISA. Data from 22 experiments (seven donors) with LPS, MPL-A, poly I:C, ssRNA40 and flagellin, and 12 experiments (six donors) with Pam3CSK4 are shown. *$p<0.05$, **$p<0.01$. **B**, IL6 ELISA. Data from 34 experiments (eight donors) with LPS and poly I:C, 17 experiments (seven donors) with MPL-A, 26 experiments (eight donors) with ssRNA40, 25 experiments (eight donors) with flagellin, and 13 experiments (five donors) with Pam3CSK4 are shown. **$p<0.01$, ***$p<0.001$. **C**, IL23 ELISA. Data from 39 experiments (eight donors) with LPS, 13 experiments (six donors) with MPL-A, 36 experiments (eight donors) with poly I:C, 22 experiments (eight donors) with ssRNA40, 24 experiments (eight donors) with flagellin, and 8 experiments (four donors) with Pam3CSK4 are shown. **$p<0.01$, ***$p<0.001$.

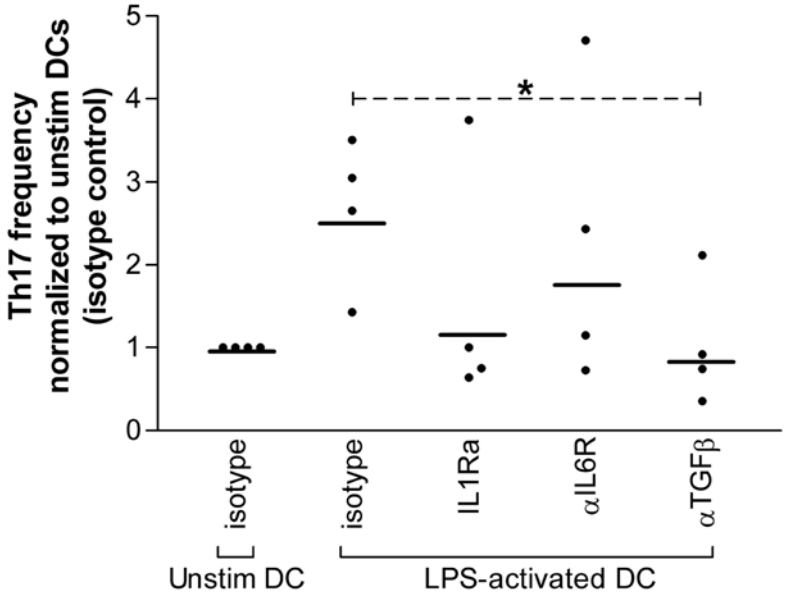

A
IL1β
pg/ml
150
70
60
40
20
0
B
IL6
200000
100000
90000
60000
30000
0
C
IL23
5000
4000
3000
2000
1000
0
Unstim
LPS
MPL-A
Poly I:C
ssRNA40
Flagellin
Pam3
*
**
***

D. Direct Evidence that TGFβ and IL1β are Required for the Th17 Differentiation in the TLR Ligand-stimulated DC/Naïve $CD4^+$ T Cell Cocultures

As described above (Figure 16), we were unable to establish the correlation between DC production of cytokines that were previously reported to be involved in Th17 differentiation of mice and/or humans. IL6 has been widely accepted to be absolutely essential to murine Th17 development in the presence of TGFβ. However, one report showed that IL6 is dispensable when IL1β is present in mice (Kryczek et al., 2007a). To directly examine the involvement of IL1β, IL6 and TGFβ in our DC/T cell cocultures, we neutralized these cytokines using IL1 receptor antagonist (IL1Ra), anti-IL6 receptor antibody (αIL6R) and anti-TGFβ antibody (αTGFβ) (Figure 17). Since the highest frequency of Th17 differentiation was observed in the cocultures using LPS-stimulated moDCs, the neutralization experiments were performed in LPS-stimulated DC/naïve $CD4^+$ T cell cocultures. As expected, αTGFβ significantly abrogated Th17 differentiation (100% inhibition) in the cocultures. IL1Ra also suppressed Th17 differentiation by 80%. In contrast, αIL6R suppressed Th17 differentiation less efficiently (40%). These results suggest that both TGFβ and IL1β integrally, whereas IL6 may only partially, contribute to human Th17 differentiation in these cocultures.

Figure 17. Neutralization of Cytokines in DC/T Cell Cocultures

Naïve $CD4^+$ T cells were cocultured with unstimulated moDCs or LPS-activated moDCs for 7 days at a 1:10 DC:T ratio in the presence of 10 μg/ml isotype control (mouse IgG_1), 1 μg/ml IL1Ra, 10 μg/ml αIL6R, or 10 μg/ml αTGFβ. The resulting differentiated T cells were restimulated with PMA/ionomycin for 10 hours and assayed for the frequency of IL17-producing cells by ELISPOT in duplicate wells. Data from four experiments and their geometric means are shown as frequency of IL17-producing cells normalized to that of coculture with unstimulated DCs and T cells in the presence of the isotype control. Statistical analysis was done using repeated measurement one-day ANOVA with Dunnett's post-test (LPS-activated DC with isotype as control). *$p<0.05$.


Th17 frequency
normalized to unstim DCs
(isotype control)
0
1
2
3
4
5
*
isotype
isotype
IL1Ra
αIL6R
αTGFβ
Unstim DC
LPS-activated DC

E. TGFβ and IL1β are Required for the Differentiation of Human Naive $CD4^+$ T Cells in an APC-free System

To determine the minimum requirements for Th17 differentiation of human naive $CD4^+$ T cells, we first depleted $CD25^+$ T cells from the naive $CD4^+CD45RO^-$ T cell population, and these $CD4^+CD25^-CD45RO^-$ T cells were activated with plate-bound anti-CD3 plus soluble anti-CD28 Abs and cultured in serum-free medium in the absence or presence of human recombinant cytokines (IL1β, IL6, IL23 and TGFβ) alone or in combinations for 7 days. The resulting differentiated T cells were restimulated with PMA/ionomycin and assayed for the frequency of IL17-producing cells by ELISPOT analysis (Figure 18A). IL1β, IL6 or IL23 alone were incapable of inducing differentiation of human Th17 cells in the APC-free cultures, whereas TGFβ alone slightly increased the frequency of Th17 cells. Whereas neither IL1β nor IL1α alone were able to induce significant Th17 differentiation, either in combination with TGFβ induced significantly increased levels of Th17 differentiation compared to TGFβ alone (Figure 18A and data not shown). Although several publications have shown that both IL6 and TGFβ are required for murine Th17 differentiation, the combination of these cytokines did not enhance Th17 frequency compared to TGFβ alone in our human cell culture system. This contrasts with the aforementioned results using TGFβ and IL1β. Furthermore, addition of IL6 did not enhance human Th17 differentiation induced by IL1β and TGFβ. This is interesting because it indicates: 1) only IL1β is required for optimal Th17 differentiation in the presence of TGFβ, whereas IL6 is not required, and 2) it confirms the finding that TGFβ is required for human Th17 differentiation. Therefore, these results demonstrate that IL1β together with TGFβ are the minimum requirements for optimal Th17

differentiation, and that a difference in the requirements for Th17 differentiation exists between mice and humans.

Because TLR ligand-activated moDCs produce very small amounts of IL1β (10-30 pg/ml), we wanted to determine whether small amounts of IL1β in the presence of TGFβ could induce Th17 differentiation in the APC-free system. As shown in Figure 18B, IL1β, at relatively low concentrations (i.e., 3 pg/ml, 30 pg/ml, 300 pg/ml), failed to induce significant levels of Th17 differentiation even in the presence of TGFβ. Interestingly, a slightly increased level of Th17 differentiation that was similar to that seen in the DC/T cell coculture was induced in the presence of TGFβ and a small amount of IL1β (30 pg/ml) when IL6 was also added to the culture. This IL6 dependence was not observed in the presence of higher concentrations of IL1β (300 pg/ml; Figure 18B and 30 ng/ml; Figure 5A). These results explain the neutralization results (Figure 17), in which IL1β and TGFβ were found to be the absolute requirements, but IL6 also partially contributed to Th17 differentiation in the cocultures where only small amounts of IL1β were available.

In the 7 day-APC-free culture system used here, IL23 did not have any detectable effect on Th17 differentiation or expansion even in the presence of other cytokines such as IL1β and TGFβ (Figures 18A and 19). This may be because IL23 acts as a maintenance/expansion, rather than differentiation, factor for Th17 as previously reported (Veldhoen et al., 2006; Zhou et al., 2007). Even when IL23 was also added at a higher concentration (i.e., 100 ng/ml) in addition to IL1β and TGFβ in the APC-free cultures, no significant increase in Th17 frequency was observed (Figure 19). Longer culture time frames perhaps would have allowed us to reveal a maintenance role of IL23 on Th17

cells. However, these results failed to reveal any role of IL23 directly in Th17 differentiation in the APC-free cultures.

Taken together, IL6 is dispensable for human Th17 differentiation if TGFβ and a high concentration of IL1β are present. However, IL6 is required to induce Th17 differentiation if IL1β is present at the lower concentrations observed in our DC/$CD4^+$ T cell cocultures.

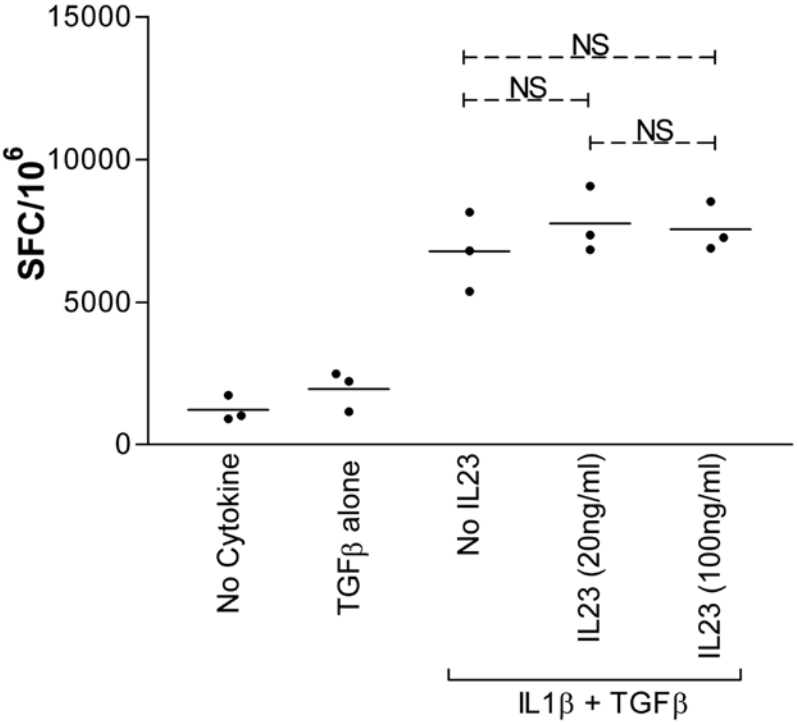
Figure 18. IL1β and TGFβ are the Minimal Requirements for Optimal Th17 Differentiation in Humans

Naïve $CD4^+CD25^-$T cells were stimulated with plate-bound anti-CD3 and soluble anti-CD28 in the absence or presence of human recombinant cytokines (30 ng/ml IL1β, 30 ng/ml IL6, 20 ng/ml IL23 and 5 ng/ml TGFβ) alone or in combinations for 7 days. The resulting differentiated T cells were restimulated with PMA/ionomycin for 10 hours and assayed for the frequency of IL17-producing cells by ELISPOT in duplicate wells. Data are shown as frequency of IL17-producing cells per one million cells ($SFC/10^6$). **A**, Numbers of experiments and donors for the data shown follow: no cytokine (11 experiments, 6 donors), IL1β (8, 6), IL6 (5, 5), IL23 (10, 6), TGFβ (11, 6), IL6 + IL1β (8, 6), IL1β + IL23 (2, 2), IL6 + TGFβ (10, 6), IL23 + TGFβ (5, 5), IL1β + TGFβ (9, 6) and IL1β + IL6 + TGFβ (9, 6). Statistical analysis was done using one-way ANOVA with Tukey post-test. ***$p<0.001$. **B**, Data from three experiments done with three different donors are shown. Statistical analysis was performed using repeated measurement one-way ANOVA with Dunnett's post-test (no cytokine as control). *$p<0.05$, ***$p<0.001$. Student's t test was also performed to compareTh17 differentiation induced by different concentrations of IL1β ± TGFβ. *$p<0.05$.

A
SFC/10^6
15000
10000
5000
0
None
IL1β
IL6
IL23
TGFβ
IL1β + IL6
IL1β + IL23
IL6 + TGFβ
IL23 + TGFβ
IL1β + TGFβ
IL1β + IL6 + TGFβ
***
***
B
15000
10000
5000
0
***
***
*
*
NS
*
NS
None
TGFβ
TGFβ + IL6
TGFβ
TGFβ + IL6
TGFβ
IL1β
3pg/ml
30pg/ml
300pg/ml
30ng/ml

Figure 19. IL23 Does Not Enhance Th17 Differentiation in Humans in the Presence of IL1β and TGFβ

Naïve CD4+CD25-T cells were stimulated with plate-bound anti-CD3 and soluble anti-CD28 in the absence or presence of human recombinant cytokines (IL1β, IL23 and TGFβ) alone or in combinations for 7 days. The resulting differentiated T cells were restimulated with PMA/ionomycin for 10 hours and assayed for the frequency of IL17-producing cells by ELISPOT in duplicate wells. Data are shown as frequency of IL17-producing cells per one million cells (SFC/106). Statistical analysis was performed using repeated measurement one-way ANOVA with Tukey post-test.


15000
10000
5000
0
SFC/10^6
NS
NS
NS
No Cytokine
TGFβ alone
No IL23
IL23 (20ng/ml)
IL23 (100ng/ml)
IL1β + TGFβ

3. Discussion

Our current study describes human Th17 differentiation in both APC-free and TLR ligand-activated DC/$CD4^+$ T cell coculture systems. The results suggest that bacterial TLR ligands, such as Pam3CSK4 (TLR1/2 ligand) and LPS (TLR4 ligand), are able to induce the production of cytokines or other factors that direct differentiation of human naïve $CD4^+$ T cells into IL17-producing T helper (Th17) cells. This agrees with previous reports that implicated Th17 responses in anti-bacterial immunity against infections with *Mycobacterium tuberculosis, Mycoplasma pneumoniae, Escherichia coli*, and *Klebsiella pneumoniae* (Matsuzaki and Umemura, 2007; Khader and Cooper, 2008). Furthermore, it has been suggested that Th17 responses are involved in inflammatory bowel diseases as a result of abnormal immune responses to commensal bacteria in the gut. This correlates well with our findings that DCs stimulated with bacterial TLR ligands are capable of directing the differentiation of Th17 cells. Another TLR4 ligand that we tested here, MPL-A, is a non-toxic monophosphoryl lipid A, which is a component of immunologically active lipid A portion of LPS and has been approved as a human adjuvant. Unlike LPS, DCs activated with MPL-A are able to induce some, but not significant, levels of Th17 differentiation. In contrast, DCs activated with flagellin (TLR5 ligand), another bacterial TLR ligand tested, also failed to induce a significant level of Th17 differentiation. It is noteworthy that, of all the bacterial TLR ligands, certain ones may be intrinsically better suited for activating DCs that subsequently induce Th17 differentiation of $CD4^+$ T cells. Unexpectedly, DCs stimulated with ssRNA40, a viral TLR ligand for TLR7/8, also induced Th17 differentiation, whereas Th17 differentiation was not induced by poly I:C-activated DCs. We have previously shown

that poly I:C-activated DCs, but not ssRNA40-activated DCs, produce significant levels of IL12p70, which is the key DC-derived cytokine that promotes Th1 differentiation of $CD4^+$ T cells (Chapter III). In that study, only poly I:C-activated DCs were able to induce heavily skewed Th1 responses in $CD4^+$ T cells, whereas more relatively balanced Th1/Th2 responses were seen using ssRNA40-activated DCs. Moreover, a recent report showed that IL12p70 can directly suppress Th17 differentiation (Hoeve et al., 2006). Collectively, these results suggest that activation of moDCs with poly I:C leads to the highest IL12p70 production among all the TLR ligands tested here, thereby resulting in heavily skewed Th1 responses. IFNγ produced by these Th1 cells, together with IL12p70, suppress both Th17 and Th2 responses. On the other hand, IL12p70 production by ssRNA40-activated DCs is insufficient to drive heavily skewed Th1 responses, allowing a relatively more balanced Th1/Th2 response, which would permit Th17 differentiation as well. Although ssRNA40 and poly I:C are both viral TLR ligands, there is a substantial difference in how they stimulate DCs, which then leads to disparate $CD4^+$ T cell responses.

We also showed that $CD4^+CD25^+$ T cells play a major role in Th17 differentiation in the DC/T cell cocultures. Although we did not directly show that these cells contribute to the differentiation by being the major producer of TGFβ, current evidence in the literature strongly suggests that $CD4^+CD25^+$ T cells are T regulatory cells with suppressive function due to their production of TGFβ (Yi et al., 2006; Cools et al., 2007). Therefore, we favor the model that $CD4^+CD25^+$ $CD45RO^-$ T cells provide TGFβ in our DC/T cell cocultures. In addition, we used serum-free medium in both APC-free and DC/T cell coculture systems, since fetal bovine serum contains bovine TGFβ, which is

identical to the human counterpart. It is possible that, in previous reports which suggested that TGFβ was not required for human Th17 differentiation (Acosta-Rodriguez et al., 2007a; Wilson et al., 2007; Chen et al., 2007b), TGFβ was already present in the cultures due to the presence of either TGFβ-producing $CD25^+$ T cells or TGFβ-containing bovine serum in the culture medium (data not shown). However, the frequencies of Th17 cells observed in our DC/T cell cocultures in the presence of FBS were decreased overall compared to those observed in serum-free culture medium. Furthermore, in the APC-free culture system, Th17 differentiation was substantially suppressed in the presence of FBS (data not shown). Therefore, unknown factor(s) in serum may suppress Th17 differentiation, and these observations agree with a previous report (Manel et al., 2008).

Manel et al. reported that RORγt-overexpressing human cord blood $CD4^+$ T cells were able to differentiate into Th17 cells in the presence of TGFβ with IL1β, IL6 or IL21 (Manel et al., 2008). However, in non-transduced cord blood $CD4^+$ T cells, the combination of TGFβ, IL1β and IL23 induced the highest levels of Th17 differentiation in their APC-free cultures. Volpe et al. also demonstrated that TGFβ, IL1β and IL23 were required for the highest levels of IL17 production by $CD4^+CD45RA^+$ T cells (Volpe et al., 2008). Furthermore, Gerosa et al. showed that IL1β was absolutely required for Th17 differentiation of $CD4^+CD45RO^-$ T cells, whereas TGFβ and IL6 were only partially required (Gerosa et al., 2008). They also showed that IL1β together with IL23 in the presence of the supernatant from a culture of TLR ligand-activated DCs induced the highest level of IL17 production by the $CD4^+CD45RO^-$ T cells. Overall, these previous reports have elucidated the cytokine requirements in and differences between human and murine Th17 differentiation, but there is no consensus on the minimum

cytokine requirements for human Th17 differentiation. In this study, we demonstrated that both IL1β and TGFβ are absolutely required for induction of Th17 differentiation of αCD3/αCD28-activated naive $CD4^+CD25^-CD45RO^-$ T cells in an APC-free system (Figure 18A). Our data also indicated that IL6 is required only when IL1β is present at relatively low concentrations, which is typically the case in DC/T cell cocultures (Figures 18B). IL23, even at a higher concentration, did not significantly increase the levels of Th17 differentiation in our APC-free culture system, which conflicts with two recent reports (Manel et al., 2008; Volpe et al., 2008) (Figure 19). However, this may be due to the differences in types of cells used (cord vs. adult $CD4^+$ T cells), durations of culture, and the readout systems used. A report by Acosta-Rodriguez et al. implicates a role for monocytes, which produce large amounts of IL1α and IL1β upon stimulation with TLR ligands, in Th17 differentiation in human naïve $CD4^+$ T cells (van Beelen et al., 2007). A previous publication has shown that monocytes are able to migrate from peripheral tissues into the T cell-zone of draining lymph nodes upon stimulation (Randolph et al., 1999). Therefore, it is possible that activated monocytes are involved in Th17 differentiation by providing large amounts of IL1 in the lymph nodes, where activated DCs can present antigens to prime T cells together with TGFβ produced by T regulatory cells to direct Th17 differentiation. Alternatively, monocytes may not be required for Th17 differentiation in the lymph nodes, since small amounts of IL1 together with IL6 produced by activated DCs in the presence of TGFβ are capable of inducing Th17 differentiation as described above. Furthermore, DCs are the predominant APC found in lymph nodes and the only cells that are capable of priming T cells efficiently. Therefore, manipulating activation of DCs with TLR ligands is an effective way to tailor human

Th17 responses by enhancing or suppressing their differentiation. Finally, recent reports on human Th17 differentiation (Manel et al., 2008; Volpe et al., 2008; Yang et al., 2008a; Gerosa et al., 2008; Acosta-Rodriguez et al., 2007a; Wilson et al., 2007; Chen et al., 2007b) have contradicted earlier reports (Acosta-Rodriguez et al., 2007a; Wilson et al., 2007; Chen et al., 2007b) that suggested that TGFβ was not required for human Th17 differentiation. Certainly, the results provided here strongly agree that TGFβ is absolutely required for human Th17 differentiation as it is in mice.

This is the first report of comprehensive analysis of the ability of TLR ligand-activated DCs to induce Th17 differentiation in naïve $CD4^+$ T cells. TLR1/2 ligands or the FDA-approved TLR4 ligand vaccine adjuvant, MPL-A, may be able to induce/enhance Th17 responses in either prophylactic vaccines or therapeutic treatments against certain bacterial, fungal, and viral pathogens. Because of the pathogenic effects of Th17 cells in autoimmune disease, extreme caution would need to be exercised in the selection of appropriate vaccine adjuvants for patients with susceptibility to or pre-existing autoimmune disease(s). For such individuals, milder Th17 inducers, such as TLR5 or TLR7/8 ligands may be used to induce Th17 responses that might be sufficient to combat infections without initiating or worsening autoimmune diseases. To induce appropriate immunity against infections with certain viruses that require a strong Th1 response, TLR3 ligands may be used to induce heavily skewed Th1 responses while hindering Th2 and Th17 responses, whereas TLR7/8 ligands may be used instead to induce anti-viral immunity against viruses that requires both Th1 and Th17 responses. Furthermore, certain TLR ligands may be used as immunomodulators to suppress autoimmune diseases by diverting Th17 differentiation. In addition, suppression of IL1,

instead of IL6, with an IL1 antagonist such as anti-IL1 or IL1Ra, may be an effective treatment for autoimmune disease in humans in combination. We conclude that these results provide a framework for the use of TLR ligands as vaccine adjuvants to induce Th17 responses against infections or treatments to suppress pathological autoimmune responses.

CHAPTER V

Summary and Future Directions

The main goal of this study was to determine the effects of TLR ligands on human DCs, as well as the subsequent effects of these TLR ligand-activated DCs on naïve $CD4^+$ T cells, to provide better understanding of the possible use of TLR ligands as vaccine adjuvants to manipulate primary $CD4^+$ T cell responses. We utilized moDCs as a model for human myeloid/conventional DCs and examined the effects of ligands for the TLRs expressed by these cells (i.e., TLR1/2, TLR3, TLR4, TLR5, TLR7/8).

Initially, the most notable difference between differentially TLR ligand-activated moDCs was seen in IL12p70 production by these cells (Figures 8 and 9). Viral TLR ligand (poly I:C and ssRNA40)-activated moDCs from not all, but certain donors, induced production of detectable levels of IL12p70, whereas bacterial TLR ligand (LPS and flagellin)-activated moDCs from all donors tested failed to do so. This agrees with a widely accepted observation that strong Th1 responses are essential to immunity against viral infection. However, it was unexpected to observe that moDCs from certain donors failed to produce detectable levels of IL12p70 when activated with viral TLR ligands. Since the discrepancy between the donors in terms of IL12p70 production induced by viral TLR ligand-activated moDCs was observed repeatedly, we arbitrarily divided the panel of donors into high responders (HRs) and low responders (LRs) for later experiments to investigate whether LR-moDCs were intrinsically distinct from HR-moDCs in terms of inducing Th1 responses in naïve $CD4^+$ T cells. In addition, we examined the upregulation of costimulatory molecules, CD80, CD86, and CD40, and the

MHC class II molecule, HLA-DR, on moDCs upon activation with LPS, a bacterial TLR ligand, or poly I:C, a viral TLR ligand. However, both LPS- and poly I:C-activated moDCs from LRs and HRs exhibited similar levels of CD80, CD86, CD40 and HLA-DR upregulation (Figure 10 and data not shown). LPS-activated moDCs in the presence of recombinant CD40 ligand also failed to produce IL12p70 (data not shown). Furthermore, activation of HR- or LR-moDCs with LPS or poly I:C did not result in differential levels of cell death within 24 hours post-stimulation (data not shown). Therefore, the differential IL12p70 production by bacterial or viral TLR ligand-activated, or at least LPS- or poly I:C-activated moDCs, is not due to differential maturation or cell death induced by these TLR ligands. IL12p70 production appears to be regulated in multiple ways. In the case of LPS vs. poly I:C activation, poly I:C activation induces upregulation of both IL12p35 and IL12p40 subunit mRNA upregulation, whereas LPS activation leads to significant IL12p40 but minimal IL12p35 mRNA upregulation in moDCs (data not shown). DCs activated with LPS in combination with IFNγ have been shown to produce large amounts of IL12p70 (Frasca et al., 2008). In the presence of IFNγ, IL12p35 mRNA was significantly upregulated in LPS-activated moDCs (data not shown), suggesting the absence of IL12p35 mRNA upregulation results in lack of detectable levels of IL12p70 production in LPS-activated moDCs. Moreover, these results suggest that regulation of IL12p35 mRNA expression determines IL12p70 production in TLR ligand-activated DCs. Finally, in the case of HR- vs. LR-moDCs, LR-moDCs produced significantly decreased amounts of IL12p40 subunit compared to that of HR-moDCs (Figure 9), suggesting that IL12p40 availability also contributes to regulation of IL12p70 production in these cells.

To determine how the differentially TLR ligand-activated moDCs influence differentiation of naïve $CD4^+$ T cells, DC/T cell cocultures were set up to allow TLR ligand-activated moDCs to directly interact with allogeneic naïve $CD4^+$ T cells to induce their differentiation into Th1 or Th2 cells (Figure 12). Several interesting observations came to light. First, HR- or LR-moDCs activated with all TLR ligands tested in these experiments (LPS, poly I:C, ssRNA40 and flagellin) induced Th1 differentiation of naïve $CD4^+$ T cells even in the absence of detectable levels of IL12p70 production. Interestingly, DCs activated with LPS have been shown to produce large amounts of IL12p70 if IFNγ is present as mentioned earlier, and this may be at least partially due to the IFNγ suppression of IL10 production by the DCs (Frasca et al., 2008). In fact, LPS-activated moDCs produced IL10, and this may explain the lack of detectable levels of IL12p70 production by LPS-activated moDCs as IL10 has been shown to inhibit IL12p70 production (de Smedt et al., 1997; Fukao et al., 2001). However, LPS-activated moDCs were unexpectedly capable of inducing Th1 differentiation of $CD4^+$ T cells. LR-moDCs activated with TLR ligands also induced similar levels of Th1 responses in $CD4^+$ T cells despite their inability to produce detectable levels of IL12p70. Second, bacterial TLR ligand-activated moDCs also induced Th2 differentiation of naïve $CD4^+$ T cells. When the levels of Th1 and Th2 differentiation were compared as Th1/Th2 ratios, it was notable that poly I:C-activated moDCs showed significantly greater Th1 skewing, whereas LPS- and flagellin-activated moDCs induced relatively more balanced Th1/Th2 responses. Third, ssRNA40-activated moDCs also induced more balanced Th1/Th2 responses despite their increased levels of IL12p70 production. We had predicted that ssRNA40-activated moDCs would induce highly Th1 skewed $CD4^+$ T cell responses,

similar to poly I:C-activated moDCs, since ssRNA40 is a viral TLR ligand derived from HIV-1 genome. The significance of the effects of ssRNA40 activation of DCs remains to be elucidated. Finally, IL12p70 is known to be the key cytokine produced mainly by APCs for Th1 differentiation of $CD4^+$ T cells. However, other cytokines, such as type I IFNs, are also capable of inducing STAT4 activation and subsequent IFNγ production by $CD4^+$ T cells in humans, but not in mice (Nguyen et al., 2000; Farrar et al., 2000). We measured IFNβ mRNA upregulation in HR- and LR-moDCs activated with LPS or poly I:C. In the presence of IFNγ, IFNβ mRNA was further upregulated in LPS-activated moDCs (data not shown). We also detected upregulation of IFNβ mRNA in both LPS- and poly I:C-activated DCs (data not shown). In addition, the frequencies of Th1 cells in the DC/T cell cocultures were significantly decreased when IL12p70 was neutralized using anti-IL12p70 antibody (Figure 13). Therefore, our results clearly shows that in all TLR ligand-activated LR-and HR-moDC/naïve CD4+ T cell cocultures, even undetectable amounts of IL12p70 plays a predominant role in Th1 differentiation. Taken together, all TLR ligands tested in these experiments induce activation of moDCs, which subsequently results in Th1 responses, whereas bacterial TLR ligand- as well as ssRNA40-activated moDCs also promoted Th2 responses, suggesting that poly I:C is a superior adjuvant for the activation of moDCs to direct highly skewed Th1 responses in $CD4^+$ T cells. Furthermore, there appears to be no functional difference between HR- and LR-moDCs in terms of their ability to induce Th1 or Th2 responses in naïve $CD4^+$ T cells. It is still unknown if there is any functional difference between HR- and LR-DCs *in vivo* or what contributes to the difference in their IL12p70 production *in vitro*. It is possible LR-moDCs failed to produce detectable levels of IL12p70 due a deficiency in

TLR3 expression in these cells. However, no significant differences in TLR3 expression between HR- and LR-DCs were observed by flow cytometric analysis (intracellular staining; data not shown). However, the anti-TLR3 antibody used does not discriminate different forms of TLR3 resulting from polymorphism. Therefore, TLR3 polymorphism may contribute to the differences in IL12p70 production by poly I:C-activated moDCs from certain donors. Nonetheless, small amounts of IL12p70 seem to be capable of inducing Th1 responses in the DC/T cell cocultures.

Differentiation of naive $CD4^+$ T cells into Th17 cells, a recently identified T helper subset distinct from Th1 or Th2, induced by TLR ligand-activated moDCs was also examined. Th17 cells have previously reported to be involved in autoimmunity and immunity against infections with many pathogens. However, the effects of differentially TLR ligand-activated moDCs on naïve $CD4^+$ T cells have not been reported. Using a similar procedure to our previous experiments, we showed that moDCs activated with bacterial TLR ligands tested (LPS, MPL-A, flagellin and Pam3CSK) induced higher levels of Th17 differentiation (Figure 14). This correlates with previous reports that Th17 responses are essential to immunity against infection by several bacteria and fungi. ssRNA40-activated moDCs also induced some $CD4^+$ T cells to differentiate into Th17 cells, whereas poly I:C –activated moDCs failed to do so. This further emphasizes our earlier findings that the TLR3 and TLR7/8 ligands, both of which are viral TLR ligands, have distinct effects on moDCs, and poly I:C-activated moDCs induce strong Th1 responses, whereas ssRNA40-activated moDCs induce more balanced Th1/Th2/Th17 responses. To correlate the cytokine profile and the levels of Th17 differentiation promoted by TLR ligand-activated moDCs, we measured production of DC-derived

cytokines that were previously reported to be involved in mouse Th17 differentiation, such as IL1β, IL6 and IL23 (Figure 16). Surprisingly, there was not a direct correlation between the patterns of cytokine production and the levels of Th17 differentiation. To better understand the cytokine requirements for human Th17 differentiation, we set up APC-free $CD4^+$ T cell culture system, in which $CD4^+$ T cells were stimulated via the TCR and CD28 using anti-CD3 and anti-CD28 antibodies in the absence or presence of exogenous recombinant cytokines (TGFβ, IL1β, IL6 and IL23) alone or in combinations. Our data clearly demonstrated that IL1β and TGFβ are the minimal requirement for human Th17 differentiation. $CD4^+CD25^+$ T regulatory cells play a major role in Th17 differentiation in our cocultures, probably by providing TGFβ (Figures 15 and 17). Unexpectedly, IL23 was not involved in Th17 differentiation, despite recent reports to the contrary (Figures 18 and 19). We used anti-IL23p19 antibody to neutralize IL23 activity in the DC/T cell cocultures but failed to observe a change in the frequencies of Th17 cells (data not shown). This observation agrees with our APC-free culture experiments, which failed to demonstrate a role for IL23 in Th17 differentiation. However, we did not have a proper positive control for IL23 neutralization, and the results need to be confirmed. In addition, IL6 is not required for Th17 differentiation in the APC-free system when high concentrations of IL1β are present. However, if only small amounts of IL1β are available in DC/T cell cocultures, IL6 works in synergy with IL1β in the presence of TGFβ to induce Th17 differentiation (Figure 18). Previous reports in both mice and humans have suggested that IL6 via STAT3 phosphorylation selectively suppresses TGFβ-mediated upregulation of the T regulatory cell-specific transcription factor, Foxp3, resulting in the expression of Th17-specific transcription factor, RORγt, though the exact mechanism for

STAT3-mediated IL6-dependant suppression of Foxp3 is unknown (Ichiyama et al., 2008; Manel et al., 2008; Yang et al., 2007). It is not known how IL1β contributes to human Th17 differentiation in our DC/T cell cocultures or APC-free cultures. Therefore, to understand the mechanisms by which, IL1β and TGFβ mediate human Th17 differentiation, it will be important to determine 1) whether TGFβ in the presence or absence of IL1β induces RORγt upregulation and/or phosphorylates STAT3, 2) whether STAT3 is required for IL1β-mediated Foxp3 downregulation, and 3) how IL1β/STAT3 selectively suppresses Foxp3 to allow RORγt expression in naïve $CD4^+$ T cells. It is possible that IL1β, at least in terms of Th17 differentiation, is a dominant STAT3-mediated inducer of Th17-specific genes, since IL1β- and IL6-dependent NFκB activation is induced via STAT3-mediated signaling pathways (Yoshida et al., 2004).

Esther de Jong's group has demonstrated that moDCs activated by TLR ligands in the presence of muramyl dipeptide (MDP), a ligand for nucleotide oligomerization domain 2 (NOD2), an intracellular PRR, induce increased levels of IL17 production by memory $CD4^+$ T cells by promoting increased IL1 production (van Beelen et al., 2007). We have measured slightly increased levels of IL1β and IL6 production by moDCs activated with TLR ligands in the presence of MDP, but these experiments need to be repeated. In addition, the effects of moDCs activated with TLR ligands in combination with MDP on naïve $CD4^+$ T cells should be determined in future experiments to demonstrate how NOD2 activation of DCs in synergy with TLR activation modulates not only Th17, but also Th1 and Th2 primary responses in naive $CD4^+$ T cells.

IL27 has been shown to suppress effector Th17 functions by inducing "suppressive" Th17 cells that also produce IL10. Since we had observed IL27p28 and

EBI3 mRNA upregulation in LPS-activated moDCs, we determined whether LPS-activated moDCs induce both "effector" Th17 and "suppressive" Th17 cells by measuring IL17 and IL10 produced by T cells in the cocultures. T cells cocultured with LPS-activated moDCs or any other TLR ligand-activated moDCs failed to produce detectable levels of IL10. IL22 was also examined in these supernatants, since IL22 was previously reported to be Th17-specific cytokine. However, the highest levels of IL22 production were detected in the supernatants from T cells activated with unstimulated allogeneic moDCs. Therefore, our results suggest that IL22 is not a Th17-specific cytokine. IL25 (IL17E) produced by Th2 cells is known to induce production of IL13 by these cells, which in return suppress Th17 functions by suppressing IL1β, IL6 and IL23 production by activated DCs (Kleinschek et al., 2007). We did not examine the production of this cytokine in the DC/T cell cocultures.

The results presented here support the use of viral TLR ligands as adjuvants for vaccines against viral infections. For example, West Nile virus (WNV) is a positive-sense RNA virus that is an emerging infectious agent in the United States and many other countries. Both ssRNA and dsRNA forms of the viral genome exist during infection, since the original positive-sense ssRNA replicates using RNA-dependent RNA polymerase yielding dsRNA. dsRNA can be detected by TLR3 in the endosomes of DCs or cytoplasmic PRR like RIG-1 and MDA5, and this process is required for cross blood-brain barrier and to enter central nervous system during WNV infection (Wang et al., 2004). Therefore, it is suggested that a TLR3 and/or RIG-1/MDA5 signaling inhibitor may be effective for treating WNV-induced encephalitis. TLR7/8 agonists may be used to boost Th1 responses against WNV infection in combination with a TLR3 signaling

inhibitor as a therapeutic treatment without activating TLR3 signaling. It is also possible that Th17 responses may contribute to successful anti-viral immunity against WNV, since Th17 responses may be involved in immunity against hepatitis C virus, which is also a positive-sense RNA virus. Since TLR7/8 ligand-activated moDCs were capable of inducing both Th1 and Th17 responses, application of TLR7/8 ligands as a vaccine adjuvant against infections by WNV and/or other viruses may lead to successful prophylaxis or therapeutic treatments of infected individuals. Furthermore, TNFα and IL6 are responsible for neuronal injury induced during the infection caused by WNV. Especially TNFα signaling was shown to disrupt blood-brain-barrier integrity and cause viral entry to the CNS (Wang et al., 2004). According to our results, ssRNA40-activated moDCs produced minimal amounts of TNFα and IL6, and this may minimize the side effects of TLR7/8 ligand used as a vaccine adjuvant *in vivo*.

TLR ligands may also be used as adjuvants for vaccines against bacterial infection such as tuberculosis. During tuberculosis caused by infection by *Mycobacterium tuberculosis* (*Mtb*), both Th1 and Th17 responses are induced. IL17 produced by Th17 cells induces chemokine production in the lungs that promotes recruitment of Th1 cells, which in return suppresses the *Mtb* growth (Khader and Cooper, 2008). Therefore, effective immunity against *Mtb* required both Th1 and Th17 responses. Alum, aluminum hydroxide, is the most widely and traditionally used vaccine adjuvant in humans (McKee et al., 2007). It has been known to promote Th2 responses but has more recently been shown to also induce Th17 responses (He et al., 2007). Alum induces TLR ligand-activated DCs to produce uric acid that acts as a "danger signal", which subsequently induce caspase-1 activation that is required for IL1β secretion (Kool et al., 2008; Li et al.,

2007a). Therefore, it would be interesting to determine whether a combination treatment with both alum and a TLR4 ligand, such as MPL-A, would induce strong Th17 as well as Th1 responses against *Mtb*.

The use of TLR ligands as vaccine adjuvants against infections caused by bacteria that are potentially introduced by bacterial bioterrorism attacks has also been proposed by others. For example, lipid A derivatives, amino-alkyl glucosaminide 4-phosphates (AGPs) used as adjuvants combined with *Yersinia pestis* (Gram-negative Enterobacteria) antigens induced protection 21 days after the first immunization when challenged with *Y. pestis* aerosol in a mouse model, whereas immunization with the antigens in the abence of the TLR ligands failed to provide protection against the infection (Airhart et al., 2008). When synthetic unmethylated CpG motifs (TLR9 ligand) were added as a vaccine adjuvant with Anthrax vaccine adsorbed (AVA), the only vaccine that is FDA-approved for pre-exposure use in humans, it promoted quicker and prolonged anti-*Bacillus anthracis* (Gram-positive facultative anaerobe) immunity and increased the magnitude of protective antibody responses in mice (Tross and Klinman, 2008). Another TLR9 ligand, phosphodiester immunostimulatory oligonucleotide R10-60, a derivative of unmethylated CpG, was shown to delay death in mice that were exposed to *B. anthracis*, which was accompanied with increased transient IL1β production (Wu et al., 2008). The results from these *in vivo* studies using mouse models still need to be successfully translated into the safe and effective use of TLR ligands as vaccine adjuvants against these infections in humans. However, it certainly implicates the beneficial activities of TLR ligands as immunomodulators in this context.

Recent publications have demonstrated that TLR ligands are also capable of modulating memory T cell responses (van Beelen et al., 2007). In fact, it has also shown that some human memory T cells are not terminally differentiated and possess functional flexibility to produce different cytokines depending on the polarizing conditions that they encounter (Ahmadzadeh and Farber, 2002; Ahmadzadeh and Farber, 2002; Grogan et al., 2001; Sallusto et al., 2004). Therefore, it may be possible to redirect autoimmune Th17 memory responses to either Th1 responses using a TLR3 ligand or balanced Th1/Th2/Th17 responses with other TLR ligands, such as either the TLR4 ligand, MPL-A or a TLR7/8 ligand.

One obstacle for the use of TLR ligands as vaccine adjuvants in humans is polymorphism in TLR responses. TLR polymorphisms are known to be linked to susceptibility to many human diseases. Such polymorphisms contribute to differences in TLR-mediated immunity and hence the effectiveness of TLR ligands as vaccine adjuvants. For instance, individuals with TLR4 polymorphisms are associated with hyporesponsiveness to LPS, and hence increased susceptibility to bacterial infections (e.g. meningococcal and pneumococcal infections) and sepsis. TLR2 polymorphisms are associated with increased susceptibility to *Staphylococcus aureus* and *Mtb* infections. TLR5 polymorphisms are associated with increased susceptibility to *Legionella pneumophila* infection and Crohn's disease. TLR7 polymorphisms are associated with increased hepatitis C viral inflammation, and TLR9 polymorphisms with HIV progression (Misch and Hawn, 2008). Polymorphisms in TIRAP, an adaptive molecule involved in MyD88-dependent TLR signaling, were also reported to be associated with

increased susceptibility to *Mtb* infection as well as pneumococcal and malaria infections (Misch and Hawn, 2008).

A single nucleotide polymorphism in a TLR3 exon and two genotypic patterns in the TLR3 gene in Japanese patients were shown to be associated with Stevens-Johnson syndrome, which is a multisystem inflammatory disorder of skin and mucous membranes (Ueta et al., 2007). Although the exact cause of this disease is unknown, previous studies have described the possible etiologic agents including viruses such as HIV, herpes simplex virus, Epstein-Barr virus, influenza virus and coxsackie virus, as well as bacterial, fungal and protozoal agents and allergies to drugs (Hazin et al., 2008). In addition to TLR3 polymorphisms, dysregulation of TLR3-mediated innate immune response is also linked to increased severity of WNV infection seen in elderly individuals (Kong et al., 2008). Macrophages isolated from younger human donors downregulated their TLR3 expression upon infection by WNV, whereas those cells from older donors failed to do so resulting in increased cytokine production by these cells and permeability of the blood-brain barrier and viral entry into the central nervous system.

Abnormal TLR signaling may be caused by deficiencies in molecules involved in the signaling pathways. Both mutations in the genes encoding for NFκB essential modulator (NEMO) and gain-of-function mutation of IκBα lead to impaired NFκB activation and result in increased susceptibility for pyogenic bacterial infections caused by bacteria such as *Streptococcus pneumoniae*, *S. aureus*, *Haemophilus influenzae*. Interleukin-1 receptor associated kinase 4 (IRAK4) deficiency caused by *IRAK4* mutations affects all TLR signaling pathways, except that of TLR3, causing increased susceptibility to *S. pneumoniae* and *S. aureus* (Turvey and Hawn, 2006). Therefore, the

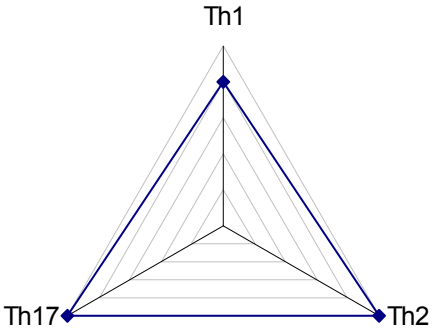
spectrum of different polymorphisms in the genes involved in human TLR responses is just beginning to be explored. The relative prevalence of these alleles in the human population and how they impact on both DCs and T cell responses could potentially jeopardize the use of TLR ligands as adjuvants in vaccines and modulators in immunotherapies for the general population.

Collectively, we successfully cataloged the patterns of human T helper cell differentiation induced by differentially TLR ligand-activated DCs (Figure 20). Bacterial TLR ligand-activated DCs induce relatively more balanced Th1/Th2/Th17 $CD4^+$ T cell responses. In addition, DCs activated with a viral ligand, poly I:C (a TLR3 ligand), induced more heavily skewed Th1 responses, whereas DCs activated with another viral TLR ligand, ssNRA40 (TLR7/8 ligand), induced balanced Th1/Th2/Th17 responses similar to that induced by bacterial TLR ligand-activated DCs. This provides a framework for the use of TLR ligands as vaccine adjuvants to provide appropriate immune responses against infection and/or to prevent or redirect inappropriate immune responses against self.

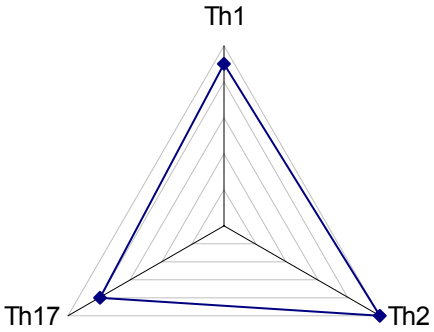
Figure 20. A Conceptual Model for T Helper Cell Differentiation Induced by TLR Ligand-activated moDCs

The levels of Th differentiation (Th1, Th2 and Th17) induced by differentially TLR ligand-activated moDCs were graphed on a scale of 0 to 5 based on the results presented herein, 5 being the highest and 0 lowest levels observed in this study relatively compared between different TLR ligand-activated DC/T cell cocultures. The highest frequencies of Th2 and Th17 responses observed in this study were always considerably lower than the Th1 frequencies observed. Th2 differentiation of $CD4^+$ T cells induced by MPL-A and Pam3CSK4-activated DCs was not determined (indicated by question marks) in this study. The level of Th2 differentiation in the cocultures with MPL-A-activated DCs was estimated based on and relative to the level of Th2 differentiation induced by LPS-activated DCs since MPL-A is a derivative of LPS. The level of Th2 differentiation induced by Pam3CSK4-activated DCs were estimated based on the previous reports demonstrating Pam3CSK4-activated DCs are capable of producing a Th2 cytokine, IL13, as well as inducing Th2 responses in a mouse *in vivo* study (Lombardi et al., 2008; Redecke et al., 2004).

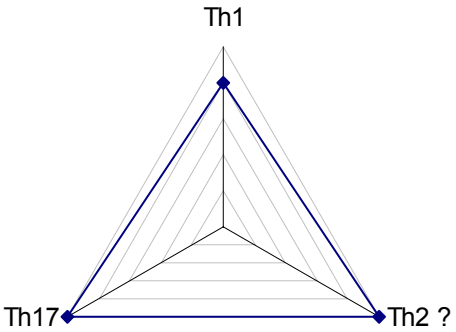
LPS



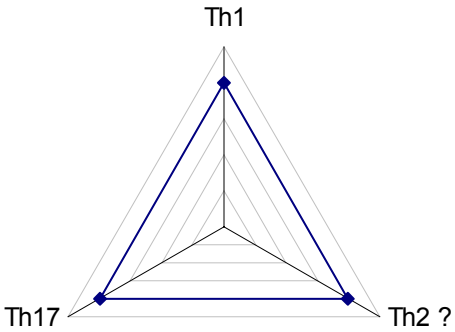
Flagellin



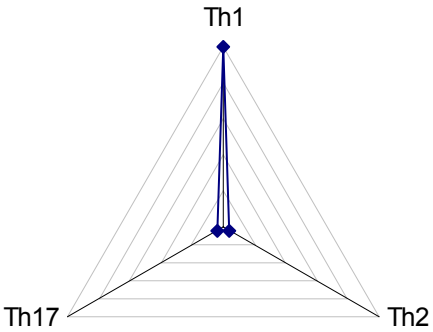
Pam3CSK4



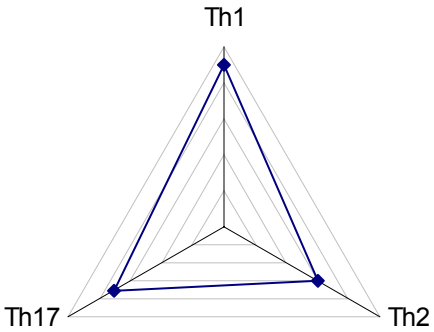
MPL-A



Poly I:C



ssRNA40



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