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ABSTRACT

IL-17A-secreting CD4 T cells (Th17 cells) have been demonstrated to play pivotal roles in modulating immune responses during various types of infectious and autoimmune diseases. While IL-17A-secreting CD8 T cells have been detected in numerous disease models, much less is known about them. In this thesis, the differentiation conditions and effector functions of IL-17A-secreting CD8 T cells have been examined. In order to differentiate naïve CD8 T cells into IL-17A secretors, TGF-β, IL-6, and neutralization of IFN-γ are required as in Th17 cells. IL-17A-secreting CD8 T cells produce the effector cytokines, IL-17A, IL-17F and IL-22, but do not produce granzyme B, implicating the lack of cytotoxicity. Furthermore, IL-17A-secreting CD8 T cells can respond to exogenous cytokines without a cognate antigen, suggesting that they can act in an innate fashion. Collectively, IL-17A-secreting CD8 T cells possess the same effector functions as Th17 cells, and thus may play as significant roles in various diseases as Th17 cells.

CHARACTERIZATION OF IL-17A-SECRETING CD8 T CELLS

THESIS

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TABLE OF CONTENTS

CHAPTER I

INTRODUCTION

A living organism constantly encounters foreign substances and microbes throughout its life. This continual exposure to foreign substances can result in beneficial effects; however, microbes can invade their host and proliferate, utilizing energy and machinery of the host and eventually destroying its biological homeostasis. The immune system has evolved to provide defense mechanisms against foreign pathogens and maintain homeostatic balance in the host. Thus a variety of infections and diseases can take place when the immune system is disrupted or malfunctions.

The immune system is comprised of unique sets of cells that are critical for protection against invading pathogens. In order to effectively regulate these specialized immune cells during infection, the host defense mechanisms have developed two arms of the immune system. One is innate immunity, which generates fast, non-specific immune responses against harmful microbes [1]. The other, adaptive immunity, develops over time and reacts in an antigen-specific manner.

Unlike the innate immune response, adaptive immunity exhibits immunological memory; it "remembers" pathogens that it previously encountered and responds to the subsequent exposure of the same pathogen more rapidly and efficiently [1].

In the adaptive immune system, T cells play a vital role in regulating immune responses against various types of infections and diseases. Among the T cells, cytotoxic CD8 T cells are specialized in the elimination of infected cells [2]. Originating from hematopoetic stem cells in the bone marrow, CD8 T cells perform cytotoxic activities by releasing granzyme and perforin molecules [2]. Perforins create pores on the lipid bilayer of target cells to generate osmotic imbalance and thus disrupt the cell structure. Granzymes activate caspase signaling pathways to induce apoptosis by cleaving procaspases and releasing catalytically active caspase molecules [2]. Interaction between Fas and Fas ligand (FasL) also contributes to apoptotic actions within the target cells: FasL on the surface of CD8 T cells bind to Fas on the surface of other cells, activating caspase molecules [2].

There is another subpopulation of T cells, namely CD4 T helper cells, which induce activation of other cell types to mount an effective immune response against foreign microbes. CD4 T cells often perform their effector functions through cytokine production. Depending on the types of secreted cytokines, CD4 T cells have

been historically broken down into two main subpopulations: T helper type 1 (Th1) and T helper type 2 (Th2) [3]. Th1 cells produce interferon gamma (IFN-γ) in order to provide protection against intracellular pathogens [3]. Th2 cells, on the other hand, secrete interleukin 4, 5, and 13 (IL-4, -5, -13), activating B cells and eliciting immune responses to helminth infection [3]. Thus CD4 T helper cells apply their specialized immune functions in correspondence to types of infection and disease conditions.

In addition to Th1 and Th2 cells, the scientific community has recently acknowledged a novel subtype of CD4 T helper cells called Th17 cells. These CD4 T cells produce IL-17A as an effector cytokine and have been implicated to provide protection against extracellular bacteria as well as to induce various autoimmune diseases, such as multiple sclerosis, rheumatoid arthritis, inflammatory bowel diseases and psoriasis [3]. Recent developments of clinical applications focus on the roles of Th17 cells in certain infections and autoimmune diseases [3;4]. It is also of great interest to understand how Th17 cells interact with other T cells and immune cells to modulate effective immune responses against foreign pathogens.

Unlike Th17 cells, IL-17A-secreting CD8 T cells have not drawn much attention within the scientific community although some infection studies have demonstrated the importance of IL-17A-secreting CD8 T cells in the clearance of

certain pathogens [5]. This thesis asks a question of how IL-17A-secreting CD8 T cells arise from naïve CD8 T cells and what the characteristics of these CD8 T cells are, in terms of their effector functions. These studies will provide insight into the mechanisms of differential development of CD8 T cells within a certain microenvironment with various cytokines. Our findings may explain the unique roles of the CD8 T cells during certain infections and autoimmunity. This, in turn, will enable us to devise effective treatment strategies when the presence of these CD8 T cells results in great difference in the pathologic outcome. This present study may also contribute to developing IL-17A-secreting CD8 T cells specific for certain types of cancer due to IL-17A involvement in anti-cancer activities [6]. In this way, the current study can further improve conventional cancer treatment and thus establish better strategic therapy programs that provide more efficient care for patients.

The Advent of Th17 cells

Before the concept of Th17 cells was brought into light, the immunology field was dominated by the theory of Th1 and Th2 cells as the main defense players in CD4 T cell-driven immunity. This Th1 and Th2 paradigm has been first proposed in 1989 to explain how the adaptive immune system of hosts responds to invasion of various

foreign pathogens [7]. According to this theory, naïve CD4 T helper cells are activated upon the first encounter of antigens presented by antigen-presenting cells (APC) and differentiate into either IFN-γ-secreting Th1 cells or IL-4-producing Th2 cells. This differentiation of naïve CD4 T cells is affected by signals from APCs, including direct contact of APCs with naïve CD4 T cells and cytokines produced by APCs [8]. These two subsets of CD4 T helper cells are required to orchestrate proper control over various types of pathogenic invasion in hosts. For instance, Th1 cells are known to enhance the cellular immunity against virus or intracellular pathogens, whereas Th2 cells are involved in clearance of helminth and enhancement of humoral immunity [9].

The Th1-Th2 theory has been very useful for understanding various infectious and allergic diseases [4]. However, this model could not fully elucidate pathogenicity of some autoimmune diseases. For example, Th1 cells had been known to induce experimental autoimmune encephalomyelitis (EAE), the murine disease model that was broadly studied for human multiple sclerosis (MS) [10]. IL-12 from APCs had been speculated to induce the differentiation of Th1 cells, and numerous studies had focused on the relationship between IL-12 and Th1 in order to understand the mechanisms of the so-called Th1-driven autoimmunity. Interestingly, mice lacking

IL-12 gene (IL-12p35 KO) had few IFN-γ-secreting Th1 cells but still suffered from a hyperacute form of EAE [10]. This experimental outcome puzzled the immunology community, and additional information was required to supplement the Th1-Th2 hypothesis in explaining mechanisms of onset and progress of certain autoimmune diseases.

In the midst of this scientific inquiry, IL-23 has been discovered and demonstrated to share one subunit with IL-12 (IL-12p40), implicating the possible pathologic role of IL-23 in Th1-driven autoimmune diseases [11]. In the meantime, IL-17A has been demonstrated to be a T-cell cytokine that possesses important functions in inflammatory responses [12]. In 2000, it was reported that mouse naïve T cells primed with lysate from *Borrelia burgdorferi* produced IL-17A, rather than Th1 or Th2 effector cytokines [13]. This result indicated that IL-17A-secreting T cells might represent a new subset of T cells. The experiment that highlighted the significance of IL-17A-secreting T cells for onset of autoimmune diseases was conducted in 2005. In this study, EAE was induced by passive transfer of IL-17A-secreting CD4 T cells into wild-type mice [14]. In addition, the lack of Th1 cells contributed to further exacerbation of EAE symptoms in those mice, indicating the

possible protective role of Th1 cells and the pathogenic role of IL-17A-producing CD4 T cells during EAE [14].

Shortly after that report, two independent groups conducted pioneering research to establish a novel, distinct subset of T helper cells that produce interleukin 17 (IL-17 or IL-17A) as an effector cytokine [15;16]. These T helper cells required transforming growth factor-beta (TGF-β) and IL-6 in order to differentiate from naïve CD4 T cells. In addition, those two groups demonstrated that IFN-γ and IL-4, the cytokines necessary for the generation of Th1 and Th2 cells respectively, inhibited the differentiation of naïve CD4 T cells into IL-17A secretors. This distinct subset of T helper cells was quickly named "Th17", and numerous studies have been performed to understand the effector role of Th17 cells in various disease models.

Characteristics of Th17 Cells

Differentiation Conditions

The general consensus in regards to the generation of mouse Th17 cells is that TGF-β and IL-6 synergistically drive the differentiation of naïve CD4 T cells into Th17 cells [4]. However, there had been considerable confusion in the literature regarding the cytokines required for human Th17 cell generation. One report has

claimed that IL-1β, instead of TGF-β, is essential for eliciting the Th17 phenotype in human [17]. Interestingly, TGF-β was not required for producing Th17 cells; in fact, TGF-β inhibited the Th17 generation, even when IL-6 was present in the culture. Therefore they concluded that IL-1β plays a major role in Th17 differentiation and that IL-6 enhances IL-1β-induced Th17 generation. Congruent with this finding, some other studies also suggested that the combination of TGF-β plus IL-6 does not drive IL-17A-producing CD4 T cells in vitro [18;19].

A recent report, however, has identified a significant problem in the experimental protocol of the Th17 generation. Manel, et al. cleverly realized that human and bovine serum might contain endogenous TGF-β and thus might lead to incorrect interpretations for some experimental results. Therefore they used serum-free medium to demonstrate that TGF-β plays a pivotal role in the differentiation of human Th17 cells [20]. Their report implicated that the previous studies that failed to induce Th17 cell differentiation from naïve CD4 T cells could possibly be explained from their use of high serum amounts, which might provide substantial amounts of endogenous TGF-β. In accordance with this report, another study demonstrated the critical role of TGF-β in Th17 cell generation, using a carefully selected fetal calf serum (Yssel's medium) that did not contain endogenous TGF-β [21]. Thus these

studies emphasized the importance of TGF-β during Th17 cell generation and claimed that TGF-β is indeed required for the expansion of the Th17 cell pool.

In addition to TGF-β and IL-6, several reports have documented the significance of IL-21 during Th17 differentiation [22;23;23;24]. In mice, IL-6 induced the production of IL-21 in CD4 T cells in a STAT3-dependent manner, and CD4 T cells from IL-21 KO mice failed to produce IL-17A [23]. However, IL-6 KO mice still developed EAE and Th17 cells in the absence of regulatory T cells, implicating that there might be IL-6-independent mechanisms that increase IL-21 expression in vivo [22]. The unique aspect about IL-21 is that IL-21 is produced by Th17 cells and further stimulates the Th17 differentiation in an autocrine fashion [23]. IL-21 is also known to regulate B cell maturation and terminal differentiation [25]. Thus future research can determine whether Th17 cells affect B cell functions and differentiation through IL-21 activities.

IL-23 has also been proposed to have an essential role in Th17 polarization. However, numerous studies suggested that IL-23 does not participate in the Th17 differentiation processes, but is instead required for proper maintenance and expansion of the Th17 cell population. For example, Veldhoen, et al. generated Th17 cells from mice without using IL-23 in an APC-free culture for 3 days and then

observed an increased percentage of IL-17A-secreting CD4 T cells when they were re-stimulated with IL-23 for 3 days [26]. Indeed, the lack of IL-23 in mice resulted in fewer infiltrating Th17 cells in EAE and other autoimmune diseases [27-29]. The study which reported that naïve T cells do not express IL-23 receptors (IL-23R) supports the idea that IL-23 participates only in the maintenance and expansion of already differentiated Th17 cells [15]. But exposure to IL-6 can increase the level of IL-23R mRNA in mouse CD4+ T cells during the early stage of the Th17 differentiation [24]. Therefore the presence of IL-23 might exert a significant, positive influence on Th17 cell generation.

Collectively, the above studies emphasize the importance of TGF-β, IL-6 and IL-21 for Th17 differentiation, whereas IL-23 is mainly involved in maintenance and expansion of already differentiated Th17 cells. In addition, TNF-α and IL-1β can further enhance the differentiation of Th17 cells [26]. Besides the stimulation from these cytokines, activation signaling from costimulatory molecules, such as CD28 and inducible costimulators (ICOS), is reported to be critical for the establishment of the Th17 phenotype [16]. Thus the optimal generation of Th17 cells involves not only stimulation from cytokines, but also cell-to-cell interactions through surface molecules.

Transcription Factors for Th17 Cell Differentiation

During Th1 and Th2 differentiation, selective members of transcription factors regulate gene transcription events [3]. For example, STAT1 (signal transducer and activator of transcription) and STAT4 are activated during Th1 differentiation, which further induce the expression of T-bet, another transcription factor indispensable for Th1 generation [15]. In developing Th2 cells, the expression of STAT6 and the subsequent activation of GATA3 transcription factors are critical for inducing and maintaining the Th2 phenotype [15]. For the establishment of the Th17 cell population, neither Th1- nor Th2-related transcription factors are required [15], thus substantiating the claim that Th17 cells represent a distinct lineage of T helper cells. It has been reported that Th17 cell generation in mice requires STAT3, RORγt (retinoic-acid-receptor-related orphan nuclear receptor), and interferon-regulatory factor 4 (IRF4) [30-33]. However, it is not clear whether those transcription factors have synergistic effects on Th17 generation at the promoter level or bind to different promoters to simultaneously express Th17-driving molecules.

Negative Regulators of Th17 Cell Differentiation

IFN-γ and IL-4, the cytokines that are essential for Th1 and Th2 differentiation respectively, inhibit Th17 polarization in both human and mice [16;17]. Therefore, neutralizing antibodies against IFN-γ and IL-4 are necessary for developing the Th17 cell subset. T-bet and STAT1, the Th1-associated transcription factors, suppress IL-17A secretion from mouse CD4 T cells [15;34]. Thus Th1-related cytokines and transcription factors prevent the differentiation of Th17 cells. STAT6, a Th2-related transcription factor, also negatively regulates Th17 cell generation in mice [15;16]. However, it is not certain whether GATA3, another Th2-cell-specific transcription factor, constrains the generation of IL-17A secretors.

Several cytokines have been reported to negatively regulate Th17 differentiation. For example, IL-2 restricts in vitro and in vivo generation of mouse Th17 cells by upregulating STAT5 in naïve CD4 T cells [30]. In addition, IL-25 (IL-17E) and IL-27 have been demonstrated to constrain the generation of Th17 cells in vivo [35;36]. The inhibition of the Th17 differentiation can also take place through interactions of surface molecules, such as OX40 (CD134) and OX40L [37]. The multiple layers of regulatory mechanisms on Th17 generation implicate that Th17 cells may exert very potent effector functions during a variety of inflammatory

diseases. Thorough understanding of this regulation would benefit future therapies that can dampen excess inflammatory responses induced by Th17 cells and their subsequently activated immune cells.

Surface Markers of Th17 Cells

Distinct receptor expression has been associated with differentiation pathways and certain phenotype of cells. For example, CXCR3 (C-X-C chemokine receptor 3) is preferentially expressed by Th1 cells, and CCR4 (C-C chemokine receptor 4) can often be found on Th2 cells isolated from humans [38]. To date, the surface molecules that are selectively expressed on Th17 cells have not been identified. The mRNA and protein level of IL-23R on human Th17 cells have been examined, but some other cells also express IL-23R [38;39]. Several reports demonstrated that CCR6 is expressed on human Th17 cells [38-43]. CCR6 mediates homing to skin and mucosal tissues [44], and pathogenic T cells in some inflammatory diseases have been shown to be recruited to various target tissues using CCR6 [43;45]. Sato, et al. demonstrated that human Th17 cells are CCR2+ CCR5- [46], but their report is contradicting with one report that observed IL-17A-secreting CD4+ CCR5+ cells in human [40]. Lim, et al. identified numerous trafficking receptors on human Th17

cells that are shared by Th1 and Th2 cells [40]. Thus Th17 cells display a wide range of the expression profile for surface receptors, and further research needs to be performed to find receptors that are preferentially expressed on Th17 cells.

Phenotypic Stability of Th17 Cells

One of the distinguishing features of the Th1 and Th2 phenotype is their phenotypic stability. Once naïve CD4 T cells are differentiated into Th1 or Th2 cells, they do not transform into other subtypes but maintain their final phenotype [3]. Regarding the phenotypic stability of Th17 cells, conflicting results have been reported. One report suggested that the phenotype of Th17 cells is not stable [26]. In this study, CD4 T cells were differentiated into Th17 cells for 3 days, rested for 3 days and then treated with IL-2 or IL-23 for 3 days, followed by 4-day rest. The treatment with IL-2 caused the secretion of IFN-γ instead of IL-17A, whereas Th17 cells that received IL-23 produced IL-17A, not IFN-γ. Therefore this study concluded that Th17 cells do not maintain their phenotype and are able to transform into another subtype under certain conditions. Congruent with this conclusion, a recent report demonstrated that Th17 cells that were generated in vitro for 15 days did not produce IL-17A when they were treated with Th1 or Th2 cytokines [47]. Therefore this report

argued that Th17 cells lose their ability to produce the effector cytokine under Th1 or Th2 conditions.

On the contrary, another study claimed that Th17 cells exhibit a stable effector phenotype. In this study [15], Th17 cells were generated for 5 days and then treated for 3 days with either Th1- or Th2-polarizing cytokines. Th17 cells still produced IL-17A under Th1- or Th2-optimal conditions and expressed more IL-17A when treated with IL-23. Therefore, this report concluded that Th17 cells exhibit a stable phenotype once they are differentiated. In addition, a recent study demonstrated that Th17 cells that were obtained from BALB/c mice ex vivo maintained their ability to produce IL-17A under Th1 or Th2-polarizing conditions [48]. Taken together, a few studies have reached conflicting conclusions with respect to the phenotypic stability of Th17 cells. Additional research should be performed to clearly understand the mechanisms and conditions for establishing the stable Th17 phenotype if it can remain stable at all.

Cytokines produced by Th17 Cells

As the name suggests, Th17 cells produce IL-17A as an effector cytokine. More specifically, Th17 cells secrete IL-17A and IL-17F, which possess similar

functions as they bind to the same IL-17R complex [49]. IL-17A and IL-17F can also heterodimerize and recruit neutrophils to inflammatory sites, but the homodimer of IL-17A exhibits more potent effects in recruiting neutrophils [49]. In addition to IL-17A and IL-17F, Th17 cells produce IL-22, which upregulates anti-microbial peptides and acute phase proteins in various epithelial and fibroblast cells [50]. During Th17 differentiation, IL-21 is produced by mouse Th17 cells and acts in an autocrine manner to increase the expression level of IL-23R [24]. Several groups have also identified inflammatory cytokines in Th17 cells, such as TNF-α, in both human and mouse [17;51]. Although various cytokines are produced in Th17 cells, IL-17A, IL-17F, and IL-22 have received the most attention due to their prominent effector functions and increased expression levels during inflammatory and autoimmune diseases. Thus in this introduction, the function of those cytokines has been explained more in depth, and this current study examined IL-17A, IL-17F and IL-22 expression levels in CD8 T cells that were differentiated under Th17-promoting conditions.

Interleukin 17 (IL-17)

IL-17, more specifically IL-17A, is one of the cytokines in the IL-17 cytokine family, which is composed of six cytokines identified to date (IL-17A through IL-

17F) [5]. Human IL-17A is composed of 155 amino acids and is 63% identical to the mouse counterpart [52]. The IL-17 superfamily shares little sequence homology with other cytokine families, and its in vivo functions have not been fully clarified [53]. IL-17A is mainly produced by activated T cells as a homodimer, and it triggers many of the same signaling pathways as innate cytokines [54]. Thus IL-17A is considered as a significant bridging molecule between innate and adaptive immunity [54].

In contrast to IL-17A, IL-17A receptors are ubiquitously expressed by numerous cell types, including epithelial cells, vascular endothelial cells, fibroblasts, and non-hematopoetic cells [4]. IL-17A receptor is composed of IL-17RA and IL-17RC although IL-17RA can exist as a homodimer [55]. Upon binding to the IL-17R complex, IL-17A increases the expression of genes that are involved in neutrophil recruitment, such as CXCL1 (growth-related oncogene α (Groα)/keratinocyte-derived chemokines (KC)), CXCL2 (macrophage-inflammatory protein-2 (MIP-2)), and CXCL5 (LPS-induced CXC chemokine (LIX)) [56;57]. IL-17A also upregulates granulocyte colony-stimulating factor (G-CSF) [58], and the overexpression of IL-17A causes neutrophilia in mice [59]. Thus IL-17A plays an important role particularly in differentiation, recruitment and mobilization of neutrophils into sites of inflammation [60].

Among the IL-17 family cytokines, IL-17F shares the most sequence homology (55%) with, and has similar functions to, IL-17A [61]. IL-17F mainly exists as a homodimer but can also form a heterodimer with IL-17A and bind to the IL-17R complex, which explains the redundancy in the functions of IL-17A and IL-17F [61]. However, a recent study demonstrated that IL-17A and IL-17F might play differential roles in humoral immunity and progression of autoimmunity [62]. In this study, splenocytes from IL-17F-deficient mice produced more IgG2a than WT or IL-17A-deficient mice. In addition, IL-17A-deficient mice exhibited greatly delayed onset and progression of EAE, whereas IL-17F deficiency did not ameliorate EAE. Moreover, IL-17A KO mice showed reduced Th2 cytokine production, but IL-17F deficiency resulted in elevated levels of Th2 cytokines and enhanced eosinophil functions [62]. Thus IL-17A and IL-17F may possess distinct functions although the two cytokines share the same receptor complex. A possible explanation for this phenomenon is that IL-17F may also bind to different receptors, inducing the expression of other genes. A further investigation should be performed to clarify the differential functions of the two cytokines in various types of inflammatory responses.

Interleukin 22 (IL-22)

IL-22 is one of the IL-10 family cytokines, and both human and mouse IL-22 cDNA encode proteins of 179 amino acids, which are 79% identical to each other [63]. Due to its similar primary structure with IL-10, this protein was first named "IL-10-related T cell-derived Inducible Factor (IL-TIF)". However, it was quickly renamed IL-22, due to its receptor complex being different from the IL-10 receptor [64]. The IL-22 receptor complex is comprised of IL-10R2 and IL-22R1 subunits [64]. IL-10R2 is known to be expressed on various types of cells, and IL-10 binds to the IL-10R2 chain. Therefore, IL-22R1 expression has been closely examined in search for cells that are responsive to IL-22 [63]. Most IL-22R1-expressing tissues contain epithelial cells of outer body barriers [63], but IL-22R1 expression has also been detected in human subepithelial myofibroblasts [65] and synovial fibroblast lines derived from patients with rheumatoid arthritis [66].

IL-22 is best known for its ability to induce antimicrobial peptides, such as β-defensins, S100-family proteins and regenerating gene (Reg)-family proteins [63]. β-defensins are very small, highly charged cationic proteins that kill bacteria, fungi and viruses by penetrating and creating pores on the microbial membrane [67]. They are mainly produced by skin and mucosal epithelia where they form a barrier against

microbial infections [67]. S100-family proteins are zinc-binding proteins that sequester zinc to kill microbes [67;68]. Similar to β-defensins, many S100 proteins are expressed in human epithelial tissues and in mouse colon [67;68]. Reg-family proteins, first identified in pancreatic islet cells, are involved in killing gram-positive bacteria through their interaction with microbial peptidoglycan carbohydrate [68]. A recent study has demonstrated that Reg-family proteins were upregulated in the colon by IL-22 in response to *Citrobacter rodentium*, an extracellular gram-negative bacterium [68]. The increased expression levels of several types of antimicrobial peptides through IL-22 reveals that IL-22 plays a pivotal role in antimicrobial activities and is therefore a potent mediator of the host defense in the epidermis.

Th17 Cells in Host Defense

To date, Th17 cells are mainly known to regulate immune responses against infections from extracellular bacteria in mucosal tissues [4]. For example, during *Klebsiella pneumoniae* lung infection in mice, IL-17A is produced by T cells upon encounter of the pathogen [5], and IL-22 from Th17 cells induces the expression of an iron-sequestering antimicrobial peptide, lipocalin-2, in mouse tracheal epithelial cells [69]. In addition, *Bacteroides fragilis* intraperitoneal inoculation resulted in

formation of peritoneal abscesses which depends on IL-17A from CD4 T cells [70]. Furthermore, the whole cell pertussis vaccine requires IL-17A from CD4 T cells and macrophages that are activated by IL-17A in order to efficiently confer resistance to *Bordetella pertussis* in mice [71].

In addition to extracellular bacteria, Th17 cells are documented to play pivotal roles in protective immunity against other types of pathogens. For instance, Th17 cells restrict the growth of intracellular bacteria *Mycobacterium tuberculosis* in the mouse lung [72]. Moreover, memory Th17 cells specific to a fungal pathogen *Candida albicans* are detected at high frequency in human, implicating the importance of Th17 cells in adaptive immunity against *C. albicans* [38]. Furthermore, Th17 cells protect mice from intestinal rotavirus in mice [73]. Thus Th17 cells demonstrate beneficial roles in protection against various types of pathogens. Several reports indicate that IL-17A or IL-22 is required for protection against numerous other pathogens, but the role of Th17 cells during those infections was not examined [54;68;74-76]. Future developments that establish clear understanding of Th17 cells during protective immunity can be applied to devise more effective treatments for the wide array of infections and diseases.

IL-17A-secreting CD8 T cells

Most studies have focused on the identification and characterization of Th17 cells, but IL-17A-secreting CD8 T cells are less understood with regards to their phenotype and function in the immune system. It has been reported that mouse CD8 T cells produce as much IL-17A as CD4 T cells during the differentiation of naïve T cells into IL-17A secretors [77], implicating that IL-17A-secreting CD8 T cells might also be important for regulating immune responses against certain types of infections and diseases. Indeed, IL-17A-secreting CD8 T cells produced more IL-17A than Th17 cells when mouse T cells were cultured with *K. pneumoniae*-pulsed DC [5] or stimulated with IL-15 and LPS (lipopolysaccharide) [78]. In addition, IL-17A-secreting CD8 T cells are implied to play a protective role during *Listeria monocytogenes* infection in mice when Th1 responses are abolished [79]. IL-17A-secreting CD8 T cells can also be found during *Mycoplasma pulmonis* infection although it is not clear whether they are pathologic or protective for the host (our unpublished data). However, potential roles of IL-17A-secreting CD8 T cells in autoimmunity have been indicated in mouse colitis and lymphocytic choriomeningitis viral infection models [80;81].

Thus IL-17A-secreting CD8 T cells can be beneficial or detrimental to host immune responses, depending on the nature of the infection and diseases. Thorough understanding of characteristics of IL-17A-secreting CD8 T cells might be imperative in order to develop effective treatments and appropriate remedies for certain stages of disease progression. To date, no studies have closely examined the effector function of IL-17A-secreting CD8 T cells. In light of previous reports delineating various characteristics of Th17 cells, I hypothesized that IL-17A-secreting CD8 T cells respond to TCR stimulus and exogenous cytokines by secreting effector molecules. In order to answer this question, I investigated the following specific aims: **1)** to determine which factors are required for the differentiation of IL-17A-secreting CD8 T cells, **2)** to determine the effector functions of IL-17A-secreting CD8 T cells, **3)** to investigate the cytokine responsiveness of IL-17A-secreting CD8 T cells, and **4)** to determine the phenotypic stability of IL-17A-secreting CD8 T cells. This research provides insight into functions of IL-17A-secreting CD8 T cells in various microenvironments governed by the actions of cytokines. With this knowledge, the role of these CD8 T cells in various types of infections and diseases can be examined. Better understanding of IL-17A-secreting CD8 T cells would, in turn, enable us to

establish strategic development of effective treatments for patients with diseases in which the IL-17A+ CD8 T cells may play a significant role.

CHAPTER II

MATERIALS AND METHODS

Mice

C57BL/6 mice were purchased from Taconic Farms (Germantown, NY) or Charles River Laboratories (Wilmington, MA). All mice used for this study were 6-10 week old and maintained in specific pathogen-free conditions according to the guidelines for animal care of our institute.

Isolation of Naïve CD8 T Cells and Culture Media

Murine lymph node (LN) cells were collected from mesenteric, inguinal, axillary, brachial and lumbar LN. Spleen was also collected for an indicated experiment. Frosted microscope glass slides (Fisher) were used to grind LN. Naïve CD8 T cells were then sorted using the Cytopia InFlux Cell Sorter, based on surface expression of CD8 and CD62L, a cell surface marker expressed at high levels on naïve T cells. For the surface staining, CD8 fluorescein isothiocyanate (FITC) (BD PharMingen) and CD62L allophycocyanine (APC) (Biolegend) antibodies were used. BD Imag^{TM} CD8 magnetic beads (BD PharMingen) were used to positively select CD8+ cells for some experiments.

All cells were cultured at 37 °C in 5% CO_2 in Roswell Park Memorial Institute (RPMI) media supplemented with 10mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (Invitrogen-Gibco), 55μM 2-mercaptoethanol (Invitrogen-Gibco), 1mM sodium pyruvate (Invitrogen-Gibco), non-essential amino acids (Invitrogen-Gibco), 10 U/ml penicillin (Invitrogen-Gibco), 10μg/ml streptomycin (Invitrogen-Gibco), L-glutamine (Invitrogen-Gibco), vitamin solution (Invitrogen-Gibco) and 10% Fetal Bovine Serum (FBS) (Atlanta Biologicals, Lawrenceville, GA). This medium is referred to as complete RPMI (cRPMI).

Preparation of APCs Treated with Mitomycin C

The spleens from B6 mice were ground with frosted microscope slides and treated with tris ammonium chloride for 5-10 minutes to lyse red blood cells. Then cells were washed twice with HBSS (Hank's Buffered Salt Solution) and were resuspended in cRPMI and treated with 100μg/ml mitomycin C (EMD Calbiochem) for 25 min at 37°C. Mitomycin C crosslinks DNA and distrupts the structure of DNA [82]. Therefore, mitomycin C-treated splenocytes do not proliferate, and all transcription activities are halted. Mitomycin C-treated splenocytes were then washed twice with cRPMI and resuspended in cRPMI for cell culture.

Differentiation Conditions of IL-17A-secreting CD8 T Cells with APCs

Naïve CD8+ CD62L+ LN cells purified with the cell sorter, or CD8+ LN cells selected with CD8 magnetic beads, were cultured in 48-well plates. Each well also received APCs (Antigen-presenting Cells) from the spleen, that were treated with mitomycin C as described above. $0.9x10^6$ CD8+ LN cells and $2x10^6$ mitomycin C-treated splenocytes were cultured in cRPMI. The cell number was obtained by counting with Beckman Coulter Counter Z1. Cells were supplemented for 3 days with TGF-β (1ng/ml; PeproTech), IL-6 (20ng/ml; PeproTech), neutralizing αIFN-γ (10μg/ml; BioXCell), and soluble αCD3 (2μg/ml; BioXCell). On Day 3, those cells were re-treated with TGF-β, IL-6, αIFN-γ and IL-23 (10ng/ml; PeproTech) for 3 days, as indicated in some experiments. Other experiments were performed for 3-day differentiation culture.

Differentiation Conditions of IL-17A-secreting CD8 T Cells without APCs

$0.9x10^6$ naïve CD8+ CD62L+ LN cells purified with the cell sorter, or $0.9x10^6$ CD8+ LN cells selected with CD8 magnetic beads, were cultured in 48-well plates with cRPMI. The cell number was obtained by counting with Beckman Coulter Counter Z1. Cells were supplemented for 3 days with TGF-β, IL-6, neutralizing αIFN-γ, 10μg/ml plate-bound αCD3, and 1μg/ml soluble αCD28 (BD PharMingen).

On Day 3, the cells were re-treated with TGF-β, IL-6, αIFN-γ and IL-23 for 3 days. This differentiation culture without APCs would be referred to as "APC-free system." Depending on the culture conditions, cells were also treated with 10ng/ml IL-21 (PeproTech), 10ng/ml IL-1β (PeproTech), or 1ng/ml TNF-α (PeproTech). Unlike some of the culture system with APCs, this APC-free culture was performed only for 3 days.

Culture Conditions for Cytokine Responsiveness Assay

IL-17A-secreting CD8 T cells were generated for 6 days as described above. On Day 6, cells were harvested and purified for activated CD8 T cells with the cell sorter, using CD8 and CD44 markers (CD8 PE-Cy7, BD PharMingen; CD44 FITC, BD PharMingen). The sorted CD8 T cells were treated for 12-16 hours with +/- 5ng/ml IL-12 (PeproTech), +/- 10ng/ml IL-18 (Medical & Biological Laboratories), and +/- 10ng/ml IL-23, depending on the culture conditions.

Surface molecules and intracellular cytokine staining

In order to perform cell staining for the flow cytometric analysis, the following antibodies were used: IL-17A phycoerythrin (PE) (Bioledgend), IL-17F AF 488 (Alexa Fluor 488) (eBioscience), IL-22 PE (R&D Systems), IFN-γ APC (Bioledgend), CD44 FITC (BD PharMingen), CD8 PE-Cy7 (BD PharMingen),

granzyme B APC (Caltag) and purified CD16/32 antibodies (BD PharMingen). Prior to the harvest, cells were treated with 50ng/ml phorbol 12-myristate 13-acetate (PMA) (Sigma) and 500ng/ml Ionomycin (EMD) for five hours (where indicated) and GolgiPlug containing brefeldin A (BD PharMingen) for the last four hours. PMA enhances the activities of protein kinase C, and Ionomycin is an ionophore that increases the intracellular calcium concentration [83]. PMA and Ionomycin have been commonly used to mimic the action of TCR stimulation [84]. Cells were then harvested and incubated with antibodies against cell surface molecules at 4 ˚C for 15 min in staining buffer (1x phosphate-buffered saline (PBS) + 2% FBS + 0.1% sodium azide) with anti-CD16/32 antibodies to prevent non-specific binding of antibodies to Fc receptors. For intracellular cytokine staining, cells were fixed and permeabilized at 4 ˚C for 20 min with Cytofix/Cytoperm solution containing 4% paraformaldehyde (BD PharMingen). After washing in 1x Perm/Wash™ Buffer (BD PharMingen), cells were incubated at 4˚C with antibodies against intracellular cytokines for 20 min. Data were acquired and analyzed using a Beckman Coulter Cytomics FC500 (Fullerton, CA). For intracellular staining of granzyme B, granzyme B APC antibodies (Caltag) were used, and the same staining protocol was used as described above.

ELISA (Enzyme-linked Immunosorbent Assay)

Differentiated CD8 T cells (CD8+ CD44+) were purified with the cell sorter and re-stimulated with various combinations of cytokines (+/- 5ng/ml IL-12, +/- 10ng/ml IL-18, +/- 10ng/ml IL-23) for 12-16 hours. Some differentiated CD8 T cells were rested for one day and re-stimulated with PMA and Ionomycin for 5 hours. Supernatant from the cell culture was then collected and stored at -20 °C. IL-17A, IL-17F and IL-22 were measured by means of sandwich ELISA. IL-17A antibodies and recombinant IL-17A for standards were purchased from BD PharMingen. IL-22 antibodies and recombinant IL-22 for standards were purchased from PeproTech. The measurement of IL-17F was done with the IL-17F ELISA kit (R&D Systems). IL-17A and IL-22 sandwich ELISA was performed as the following: Purified capture antibodies were coated on 96-well plates for 12-16 hours. Then the plates were washed 3 times with wash buffer (1x PBS + 0.05% Tween20). Blocking wells with assay diluent (1x PBS with 10% fetal calf serum) took 1 hour. Standards and samples were added after the blocking step, and the plates were incubated at room temperature for 2 hours. After the plates were washed 5 times with wash buffer, biotinylated detection antibodies + SA HRP (streptadivin horse radish peroxidase) were added to each well. The plates were incubated with the detection antibodies and enzymes for 1

hour. Then the plates were washed 7 times with wash buffer, and the 1:1 mixture of substrate solution (hydrogen peroxide + 3'3, 5'5 tetramethylbenzidine) was added to each well. IL-17F ELISA was performed according to the manufacture's protocol. The optical density was read by BioTek ELISA plate reader EL808, and Gen5 software (BioTek) was used to analyze IL-17A, IL-17F and IL-22 ELISA data.

Statistical Analysis

Where appropriate, analyses of variances (ANOVAs) were conducted on the data. Bonferroni t-tests were used for post-hoc analyses. A p value of 0.05 or less was considered significant in all cases.

CHAPTER III

DIFFERENTIATION OF IL-17A-SECRETING CD8 T CELLS

Th17 cells have received much attention for their potent elicitation of immune responses during a variety of infections and diseases. Due to their pivotal roles in host immunity, the differentiation conditions of Th17 cells have been intensely investigated in an attempt to increase or lessen Th17 responses depending on the types of diseases. Now it is a general consensus that the generation of Th17 cells from naïve mouse CD4 T cells requires TGF-β and IL-6 [3]. Neutralization of IFN-γ and IL-4 is also important to block the Th1 and Th2 differentiation from naïve CD4 T cells [3]. CD8 T cells that secrete IL-17A were also implicated to have significant roles during infections with certain pathogens [5], but unlike Th17 cells, the differentiation conditions of IL-17A-secreting CD8 T cells have not been elucidated. We examined whether naïve CD8 T cells also require TGF-β, IL-6, neutralizing anti-IFN-γ (αIFN-γ) and anti-IL-4 (αIL-4) antibodies to differentiate into IL-17A secretors. In order to determine the differentiation conditions of IL-17A-secreting CD8 T cells, CD8+ cells from spleen or LN were purified using CD8 magnetic beads. Purified

CD8+ cells were then cultured with mitomycin C-treated splenocytes and treated with soluble αCD3, +/- TGF-β, +/- IL-6 and +/- αIFN-γ for 3 days. On day 3, cells were stimulated with PMA and Ionomycin for 5 hours. The flow cytometric data demonstrated that CD8 T cells are capable of producing IL-17A when they are cultured with mitomycin C-treated splenocytes and treated with TGF-β, IL-6 and αIFN-γ (Figure 1). Neutralization of IL-4 was not necessary for the differentiation of IL-17A-secreting CD8 T cells (data not shown). However, the neutralizing αIFN-γ antibody was indispensable for the optimal differentiation of IL-17A-secreting CD8 T cells (Figure 1).

Although mitomycin C hinders cell proliferation by causing DNA damage, other unknown factors from the mitomycin C-treated APCs may influence the differentiation of the naïve CD8 T cells, such as interaction between cell surface molecules. In order to establish the differentiation system without any possible factors from APCs, CD8+ cells were isolated from lymph nodes using CD8 magnetic beads and provided the Th17-favoring factors (TGF-β, IL-6, αIFN-γ) in the absence of APCs (with plate-bound αCD3 and soluble αCD28). Production of IL-17A was dramatically reduced in CD8 T cells cultured without APCs (Figure 2). The same result was observed when CD8+ CD62L+ naïve T cells were purified using the

Cytopeia InFlux cell sorter and cultured in Th17-promoting conditions (Figure 3). On the other hand, the percentage of cells that produced IFN-γ was increased (Figure 2 and 3), indicating that some CD8 T cells obtained the conventional phenotype characterized by IFN-γ secretion (Tc1 phenotype). It is interesting to observe that CD8 T cells tend to develop Tc1 phenotype even in the absence of Tc1 differentiation cytokine (IL-12).

The failure to establish IL-17A secretors from naïve CD8 T cells in the APC-free culture indicated that other factors are involved with the differentiation in addition to TGF-β, IL-6 and αIFN-γ. It has been reported that IL-1β is beneficial for the differentiation of Th17 cells by upregulating the expression of RORγt in mice [77]. In addition, tumor necrosis factor alpha (TNF-α) and IL-1β can further enhance the generation of Th17 cells in the presence of TGF-β and IL-6 [26]. Therefore, TNF-α and IL-1β were added in the Th17-polarizing, APC-free culture (with plate-bound αCD3 and soluble αCD28). The percentage of activated IL-17A+ CD8+ T cells was indeed increased, highlighting the importance of TNF-α and IL-1β for the optimal differentiation of IL-17A-secreting CD8 T cells (Figure 4). However, TNF-α or IL-1β by itself did not increase the percentage of IL-17A+ CD8+ cells in the Th17-favoring, APC-free culture (data not shown).

IL-23 has been reported to have a pivotal role in maintaining and expanding the Th17 cell population [15], but less is known about its role during the differentiation of IL-17A-secreting CD8 T cells. Therefore, in order to determine the role of IL-23 during the differentiation of IL-17A-secreting CD8 T cells, naïve CD8 T cells from LN were obtained with the cell sorter, and IL-23 was added in the APC-free culture together with TGF-β, IL-6 and αIFN-γ. The percentage of activated IL-17A+ CD8+ T cells was slightly increased when naïve CD8 T cells were cultured with IL-23 and the Th17-promoting factors (TGF-β, IL-6, αIFN-γ) (Figure 5). To determine if IL-23 still increases IL-17A production when TNF-α and IL-1β are present, naïve CD8 T cells were sorted using the cell sorter and treated with IL-23, the Th17-driving factors, TNF-α and IL-1β for 3 days in the APC-free culture. In the presence of TNF-α and IL-1β, however, IL-23 did not have any effect on the differentiation (Figure 5). Thus, IL-23 may play a minor role in the early stage of the differentiation, but its effect may not be observed in the inflammatory milieu abundant with TNF-α and IL-1β. Similar to Th17 cells, IL-23 may play more of a role in maintaining the phenotype of IL-17A-secreting CD8 T cells than inducing the differentiation. This possible role of IL-23 in the stabilization of IL-17A-secreting CD8 T cells would require further investigation.

IL-21 is a unique cytokine that acts in an autocrine fashion to enhance the differentiation of Th17 cells [3]. Since IL-21 is involved in the polarization of Th17 cells, IL-21 may also contribute to the development of IL-17A-secreting CD8 T cells. However, it is not certain whether IL-17A-secreting CD8 T cells produce endogenous IL-21. It has been shown that IL-21 receptors are expressed on CD8 T cells [85], suggesting that IL-21 may positively influence the differentiation of IL-17A-secreting CD8 T cells. However, IL-21 can also upregulate the expression of IFN-γ and IL-2 in mouse CD8 T cells [86], hence possibly inhibiting the establishment of IL-17A-secreting CD8 T cells. To determine the role of IL-21 in the differentiation of IL-17A-secreting CD8 T cells, purified naïve CD8 T cells were treated for 3 days with IL-21 under Th17-polarizing conditions. There was a slight increase in the percentage of IL-17A+ CD8+ cells when IL-21 was added (Figure 6). In order to examine whether IL-21 still increases IL-17A production in the presence of TNF-α and IL-1β, naïve CD8 T cells were purified from LN and treated with IL-21, the Th17-driving factors, TNF-α and IL-1β in the APC-free culture. In the presence of TNF-α and IL-1β, however, IL-21 did not enhance the secretion of IL-17A. Thus, IL-21 appears to be dispensable for the polarization of IL-17A-secreting CD8 T cells and does not negatively regulate the production of IL-17A from CD8 T cells that are

differentiating into IL-17A secretors. IL-21, however, may be produced by IL-17A-secreting CD8 T cells and act as an autocrine growth factor, as in Th17 cells. Thus it requires further investigation to examine whether IL-21 is produced by IL-17A-secreting CD8 T cells and plays a significant role during the differentiation process.

In summary, TGF-β and IL-6 are necessary for naïve CD8 T cells to differentiate into IL-17A secretors. Neutralization of IFN-γ is also required for the differentiation of IL-17A-secreting CD8 T cells, but IL-4 does not have to be neutralized. TNF-α and IL-1β further enhance the generation of IL-17A-secreting CD8 T cells in the presence of TGF-β and IL-6. However, IL-23 and IL-21 do not play a significant role during the differentiation. It is still unknown whether IL-23 plays a role in the maintenance of IL-17A-secreting CD8 T cells, or IL-21 is endogenously produced by IL-17A-secreting CD8 T cells. The APC system augmented the percentage of IL-17A-secreting CD8 T cells, compared to the APC-free system even with TNF-α and IL-1β (Figure 2, 3, 5, 6). This observation indicated that the cellular environment for the generation of IL-17A-secreting CD8 T cells may require the physical interaction between APCs and CD8 T cells. Therefore, naïve CD8 T cells were cultured with mitomycin-C treated splenocytes for subsequent experiments in order to obtain a large percentage of IL-17A secretors.

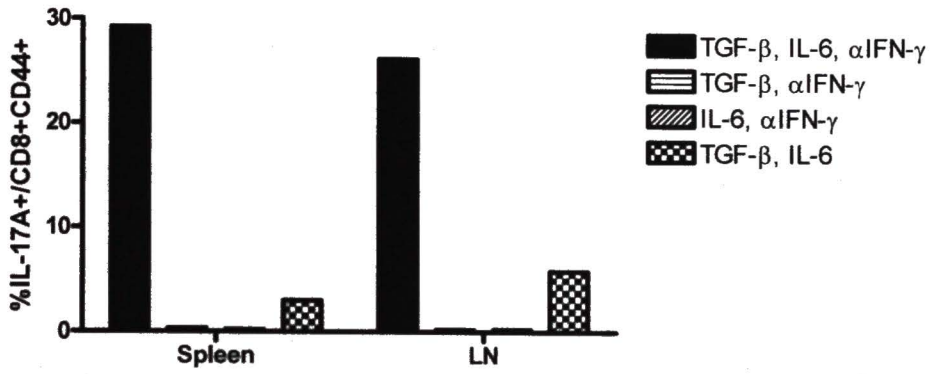

Figure 1. CD8+ cells are capable of producing IL-17A in the presence of TGF-β, IL-6 and αIFN-γ.

CD8+ cells were sorted from spleen or lymph nodes of C57BL/6 mice, using CD8 magnetic beads. Then cells were cultured with mitomycin C-treated splenocytes and treated with +/- TGF-β, +/- IL-6, +/- αIFN-γ and soluble αCD3 for 6 days. On day 6 of culture, cells were stimulated with PMA and Ionomycin for 5 hours and stained for CD8, CD44 and IL-17A. The bar graph from flow cytometric analysis represents the percentage of IL-17A-secreting cells gated on CD8+ CD44+. This data is representative of two independent experiments. The confirmatory experiment found the same pattern of results. *LN = lymph nodes

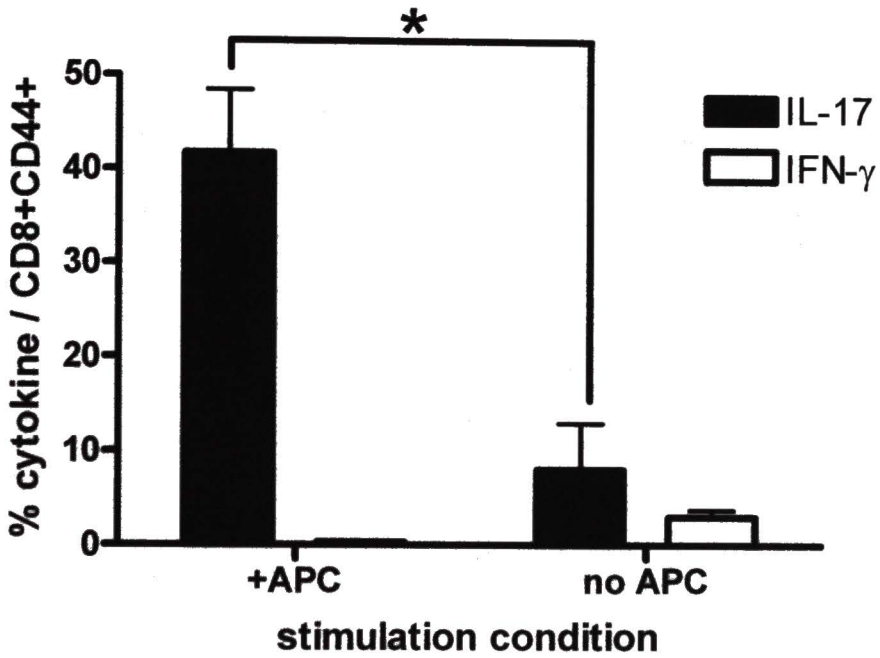

Figure 2. IL-17A-secreting CD8 T cells develop more in the presence of APCs than in the absence of APCs. (magnetic bead-sorted CD8+ cells)

CD8+ cells were obtained from lymph nodes of C57BL/6 mice using CD8 magnetic beads and cultured with APCs (mitomycin C-treated splenocytes, soluble αCD3, TGF-β, IL-6 and αIFN-γ) or without APCs (plate-bound αCD3, soluble αCD28, TGF-β, IL-6 and αIFN-γ) for 3 days. On day 3 of culture, cells were stimulated with PMA and Ionomycin for 5 hours and stained for CD8, CD44, IL-17A and IFN-γ. The bar graph from flow cytometric analysis represents the percentage of cytokine-producing cells gated on CD8+ CD44+. These data are expressed as the mean $\pm$ SEM of two independent experiments. An * denotes the APC group differs from the no APC group for IL-17A production, $p < 0.05$.

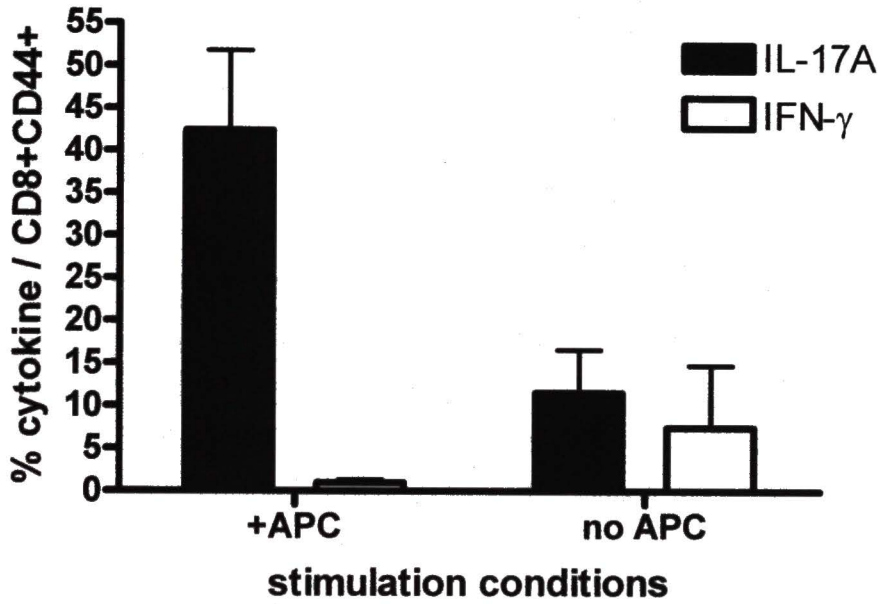

Figure 3. IL-17A-secreting CD8 T cells develop more in the presence of APCs than in the absence of APCs. (CD8+ CD62L+ cells sorted from the cell sorter)

Naïve CD8+ CD62L+ cells were purified from lymph nodes of C57BL/6 mice using the Cytopeia InFlux cell sorter and cultured with APCs (mitomycin C-treated splenocytes, soluble αCD3, TGF-β, IL-6 and αIFN-γ) or without APCs (plate-bound αCD3, soluble αCD28, TGF-β, IL-6 and αIFN-γ) for 3 days. On day 3 of culture, cells were stimulated with PMA and Ionomycin for 5 hours and stained for CD8, CD44, IL-17A and IFN-γ. The bar graph from flow cytometric analysis represents the percentage of cytokine-producing cells gated on CD8+ CD44+. These data are expressed as the mean ± SEM of two independent experiments. While statistical significance was not reached, the same pattern of results was obtained for both independent experiments, and replicated the data in Figure 2.

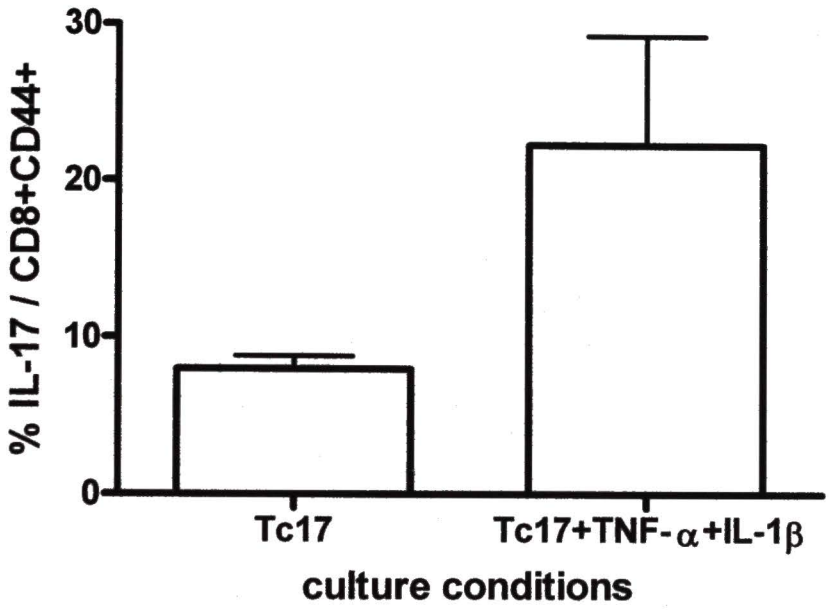

Figure 4. TNF-α and IL-1β are required for the optimal differentiation of IL-17A-secreting CD8 T cells.

Naïve CD8+ CD62L+ cells were purified from lymph nodes of C57BL/6 mice using the Cytopeia InFlux cell sorter and treated with plate-bound αCD3, soluble αCD28, TGF-β, IL-6, αIFN-γ, +/- TNF-α and IL-1β for 3 days. On day 3 of culture, cells were stimulated with PMA and Ionomycin for 5 hours and then stained for CD8, CD44 and IL-17A. The bar graph from flow cytometric analysis represents the percentage of IL-17A-secreting cells gated on CD8+ CD44+. These data are expressed as the mean ± SEM of two independent experiments.

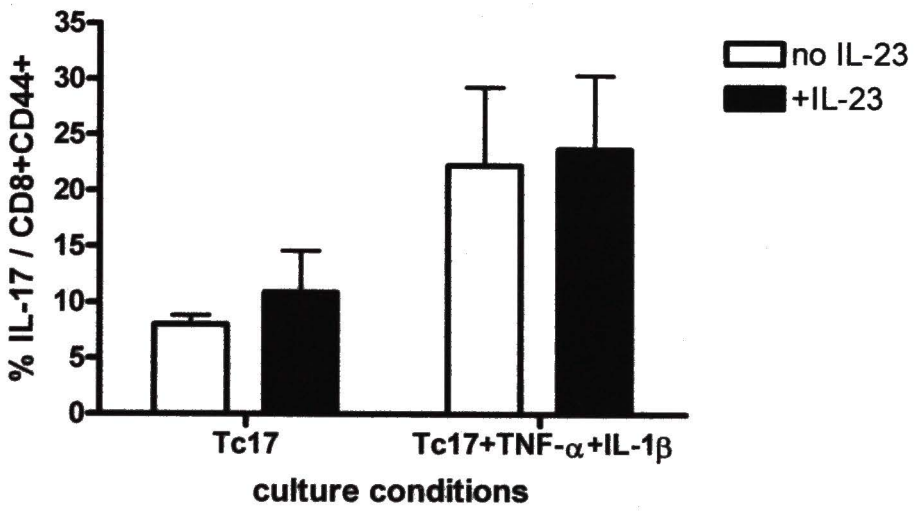

Figure 5. IL-23 is not necessary for the optimal differentiation of IL-17A-secreting CD8 T cells in the presence of TNF-α and IL-1β.

Naïve CD8+ CD62L+ cells were purified from lymph nodes of C57BL/6 mice using the Cytopeia InFlux cell sorter and treated with plate-bound αCD3, soluble αCD28, TGF-β, IL-6, αIFN-γ, +/- TNF-α and IL-1β, +/- IL-23 for 3 days. On day 3 of culture, cells were stimulated with PMA and Ionomycin for 5 hours and then stained for CD8, CD44 and IL-17A. The bar graph from flow cytometric analysis represents the percentage of IL-17A-secreting cells gated on CD8+ CD44+. These data are expressed as the mean ± SEM of two independent experiments. While statistical significance was not reached, the same pattern of results was obtained for both independent experiments.

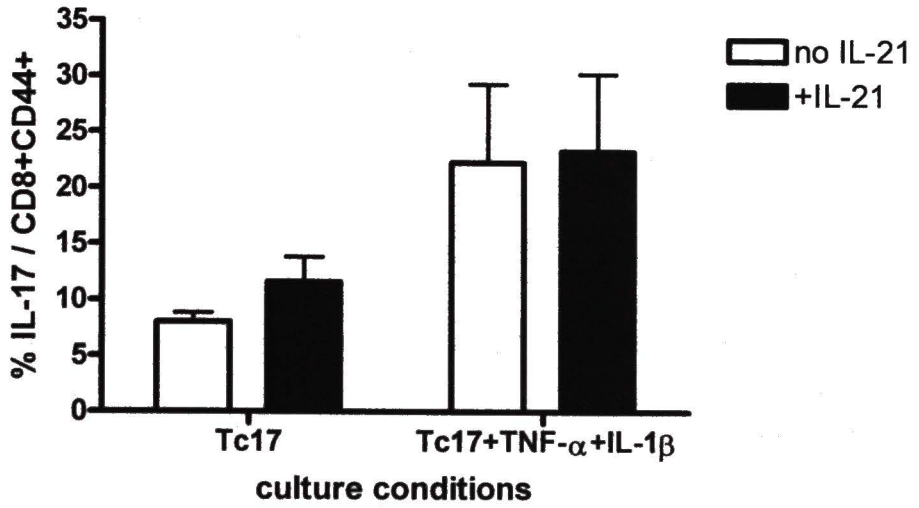

Figure 6. IL-21 is not necessary for the optimal differentiation of IL-17A-secreting CD8 T cells in the presence of TNF-α and IL-1β.

Naïve CD8+ CD62L+ cells were purified from lymph nodes of C57BL/6 mice using the Cytopeia InFlux cell sorter and treated with plate-bound αCD3, soluble αCD28, TGF-β, IL-6, αIFN-γ, +/- TNF-α and IL-1β, +/- IL-21 for 3 days. On day 3 of culture, cells were stimulated with PMA and Ionomycin for 5 hours and then stained for CD8, CD44 and IL-17A. The bar graph from flow cytometric analysis represents the percentage of IL-17A-secreting cells gated on CD8+ CD44+. These data are expressed as the mean ± SEM of two independent experiments. While statistical significance was not reached, the same pattern of results was obtained for both independent experiments.

CHAPTER IV

EFFECTOR FUNCTIONS OF IL-17A-SECRETING CD8 T CELLS

Th17 cells produce IL-17A, IL-17F and IL-22 during host defense against invasive pathogens [4]. IL-17A-secreting CD8 T cells may also secrete the same cytokines for their effector functions, but this had yet to be shown. In addition to the production of effector cytokines, CD8 T cells are known to perform cytotoxic activities to eliminate malformed, infected or autoimmune cells [2]. Therefore, it is of great interest to examine whether IL-17A-secreting CD8 T cells possess cytotoxic ability.

Although the investigation of the differentiation conditions necessary for IL-17A-secreting CD8 T cells was conducted on day 3 of culture in Chapter III, the remaining experiments utilized a 6-day period, which is commonly used to ensure a stabilized phenotype of IL-17A secretors [20;48]. In order to determine if IL-17A, IL-17F and IL-22 are produced by CD8 T cells, naïve CD8 T cells were sorted from lymph nodes and cultured with TGF-β, IL-6, αIFN-γ, αCD3 and mitomycin-C treated splenocytes for 3 days. On day 3, cells were re-treated with TGF-β, IL-6 and αIFN-γ

to further differentiate CD8 T cells into IL-17A-secreting CD8 T cells. Furthermore, IL-23 was also added on day 3 to stabilize already differentiated IL-17A-secreting CD8 T cells. Previous reports have stated that IL-23, while not necessary for the differentiation of Th17 cells, helps stabilize the Th17 phenotype [24;32]. On day 6, intracellular cytokine staining was performed for IL-17A, IL-17F and IL-22. The flow cytometric analysis indicated that IL-17A-secreting CD8 T cells produce IL-17A but not IL-17F or IL-22 (Figure 7). In order to confirm the flow cytometric data, ELISA was performed to measure IL-17A, IL-17F and IL-22 in culture supernatants. As described above, naïve CD8 T cells were differentiated into IL-17A secretors for 6 days, and cells were sorted for CD8 and CD44 expression to obtain activated IL-17A-differentiated CD8 T cells. Purified CD8+ CD44+ cells were then rested for 12-16 hours in the absence of mitomycin C-treated splenocytes, and some of the cells were stimulated with PMA and Ionomycin for 5 hours. Therefore, any cytokine detected in the culture media would be originated from the activated CD8+ CD44+ cells. The ELISA data, however, detected the presence of IL-17F and IL-22 from the culture media of the unstimulated cells or cells stimulated with PMA and Ionomycin (Figure 8). The ELISA experiment, therefore, indicated that IL-17A-secreting CD8 T cells produce IL-17F and IL-22.

The discrepancy of the results might have been caused by ineffective binding of the flow cytometric antibodies (IL-22 PE and IL-17F AF 488) to the target intracellular cytokines. The effectiveness of the antibodies was questionable due to the large amounts of the cytokines measured in the ELISA experiment. Other clones of the IL-17F and IL-22 antibodies do exist [50], but they are not commercially available. From the flow cytometric and ELISA data, it was concluded that IL-17F AF 488 and IL-22 PE antibodies were not functional, and that IL-17A-secreting CD8 T cells do produce IL-17F and IL-22.

Conventional cytotoxic CD8 T cells secrete granzyme B during their cytotoxic activities [2]. However, it was not certain whether IL-17A-secreting CD8 T cells produce granzyme B. In order to determine the expression of granzyme B in IL-17A-secreting CD8 T cells, naïve CD8 T cells were differentiated into IL-17A secretors as described above. On day 6, cells were stimulated with PMA and Ionomycin, and flow cytometry was performed to detect the intracellular expression level of granzyme B. The flow cytometric analysis revealed that IL-17A-secreting cells are not positive for the granzyme B expression, indicating that IL-17A-secreting CD8 T cells may not be involved in cytotoxicity (Figure 9). The isotype control assay was performed in order to detect any non-specific binding of granzyme B antibodies. However, the data

indicated that non-specific binding of granzyme B antibodies did not occur (data not shown).

In summary, IL-17A-secreting CD8 T cells produce IL-17A, IL-17F and IL-22 as effector cytokines, which are also secreted by Th17 cells. However, IL-17A-secreting CD8 T cells differ with conventional CD8 T cells in that they do not produce granzyme B. This result suggests that IL-17A-secreting CD8 T cells may not engage in cytotoxic activities. Expression of other types of granzyme molecules, as well as perforins and Fas ligands, needs to be examined to confirm the lack of cytotoxic ability of IL-17A-secreting CD8 T cells. Killing assays can be also performed to further support the idea that IL-17A-secreting CD8 T cells are not cytotoxic.

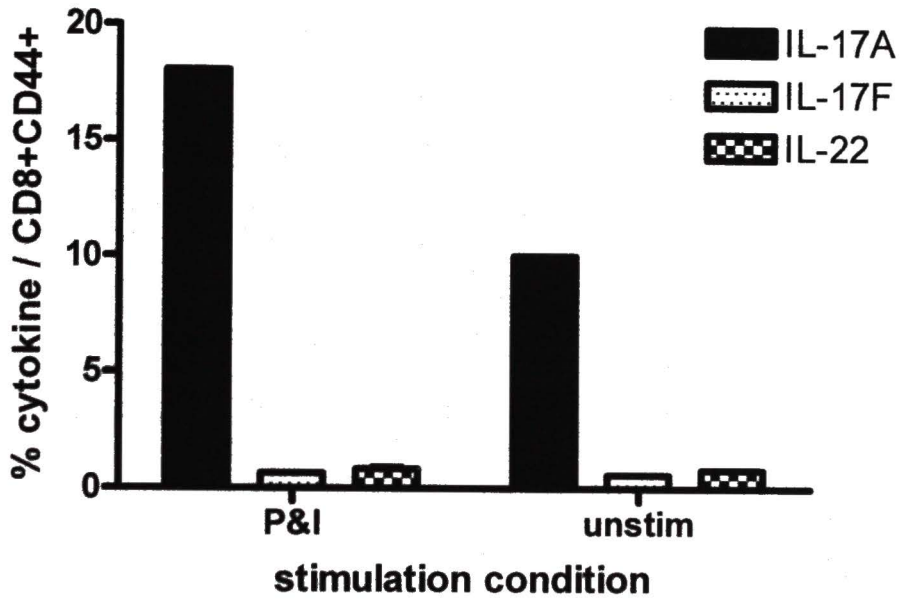

Figure 7. IL-17F+ and IL-22+ cells are not detected from the population of IL-17A-differentiated CD8 T cells.

CD8+ CD62L+ cells were purified from lymph nodes of C57BL/6 mice using Cytopeia InFlux cell sorter and cultured with mitomycin C-treated splenocytes, TGF-β, IL-6, αIFN-γ and soluble αCD3 for 3 days. On day 3 of culture, cells were re-treated with TGF-β, IL-6, αIFN-γ and IL-23. On day 6, some cells were stimulated with PMA and Ionomycin for 5 hours and then stained for CD8, CD44 and intracellular cytokines shown in the graph above. The bar graph from flow cytometry analysis represents the percentage of IL-17A, IL-17F or IL-22-secreting cells gated on CD8+ CD44+. This data is representative of two independent experiments. The confirmatory experiment found the same pattern of results. *P&I – PMA and Ionomycin *unstim = unstimulated (not stimulated with PMA and Ionomycin)

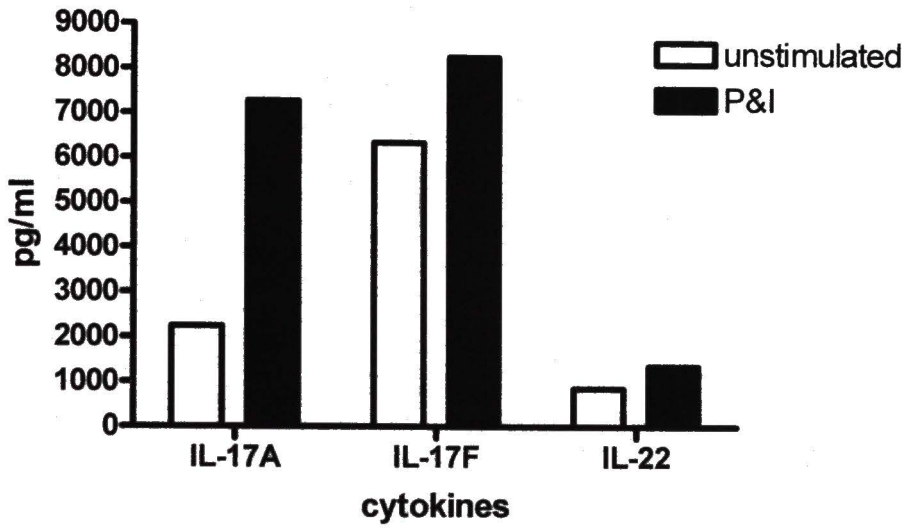

Figure 8. IL-17A-differentiated CD8 T cells produce IL-17A, IL-17F and IL-22.

CD8+ CD62L+ cells were purified from lymph nodes of C57BL/6 mice using Cytopeia InFlux cell sorter and cultured with mitomycin C-treated splenocytes, TGF-β, IL-6, αIFN-γ and soluble αCD3 for 3 days. On day 3 of culture, cells were re-treated with TGF-β, IL-6, αIFN-γ and IL-23. On day 6, cells were harvested and sorted for CD8+ CD44+ cells. Those sorted cells were rested for 12-16 hours and then stimulated with PMA and Ionomycin for 5 hours. Some sorted cells were not stimulated with PMA and Ionomycin. The bar graph of ELISA represents cytokine levels present in the overnight culture media. This data is representative of two independent experiments. The confirmatory experiment found the same pattern of results. *P&I – PMA and Ionomycin *unstimulated = not stimulated with PMA and Ionomycin

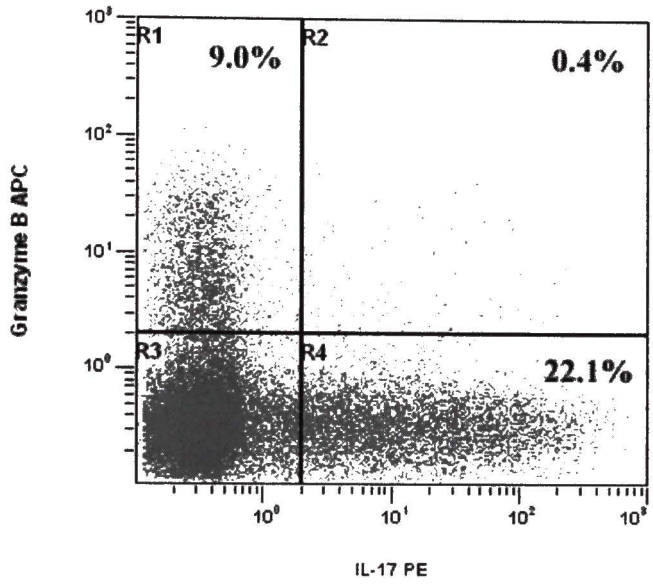

Figure 9. IL-17A-secreting cells do not express granzyme B.

CD8+ CD62L+ cells were purified from lymph nodes of C57BL/6 mice using Cytopeia InFlux cell sorter and cultured with mitomycin C-treated splenocytes, TGF-β, IL-6, αIFN-γ and soluble αCD3 for 3 days. On day 3 of culture, cells were re-treated with TGF-β, IL-6, αIFN-γ and IL-23. On day 6, cells were stimulated with PMA and Ionomycin for 5 hours and then stained for CD8, CD44, granzyme B and IL-17A. The dot plot from flow cytometric analysis represents the percentage of IL-17A and/or granzyme B-producing cells gated on CD8+ CD44+. This data is representative of two independent experiments. The confirmatory experiment found the same pattern of results.

CHAPTER V

CYTOKINE RESPONSIVENESS OF IL-17A-SECRETING CD8 T CELLS

Cytokine responsiveness refers to a cell's ability to respond to stimuli from different combinations of exogenous cytokines. The measurement of cytokine responsiveness is often demonstrated by the cytokines produced by the responding cell. For example, Th1 cells secrete IFN-γ in response to IL-12 and IL-18 [34]. In addition, IL-23-primed CD4+ T helper cells produce IL-17A when cultured with IL-23 and IL-18, whereas those cells secrete IFN-γ after their exposure to IL-12 and IL-18 [34]. Moreover, Berg, et al. have described the ability of effector and memory CD8 T cells to secrete IFN-γ in response to IL-12 and IL-18 [87]. Thus IL-17A-secreting CD8 T cells may produce either their known effector cytokines or other types of cytokines, such as IFN-γ, when cultured with different combinations of IL-12, IL-23 and IL-18.

IL-12, IL-23 and IL-18 are predominantly produced by activated dendritic cells and macrophages and have pleiotropic effects on various types of cells in both innate and adaptive immunity [88]. IL-12 and IL-23 often

exhibit greater functional outcomes or synergistic effects in the presence of IL-18 [88]. While IL-12 and IL-23 share a common p40 subunit, they often induce a discrete array of cytokines and molecules [88]. Due to similar structures or synergistic effects, IL-12, -23 and -18 have been closely examined in regards to their functions during host defense and autoimmune reactions.

In order to determine the cytokine responsiveness of IL-17A-secreting CD8 T cells, purified naïve CD8 T cells were differentiated into IL-17A secretors for 6 days, as described in Chapter II and IV. Following the differentiation procedures, activated CD8+ CD44+ cells were further purified using the cell sorter and cultured with different combinations of IL-12, IL-23 and IL-18 for 12-16 hours. This culture system of purified, activated CD8 T cells does not contain mitomycin C-treated APCs. The cytokine measured, therefore, would solely come from the CD8+ CD44+ cell population.

The flow cytometric analysis revealed that IL-12 and IL-18 significantly increased the percentage of IL-17A-differentiated CD8 T cells that also produced IFN-γ (Figure 10). However, IL-23 and IL-18 augmented IL-17A production from IL-17A-differentiated CD8 T cells (Figure 10). Cells that were not treated with any exogenous cytokines still produced IL-17A but not IFN-γ, indicating that the cells

were differentiated into IL-17A secretors (Figure 10). Thus, this data demonstrated that IL-17A-differentiated CD8 T cells produce IFN-γ in response to IL-12 and IL-18, whereas they enhance the production of IL-17A in response to IL-23 and IL-18.

Intracellular cytokine staining for IL-17F and IL-22 was not performed due to the potential inefficient binding of the antibodies to the target cytokines (Figure 7, 8). Instead, ELISA was utilized to determine the levels of IL-17F and IL-22 in the culture media of IL-17A-differentiated CD8 T cells treated with IL-12+IL-18 or IL-23+IL-18. Naïve CD8 T cells from LN were purified with the cell sorter and then differentiated into IL-17A secretors for 6 days, as described earlier. On day 6 of culture, CD8+ CD44+ cells were further purified to obtain activated IL-17A-differentiated CD8 T cells, and they were treated with different combinations of IL-12, IL-23 and IL-18 for 12-16 hours.

Congruent with the flow cytometric data, the ELISA experiment demonstrated that IL-23 and IL-18 enhanced the production of IL-17A from IL-17A-differentiated CD8 T cells (Figure 11). Interestingly, IL-12 and IL-18 also increased the production of IL-17A although not in the same degree as IL-23 and IL-18 did (Figure 11). Since both treatment conditions (IL-12+IL-18 and IL-23+IL-18) increased the secretion of IL-17A, the role of IL-18 in the cytokine responsiveness was investigated. IL-18

enhanced the production of IL-17A, but the production level was lower, compared to IL-12+IL-18-treated or IL-23+IL-18-treated cells (Figure 11). Thus, while IL-18 was able to induce IL-17A secretion on its own, it also worked synergistically with IL-23 and, to a slight degree, with IL-12, to enhance IL-17A production from IL-17A-differentiated CD8 T cells.

Similar to IL-17A, the production of IL-22 was increased in response to IL-23 and IL-18 (Figure 12). IL-12 and IL-18 also induced the increase in the secretion of IL-22 albeit with a less degree than IL-23 and IL-18 (Figure 12). The role of IL-18 in the production of IL-22 could not be investigated due to insufficient numbers of purified IL-17A-differenitated CD8 T cells. IL-18, however, is expected to moderately increase IL-22 production.

Like IL-17A and IL-22, the production level of IL-17F was also increased upon the exposure to IL-23 and IL-18 (Figure 13). IL-12 and IL-18 demonstrated the enhancement of IL-17F secretion, but not more than IL-23 and IL-18 (Figure 13). IL-18 alone elicited the slight increase in the production of IL-17F from IL-17A-differentiated CD8 T cells (Figure 13). The high levels of IL-17F, compared to IL-17A or IL-22, might indicate that IL-17A-differentiated CD8 T cells constitutively produce IL-17F (Figure 11, 12, 13).

In summary, IL-17A-differentiated CD8 T cells produced Th17 effector cytokines (IL-17A, IL-17F, IL-22) when they were treated with IL-23 and IL-18. However, those CD8 T cells produced IFN-γ in response to IL-12 and IL-18. IL-12 and IL-18 also increased the production of IL-17A, IL-17F and IL-22, but not more than IL-23 and IL-18. IL-18 moderately induced the production of IL-17A and IL-17F, but it also demonstrated the synergistic effects with IL-23, and to a less degree, with IL-12. Collectively, these data suggest that IL-17A-differentiated CD8 T cells are capable of secreting a distinct array of effector cytokines in accordance with certain exogenous stimulus, independently of TCR stimulation. This ability to secrete multiple effector cytokines can be further examined to determine the roles these CD8 T cells play in the inflammatory milieu where numerous cytokines are secreted by various types of cells.

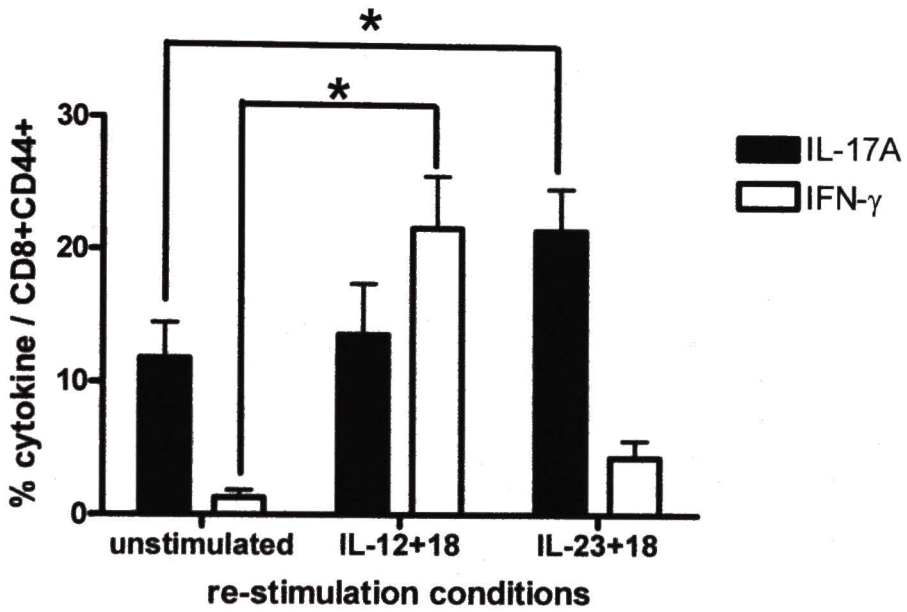

Figure 10. IL-17A-differentiated CD8 T cells secrete IFN-γ or IL-17A in response to IL-12 and IL-18, or IL-23 and IL-18, respectively.

Purified CD8+ CD62L+ cells were differentiated into IL-17A-secreting CD8 T cells for 6 days, as described in Chapter II, and CD8+ CD44+ cells were purified on day 6 using the Cytopeia InFlux cell sorter. The sorted CD8+ CD44+ cells were then treated with +/- IL-12 and IL-18, +/- IL-23 and IL-18 for 12-16 hours in the absence of APCs. The intracellular cytokine staining was performed. The bar graph of flow cytometric analysis represents the percentage of cells that secrete IL-17A or IFN-γ, gated on CD8+ CD44+. These data are expressed as the mean $\pm$ SEM of two independent experiments. An * denotes IL-12+18 group differs from the unstimulated group for IFN-γ production, and IL-23+18 group differs from the unstimulated group for IL-17A production, $p < 0.05$.

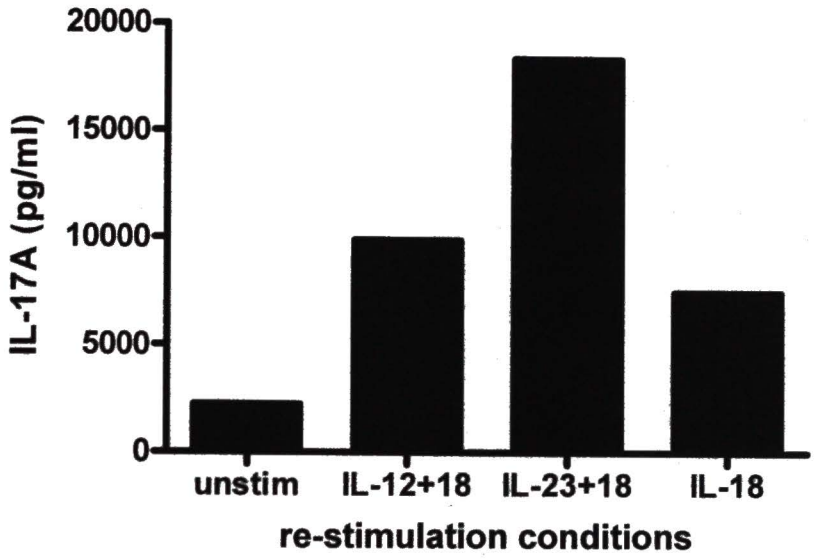

Figure 11. IL-17A-differentiated CD8 T cells secrete IL-17A in response to IL-23 and IL-18.

Purified CD8+ CD62L+ cells were differentiated into IL-17A-secreting CD8 T cells for 6 days, as described in Chapter II, and CD8+ CD44+ cells were purified on day 6 using the Cytopeia InFlux cell sorter. The sorted CD8+ CD44+ cells were then treated with +/- IL-12 and IL-18, +/- IL-23 and IL-18, +/- IL-18 for 12-16 hours in the absence of APCs. The bar graph of ELISA analysis represents the amount of IL-17A measured from the culture media of IL-17A-differentated CD8 T cells. This data is representative of two independent experiments. The confirmatory experiment found the same pattern of results. *unstim = unstimulated

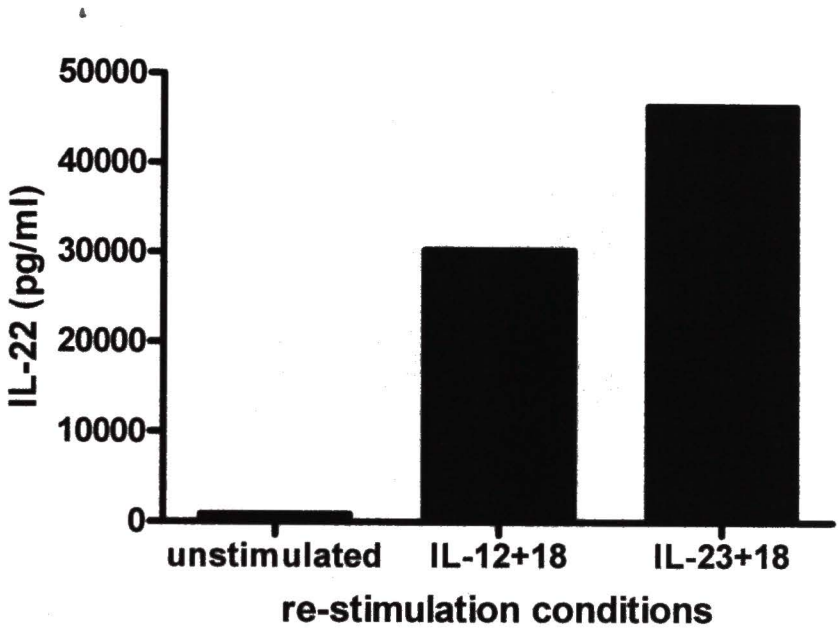

Figure 12. IL-17A-differentiated CD8 T cells increase the production of IL-22 in response to IL-23 and IL-18.

Purified CD8+ CD62L+ cells were differentiated into IL-17A-secreting CD8 T cells for 6 days as described in Chapter II, and CD8+ CD44+ cells were purified on day 6 using the Cytopeia InFlux cell sorter. The sorted CD8+ CD44+ cells were then treated with +/- IL-12 and IL-18, +/- IL-23 and IL-18 for 12-16 hours in the absence of APCs. ELISA was performed to determine the concentration of IL-22 in the culture media of IL-17A-differentiated CD8 T cells. This data is representative of two independent experiments. The confirmatory experiment found the same pattern of results, but a standard curve was unable to be generated.

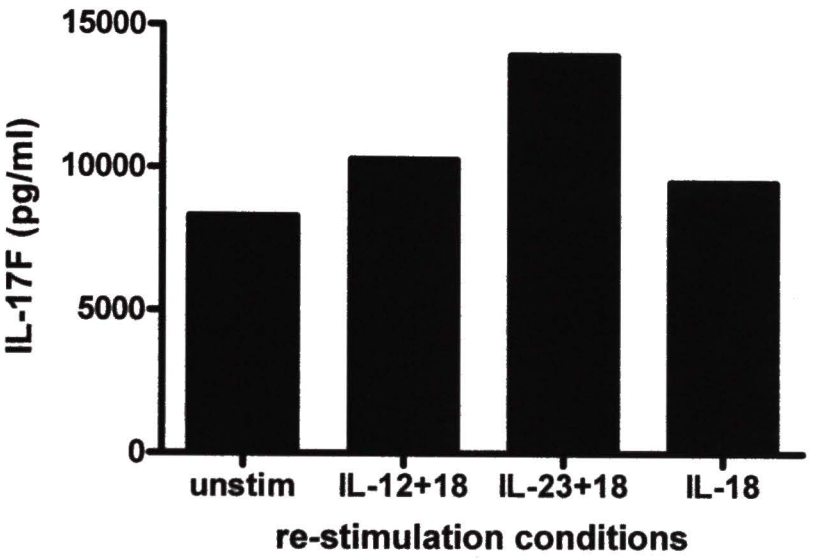

Figure 13. IL-17A-differentiated CD8 T cells increase the production of IL-17F in response to IL-23 and IL-18.

Purified CD8+ CD62L+ cells were differentiated into IL-17A-secreting CD8 T cells for 6 days as described in Chapter II, and CD8+ CD44+ cells were purified on day 6 using the Cytopeia InFlux cell sorter. The sorted CD8+ CD44+ cells were then treated with +/- IL-12 and IL-18, +/- IL-23 and IL-18, +/- IL-18 for 12-16 hours in the absence of APCs. ELISA was performed to determine the concentration of IL-17F in the culture media of IL-17A-differentiated CD8 T cells. This data is representative of two independent experiments. The confirmatory experiment found the same pattern of results. *unstim = unstimulated

CHAPTER VI

PHENOTYPIC STABILITY OF IL-17A-SECRETING CD8 T CELLS

One of the hallmarks about the Th1 and Th2 cells is their phenotypic stability. Once the final phenotype is established, Th1 and Th2 cells do not transform into another subtypes [3]. The phenotype stability of T helper cells is mainly determined by effector cytokines they produce under different differentiation conditions. For instance, Th1 cells secrete their effector cytokine, IFN-γ, under Th2-favoring conditions, whereas Th2 cells produce IL-4, -5 and -13 under Th1-polarizing conditions [3]. The phenotypic stability of Th17 cells, therefore, can be assessed from their ability to secrete IL-17A under Th1 or Th2 generation conditions.

As mentioned in Chapter I, conflicting reports have been published in regards to the phenotypic stability of Th17 cells. In addition, the discovery of CD4 T cells that simultaneously produce both IL-17A and IFN-γ further adds more complexity to this issue [39]. Th1 and Th17 cells may share a common differentiation stage which provides the ability to produce both IFN-γ and IL-17A before reaching their final phenotype. Alternatively, Th17 cells may not exhibit phenotypic stability and thus

secrete IFN-γ in response to certain stimuli. As shown in Chapter V, IL-17A-secreting CD8 T cells produced IFN-γ in response to IL-12 and IL-18, without losing their ability to produce IL-17A (Figure 10). This data suggests that IL-17A-secreting CD8 T cells either do not demonstrate phenotypic stability or obtain the ability to produce both Th1 and Th17 cytokines and require appropriate stimuli to induce either one. In order to clearly determine the phenotypic stability of Th17 cells, additional information is required in regards to surface molecules that are preferentially expressed on IL-17A-secreting CD8 T cells. To date, no reports have showed Th17-specific surface markers on IL-17A-secreting CD8 T cells.

In addition, almost perfect purification of the CD8 T cells is necessary in order to determine the phenotypic stability of IL-17A-secreting CD8 T cells. A few effector and memory cells that are not differentiated into IL-17A-secreting CD8 T cells would hinder the interpretation of the data. Purification of IL-17A-secreting CD8 T cells based on CD8 and CD44 expression would recover some of the IL-17A-secreting CD8 T cells. However, a few activated Tc1 cells (CD8 T cells with Th1 phenotype) would be also sorted out. Several reports demonstrated that human Th17 cells express CCR2, CCR4 and CCR6 [40;46], and these receptors could be utilized for selecting IL-17A-secreting CD8 T cells. However, antibodies against CCR2, CCR4 and CCR6

are commercially available only for human cells, and no information is available for the cross-reactivity of those antibodies with mouse. Moreover, those chemokine receptors are also expressed by other types of cells [40]; therefore, additional surface markers are necessary to purify IL-17A-secreting CD8 T cells.

Since Th17 cells express IL-23R [39], the expression of IL-23R on IL-17A-secreting CD8 T cells was examined with flow cytometry. CD8 T cells with high expression of CD44 and IL-23R could be better defined as IL-17A-secreting CD8 T cells. In order to examine IL-23R on IL-17A-differentiated CD8 T cells, naïve CD8 T cells from LN were purified with the cell sorter and differentiated into IL-17A-secreting CD8 T cells for 6 days, as described in Chapter II and IV. On day 6 of culture, CD8+ CD44+ cells were further purified to obtain IL-17A-differentiated CD8 T cells. Following the purification, cells were rested for 12-16 hours and stimulated with PMA and Ionomycin for 5 hours. The flow cytometric data revealed the cell population positive for IL-23R expression (Figure 14).

In order to test the non-specific binding of the antibodies, the isotype control assay was performed. The isotype control assay estimates the non-specific binding of primary antibodies to cell surface antigens. Non-specific binding occurs due to binding of primary antibodies to Fc receptors or other proteins. In the assay, isotype

control monoclonal antibodies are used, which have the same isotype as the primary antibodies. Therefore, if non-specific binding occurs, then false positive cells appear in the flow cytometric dot plots.

The isotype control assay demonstrated that cells stained with isotype control antibodies had a similar percentage of positive cells, compared to cells that were stained with IL-23R antibodies (Figure 14). This result indicated that IL-23R antibody showed non-specific binding and thus cannot be used for selecting IL-17A-secreting CD8 T cells. Due to the lack of reagents to sort out IL-17A-secreting CD8 T cells with 100% purity, this aim remains yet to be answered.

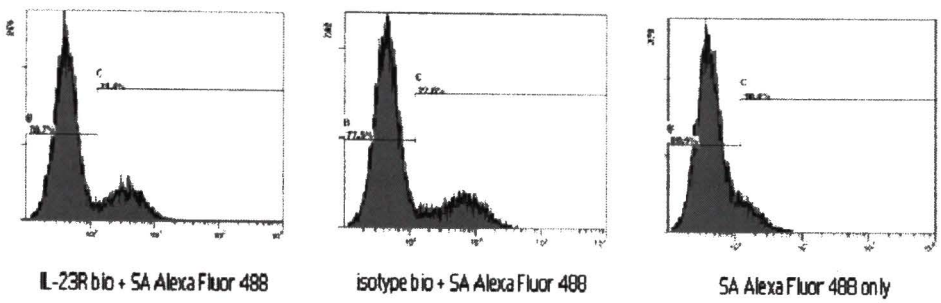

Figure 14. IL-23R and the isotype control staining showed non-specific binding of IL-23R antibody.

CD8+ CD62L+ cells were purified from lymph nodes of C57BL/6 mice using Cytopeia InFlux cell sorter and cultured with mitomycin C-treated splenocytes, TGF-β, IL-6, αIFN-γ and soluble αCD3 for 3 days. On day 3 of culture, cells were re-treated with TGF-β, IL-6, αIFN-γ and IL-23. On day 6, cells were harvested and sorted for CD8+ CD44+ cells. Those cells were rested for 12-16 hours and then stimulated with PMA and Ionomycin for 5 hours. The numbers in the histograms represent the percentage of positive or negative cells with given antibodies shown above (out of all the live-gated events). *bio = biotin, SA = streptadivin

CHAPTER VII

DISCUSSION

Th17 cells have recently emerged as a new subset of T helper cells that modulate inflammatory responses during a variety of infections and diseases. Although effector functions and characteristics of Th17 cells have been intensely investigated, IL-17A-secreting CD8 T cells have not drawn much attention. In this study, the differentiation conditions and effector functions of IL-17A-secreting CD8 T cells were demonstrated. Similar to Th17 cells, naïve CD8 T cells required TGF-β and IL-6 in order to obtain the ability to produce IL-17A. Inflammatory cytokines, such as TNF-α and IL-1β, further enhanced the development of IL-17A-secreting CD8 T cells. IFN-γ negatively affected the polarization of naive CD8 T cells into IL-17A secretors, but IL-4 did not show any negative effects on the differentiation of IL-17A-secreting CD8 T cells. This finding implicates that the generation of IL-17A-secreting CD8 T cells is impeded more in Th1 than Th2 environments. Thus, IL-17A-secreting CD8 T cells may be more easily generated during Th2 responses, potentiating their effector functions. The role of IL-17A-secreting CD8 T cells in

Th2-driven responses is of great interest to the understanding of the relationships between the two subsets of effector T cells, Th2 and IL-17A+ CD8 T cells, during the host defense against certain pathogens.

In order to determine effector functions of IL-17A-secreting CD8 T cells, the expression of granzyme B was examined, which is a measure of the cytotoxic ability of CD8 T cells. Surprisingly, the flow cytometric data showed no expression of granzyme B from IL-17A secretors. This finding implicates that IL-17A-secreting CD8 T cells are not cytotoxic, unlike conventional CD8 T cells. Thus IL-17A-secreting CD8 T cells can be efficient for immune responses against extracellular pathogens, whose clearance does not require the elimination of infected cells. To further support this observation, the expression of other granzymes and perforin molecules can be evaluated. Killing assays, such as chromium release assays, can be also performed to determine cytotoxicity of IL-17A-secreting CD8 T cells.

In addition to granzyme B, effector cytokines of IL-17A-secreting CD8 T cells was examined to determine their effector functions. Intracellular cytokine staining data indicated that IL-17A-secreting CD8 T cells produced IL-17A, and the ELISA data confirmed the production of IL-17F and IL-22 from IL-17A-secreting CD8 T cells. These results demonstrate that IL-17A-secreting CD8 T cells are involved in

the generation and recruitment of neutrophils and the production of antimicrobial peptides. Neutrophils are generally the first to arrive at inflammatory cites during innate immunity, and antimicrobial peptides are produced by various types of cells during innate immune responses. Thus IL-17A-secreting CD8 T cells can further strengthen innate immunity through the actions of their effector cytokines.

During adaptive immune responses, T cells perform their effector functions via activation signals delivered through TCR (T cell receptor) and costimulatory molecules. It was demonstrated that IL-17A-secreting CD8 T cells produce their effector cytokines via TCR signaling. However, IL-17A-secreting CD8 T cells also secreted their effector cytokines when they were treated with IL-23 and IL-18. This finding indicates that IL-17A-secreting CD8 T cells can respond to signals from exogenous cytokines, independently of TCR stimulation. In other words, they can perform their effector functions in an innate fashion as various cytokines from APCs and other cells bind to cell surface receptors. IL-17A-secreting CD8 T cells, thus, can participate in innate immune protection in the absence of a cognate antigen. To confirm this argument, future studies using certain disease models can evaluate the production of the effector cytokines from IL-17A-secreting CD8 T cells that are generated in vitro and transferred into mice. Another future experiment can examine

the levels of the effector cytokines in mice challenged with a particular antigen and then injected with exogenous IL-23 and IL-18.

The cytokine responsiveness studies demonstrated that IL-17A-secreting CD8 T cells responded to IL-12 and IL-18 by producing IFN-γ. However, they did not lose their ability to secrete IL-17A. This observation suggests that IL-17A-secreting CD8 T cells can serve dual effector functions, depending on the exogenous cytokines they encounter. For example, IL-17A-secreting CD8 T cells can secrete IFN-γ and stimulate macrophages, whereas IL-17A from those CD8 T cells can facilitate the recruitment of neutrophils into infection sites. Thus IL-17A-secreting CD8 T cells can exhibit differential effector functions in response to external stimuli. Depending on the nature of the infection and the stage of the diseases, IL-17A-secerting CD8 T cells can mediate immune responses by producing distinct effector cytokines that are appropriate for the clearance of particular pathogens they are encountering.

In addition to IL-17A, IL-17F and IL-22, Th17 cells are known to produce IL-21 [22;23;23;24]. IL-21 enhances Th17 differentiation as an autocrine growth factor. This current study did not measure IL-21 from IL-17A-secreting CD8 T cells because IL-21 transcripts are reported to be absent in peripheral CD8 T cells [85]. However, it is possible that IL-17A-secreting CD8 T cells produce IL-21 during the differentiation

stage as in Th17 cells. One recent report demonstrated that IL-21 differentiates naïve CD8 T cells into effector CD8 T cells characterized by the lack of cytotoxicity but efficient anti-tumor activities [89]. This description of effector CD8 T cells is congruent with the one seen in IL-17A-secreting CD8 T cells with the lack of granzyme B expression. I demonstrated that IL-21 plays a minor role in the differentiation of IL-17A-secreting CD8 T cells. It may be the case that IL-17A-secreting CD8 T cells produce enough endogenous IL-21 during the differentiation and as a consequence, exogenous IL-21 is not necessary. Experiments with IL-21-deficient mice or neutralizing IL-21 antibody would determine the existence of endogenous IL-21 from IL-17A-secreting CD8 T cells and the importance of IL-21 during the differentiation of IL-17A-secreting CD8 T cells.

Th17 cells produce IL-21 during the differentiation, but it is not certain whether IL-21 can act as another Th17 effector cytokine. IL-21 is known to be critical for humoral responses, especially during normal Ig production and terminal B cell differentiation into plasma cells [90]. Thus, if IL-17A-secreting CD8 T cells secrete IL-21, a subsequent future experiment can determine whether IL-17A-secreting CD8 T cells can influence humoral responses via IL-21.

In addition to humoral responses, IL-17A-secreting CD8 T cells may influence cellular responses. Nakae, et al. demonstrated the importance of IL-17A for optimal induction of Th1 and Th2 cells [91]. In this report, IL-17A-deficient mice failed to exhibit antigen-specific T cell sensitization during Th1-driven delayed-type hypersensitivity and Th2-mediated airway hypersensitivity response. Moreover, the impairment of Th1 induction has been reported in the absence of IL-17A and IL-23 during *Mycobacterium tuberculosis* infection [72]. This report indicated that IL-17A from Th17 cells induced the production of CXCL9, 10 and 11 from the lung. These chemokines are the ligands for CXCR3, which are preferentially expressed on Th1 cells [72]. Thus Th17 cells can promote the activation and recruitment of Th1 cells into infection sites through IL-17A's induction of Th1-recruiting chemokines.

The possible involvement of Th17 cells for recruiting Th2 cells has not been investigated. However, IL-17A and IL-13 synergistically induce IL-19 in human airway epithelial cells and eventually lead to asthma [92]. IL-19 is also known to increase the production of IL-4 and IL-13 from Th2 cells. Thus it is possible that IL-17A-secreting CD8 T cells can indirectly induce the Th2 effector cytokines via IL-17A and the subsequent production of IL-19 from epithelial cells. Aujla, et al. demonstrated the increase in the gene transcription for IL-19 when primary human

bronchial epithelial cells were treated with IL-17A and IL-22 [69]. This observation further confirms the notion that IL-17A-secreting CD8 T cells, along with other IL-17A secretors, may participate in the activation and possibly recruitment of Th2 cells. As already mentioned, the differentiation of IL-17A-secreting CD8 T cells is not affected by IL-4. Thus during Th2-driven pathology, IL-17A-secreting CD8 T cells may show detrimental effects by further activating Th2 effector functions.

Although the phenotype of Th1 and Th2 cells is known to be stable, it is not certain whether Th17 cells show phenotypic stability. A few reports argued that Th17 cells possess a stable phenotype although the opposing argument has also been made [15;26;48]. This current study attempted to investigate the phenotypic stability of IL-17A-secreting CD8 T cells. Due to the lack of reagents that purify the sole population of IL-17A-secreting CD8 T cells, this question remains yet to be answered. The observation that IL-23 plays a minor role during the differentiation of IL-17A-secreting CD8 T cells indicates that IL-17A-secreting CD8 T cells express IL-23R. The staining of IL-23R, however, was unsuccessful due to the non-specific binding of IL-23R antibodies. We found that IL-17A capture assay kits just recently became commercially available (Miltenyi Biotec). These reagents would be useful to purify IL-17A-secreting CD8 T cells and determine the phenotypic stability of IL-17A-

secreting CD8 T cells. Moreover, the purification of IL-17A-secreting CD8 T cells is necessary in order to perform adoptive transfer studies and investigate the role of IL-17A-secreting CD8 T cells in various disease models. It might also be of great interest to investigate the cellular mechanism of IL-17A-secreting CD8 T cells during anti-tumor immunity, as already reported that IL-21-primed CD8 T cells lack cytotoxicity but enhance regression of melanoma cells in mice [89]. Collectively, adoptive transfer studies of IL-17A-secreting CD8 T cells in a variety of infections and disease models will contribute to further understanding of the roles that IL-17A+ CD8 T cells play during host defense.

In conclusion, this thesis demonstrated that naïve CD8 T cells can develop into IL-17A secretors without cytotoxic ability and produce Th17 effector cytokines upon TCR stimulation and signals from exogenous cytokines. The present study also demonstrated that IL-17A-secreting CD8 T cells are capable of producing a Th1 cytokine when distinct exogenous cytokines are provided. These observations suggest that IL-17A-secreting CD8 T cells perform a wide array of effector functions that are essential for clearance of invasive pathogens. This study would be useful in obtaining knowledge to perform adoptive transfer experiments, which should raise the

possibility for new therapies to limit or prevent certain infections and diseases, including cancer.

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