Targeting Th (Th17 and Th2) suppressive and stimulatory effect on cytotoxic T cells

A thesis submitted to the College of

Graduate Studies and Research

in partial fulfillment of the requirements

for the degree of Doctor of Philosophy

In the Department of Pathology and Laboratory Medicine

University of Saskatchewan

Saskatoon

By

Manjunatha Ankathatti Munegowda

PERMISSION TO USE:

In presenting this thesis in partial fulfilment of the requirements for a postgraduate degree from the University of Saskatchewan, I agree that the libraries of this University may make it freely available for inspection. I further agree that permission for copying of this thesis in any manner, in whole or in part, for scholarly purposes may be granted by me or Dr. Jim Xiang, in his absence, by the Head of the Department or the Dean of the college in which my thesis work was done. It is understood that any copying or publication or use of this thesis or parts thereof for financial gain shall not be allowed without my written permission. It is also understood that due recognition shall be given to me and to the University of Saskatchewan in any scholarly use which may be made of any material in my thesis.

Requests for permission to copy or to make other use of material in this thesis in whole or part should be addressed to:

The Department of Pathology and Laboratory Medicine
Room 2841 Royal University Hospital, 103 Hospital Drive
University of Saskatchewan
Saskatoon, Saskatchewan, S7N 0W8

ABSTRACT

As the name indicates, T-helper cells are shown to help in primary and secondary cellular and humoral immune responses. They behave as conductors of immune responses. Conferring immunity to various kinds of antigens, the immune system has evolved different cell types. There are different terminally differentiated helper cells such as Th1, Th2, TFh, Th17, Treg, Th9, and Th22 cells tailored to combat different pathogens. Production of any subtype of cells depends on the type of antigen, dose of antigen, mode of entry, and cytokine milieu in the microenvironment. An infection or an aberrant growth of tumor cells or an autoimmunity occurs when there is an imbalance in immune responses. Since $CD4^+$ T cells are the conductors controlling different arms of immune-responses, the most frequent imbalances of immune response in the above conditions occur from deregulated $CD4^+$ T cell responses. Because of the importance associated with $CD4^+$ T cells, understanding the patho-physiology and biology associated with $CD4^+$ T cells is crucial. Our study addresses the role of $CD4^+$ Th17 cells in tumor immunity, in autoimmune type 1 diabetes (T1D), and in experimental autoimmune encephalitis (EAE). We have also considered the biology associated with $CD4^+$ Th2 cells.

In tumor immunity, it was demonstrated by various studies that $CD4^+$ Th17 cells induce antitumor immunity, leading to the eradication of established tumors. However, the mechanism of $CD8^+$ CTL activation by $CD4^+$ Th17 cells and the distinct role of $CD4^+$ Th17 and $CD4^+$ Th17 activated $CD8^+$ CTLs in antitumor immunity were still elusive. In this study we have demonstrated that $CD4^+$ Th17 cells acquired pMHC-I and expressed RORγt, IL-17 and IL-2. $CD4^+$ Th17 cells did not have any direct in vitro tumor cell killing activity, but still were able to stimulate $CD8^+$ CTL responses via IL-2 and pMHC-I, but not IL-17 signalling. The therapeutic effect of $CD4^+$ Th17 cells was shown to be associated with IL-17, but not IFN-γ, and was mediated by $CD4^+$ Th17-stimulated $CD8^+$ CTLs via the perforin pathway, which were recruited into B16 melanoma via $CD4^+$ Th17-stimulated CCL20 chemoattraction. These results elucidated distinct roles of $CD4^+$ Th17 and $CD4^+$ Th17-stimulated $CD8^+$ CTLs in the induction of preventive and therapeutic antitumor immunity, which may greatly impact the development of $CD4^+$ Th17-based cancer immunotherapy.

In autoimmunity, earlier studies showed that both $CD4^+$ Th17 cells and $CD8^+$ CTLs were involved in T1D and EAE. However, their relationship in pathogenesis of autoimmune diseases was still elusive. In this study, we found that $CD4^+$ Th17 cells stimulated OVA- and MOG-specific $CD8^+$ CTL responses, respectively, in mice. When $CD4^+$ Th17 cells were transferred into (i) transgenic RIP-mOVA or (ii) RIP-mOVA mice treated with anti-CD8 antibody to eliminate Th17-stimulated $CD8^+$ T cells, we found that OVA-specific $CD4^+$ Th17-stimulated $CD8^+$ CTLs, but not $CD4^+$ Th17 cells themselves, induced diabetes in RIP-mOVA. In cases of mice injected with MOG-specific $CD4^+$Th17 lymphocytes, $CD4^+$ Th17 but not $CD4^+$ Th17-activated $CD8^+$ CTL induced EAE in C57BL/6 mice. These results demonstrate the distinct roles of $CD4^+$ Th17 and $CD4^+$ Th17-stimulated $CD8^+$ CTLs in the pathogenesis of autoimmune diseases, which may have great impact on the overall understanding of $CD4^+$ Th17 cells in the pathogenesis of autoimmune diseases.

To study the functional conversion of naive $CD4^+$ T-helper cells into Th1 or Tr1 cells under Th2 differentiation culture conditions, we generated OVA-specific wild-type (WT) Th2, and Th2(IL-5 KO), or Th2(IL-5 KO), or Th2(IL-6 KO), or Th2(IL-10 KO) cells, and assessed their capacity in modulating DC_{OVA}-induced $CD8^+$ cytotoxic T lymphocyte (CTL) responses and antitumor immunity in WT C57BL/6 mice. We demonstrated that GATA-3-expressing Th2 cells enhanced DC_{OVA}-induced CTL responses via IL-6 secretion. We also showed that IL-6 and IL-10 gene deficient Th2(IL-6 KO) and Th2(IL-10 KO) cells, but not IL-4 and IL-5 gene deficient Th2(IL-4 KO) and Th2(IL-5 KO) cells, behaved like functional Tr1 and Th1 cells by inhibiting and enhancing DC_{OVA}-induced OVA-specific $CD8^+$ CTL responses and antitumor immunity, respectively. We further demonstrated that inhibition and enhancement of DC_{OVA}-induced OVA-specific CTL responses by Th2(IL-6 KO) and Th2(IL-10 KO) cells were mediated by their immune suppressive IL-10 and pro-inflammatory IL-6 secretions, respectively. Taken together, our results suggest that deletion of a single cytokine gene IL-6 and IL-10 converts $CD4^+$ Th2 cells into functional $CD4^+$ Tr1 and Th1 cells under Th2 differentiation condition. Our data thus not only provide new evidence for another type of $CD4^+$ T cell plasticity, but also have a potential to impact the development of a new direction in immunotherapy of allergic diseases.

AKNOWLEDGEMENTS

It gives me an immense pleasure to thank my supervisor Dr. Jim Xiang, who has been a back bone of my PhD programme, his unconditional and timely advice has made it possible for me to contribute this knowledge to the humankind. I thank my advisory committee members Dr. Joseph Blondeau, Dr. Qiang Liu, Dr. Qingyong Xu, Dr. Anurag Saxena, and Prof. A. Mabood Qureshi for their advice in this research. I thank Dr Rajni Chibbar for helping me with histopathology slide reading. I thank Dr. Sylvia and Marlene Snider for helping me with some experiments. I thank our technician Mark Boyd for his help in flowcytometric analysis. I thank my lab members Jenny, Shulin, John, Ash, Yufeng, Li, Chans, Lu, Roopa, Iris and Rita for their cooperation and constructive discussions. I thank members of cancer research unit, Saskatoon Cancer Centre for their kindness.

I thank University of Saskatchewan for hosting me as a graduate student and I also thank various granting agencies (Canadian Institute of Health Research, Canadian Breast Cancer Foundation, Canadian Cancer Society, Saskatchewan Cancer Agency, College of Graduate Studies, College of Medicine, VWR International Scholarship) for helping me to pursue my goal.

My acknowledgements would be incomplete if I don't thank my buddies/mentors/well-wishers/caregivers. I don't find right word/words to thank them, namely Dr. Medhamurthy, Dr. Renuka prasad, Dr. Sujatha Prasad, Dr. Janardhan, Dr. Chandru, Srikanth, Srinivas, Prabhakar, Tara, Kiran, Vijay, Nagarjun, Ragu, Naveen, Shankar, Keshav, Suneetha, Naidu, Venkat, Sudhakar, Gouda, Shashi, Dinesh, Paddi, Parmi, Nagu, Amitha, Suneetha, Suma, Lakshmi and their families.

I thank my family for being with me through all my endeavours.

DEDICATION

This work is dedicated to my beloved parents Mr. Munegowda and Mrs. Munirathnamma , whose unconditional love has sculpted me into what I am, and to my love, Suguna and my little prince, Aarush.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Ab.....Antibody

Ahr.....Aryl hydrocarbon receptor

APC.....Antigen presenting cell

BL6-10_{OVA}..... OVA-expressing B16 melanoma

CCL.....Chemokine (C-C motif) ligand

CCR.....C-C-chemokine receptor

CD.....Cluster differentiation

cDC.....Conventional dendritic cells

CFA.....Complete freund's adjuvant

CFSE..... Carboxyfluorescein succinimidyl ester

CIA..... Collagen-induced arthritis

CMA.....Concanamycin A

CTLA-4..... Cytotoxic T-lymphocyte antigen 4

CXCR.....C-x-c chemokine receptor

DC.....Dendritic cell

DC_{MOG}.....MOG specific DCs

DC_{OVA}.....OVA specific DCs

DT.....Diphtheria toxin

EAE.....Experimental autoimmune encephalitis

FCS.....Foetal calf serum

FITC..... Fluorescein isothiocyanate

ELISA.....Enzyme linked immunosorbent assay

Foxp3.....Forkhead box p3

GATA.....GATA binding protein

GITR .....Glucocorticoid-induced TNF receptor

GMCSF..... Granulocyte-macrophage colony-stimulating factor

H&E.....Haematoxylin and eosin

HLA.....Human leukocyte antigen

ICAM.....Intracellular adhesion molecule

IFN.....Interferon

Ig.....Immunoglobulin

IL.....Interleukin

IRF.....Interferon regulatory factor

iTreg.....Induced T regulatory cell

KO..... Knockout

LCMV.....Lymphocytic choriomeningitis virus

MBP..... Myelin basic protein

MOG.....Myelin oligodendrocyte glycoprotein

MS.....Multiple sclerosis

NK.....Natural killer

NOD.....Non obese diabetic

nTreg.....Natural T regulatory cell

OTI.....Transgenic mice with $CD8^+$ T cells specific to OVA

OTII.....Transgenic mice with $CD4^+$ T cells specific to OVA

OVA.....Ovalbumin

PBS.....Phosphate buffered saline

PD-1 .....Programmed death 1

pDC.....Plasmacytoid dendritic cells

PE.....R- phycoerythrin

pMHC.....Major histocompatibility complex/peptide

PPR.....Pattern recognition receptors

RAG-1..... Recombination-activating gene 1

RBC.....Red blood cell

RIP.....Rat insulin promoter

Ror.....Retinoid orphan receptor

ROS….....……Reactive oxygen species

RT-PCR.....Reverse transcription polymerase chain reaction

STAT.....Signal transducers and activators of transcription

T1D.....Type 1 diabetes

T-bet.....T box containing protein

TCR.....T cell receptor

TFh.....T follicular helper cell

TGF.....Transforming growth factor

Th.....T helper

TLR.....Toll like receptor

TNF.....Tumor-necrosis factor

Tr/Treg.....T regulatory cell

VCAM.....Vascular cell adhesion molecule

CHAPTER 1

REVIEW OF LITERATURE, INTRODUCTION AND OBJECTIVES

Review of Literature and Introduction

1.1 General overview

All the chapters of this thesis are presented as either published manuscripts or manuscript in press, so the relevant reviews of literature and introductions are provided within each chapter. The purpose of this general review of literature, introduction, and objectives is to give a brief overview of the subject matter that will be covered in the context of the thesis as whole. Mainly $CD4^+$ T helper cell subsets comprise the subject matter of this thesis; therefore, all available information on different currently identified subsets of $CD4^+$ T helper cells is discussed. We have also discussed different disease models with reference to $CD4^+$ T helper cell involvement. Dendritic cells (DCs) being central antigen presenting cells, we have compared DC_{OVA} induced immune response with Th17 induced response and evaluated Th2 cell influence on DC_{OVA} induced immune response. To understand the biology associated with DCs we have discussed briefly the DC and DC subsets.

1.2 Dendritic cells

Pathogen recognition, activation of primary and memory immune responses, and preservation of tolerance to self-antigens are central to maintaining health. These important functions are performed by DCs. DCs are central to the immune system; they are the potent professional antigen presenting cells (APCs) of the immune system. They up take antigens by sensor receptors expressed on DCs and process the antigens on to MHC complexes. Through chemokine-guided mechanisms, they migrate to the T-cell areas of lymph nodes to present processed antigen for inducing T- cell activation. There are extensive studies showing that DCs are critical APCs for priming immune responses and also crucial in the regulation of T- cell mediated immune reposes (1). In addition to their role in adaptive immune responses, they also play an important role in innate immune responses by serving as sentinels in identifying the invading pathogens through pattern recognition receptors (PPRs). When they sense dangers, they

secrete host defence peptides and proinflammatory cytokines, eliciting the host defence by linking both innate and adaptive immune responses (2).

Initially, the presence of DCs was discovered by Paul Langerhans in 1868; he discovered DCs in skin (Langerhans cells). Later, in 1973 Steinman and Cohn identified a morphologically distinct cell type with dendrites from peripheral lymphoid organs; they named these cells as DCs and showed that they are potent stimulators of primary immune responses (3). Similar kinds of leukocytes were observed both in humans and rodents in lymphoid and non-lymphoid organs (4). There was a lack of information on DCs until the last decade because of the low frequency of these cells in the body (1-2% of total leukocytes) (3), the lack of distinct markers for DCs, and deficiency in protocols to purify DCs (5). From the last decade, with the invention of various cocktails of cytokines for in vitro culture of DCs along with monoclonal antibody development, research has led to extensive study of phenotypic and functional characterization of DCs. It has been shown that DCs follow various haematopoietic pathways of differentiation and maturation into multiple heterogeneous subsets of DCs with different marker expression (1, 6). They play both stimulatory and suppressor roles on immune responses. Upon sensing the danger signals, DCs produce various proinflammatory cytokines, stimulating innate and adaptive immune responses. On the other hand, DCs also induce immunological tolerance, as in cases of clonal deletion of self-reactive T cells in thymus (central tolerance), or of clonal deletion and active suppression by inducing T regulatory cells (peripheral tolerance) (7). These diverse functions of DCs reflect the presence of various subsets of DCs.

A proper understanding of developmental lineages, precursors and inducing factors of each subset of DCs would help in generating or activating specific DC subsets in in vitro or in vivo to potentially target various disease conditions and cancers for favourable immune responses. Because of DCs' functional similarities with macrophages and also as many in vitro studies used monocytes or GM-CSF (important myeloid growth factor) for the culture of DCs, DCs were originally thought to be myeloid in lineage. However, there is a considerable evidence showing the development of DCs by lymphoid lineage (8).

1.2.1 Dendritic cell subsets

There are two categories of DCs based on their origin namely blood derived and tissue derived. Blood derived DCs are derived from bone marrow and are delivered via peripheral blood, so they are named as myeloid DCs. These blood derived DC subsets appear to develop in lymphoid organs from precursors of DCs (pre-DCs) generated in bone marrow (9). Tissue derived forms are the DCs arrive by migration via lymphatic afferent vessels, once they uptake antigens. Blood derived DCs can be divided into two sub-populations, conventional DCs (cDCs) and plasmacytoid DCs (pDCs) (9).

Various DC subsets have been defined in mouse lymphoid organs on the basis of cell surface markers expression. Mouse DCs are basically differentiated based on their expression of CD11c and MHC class II in combination with CD4, CD8α, CD11b, and CD205 (DEC 205). The T cell markers CD4 and CD8 are useful in differentiating DC subsets. CD8 on the DC is in the form of αα-homodimer rather then αβ-heterodimer which is typically seen in $CD8^+$ T cells. Other markers that are useful for segregating mouse DC subsets include myeloid cell marker CD11b and interdigitating DC marker CD205 (DEC205)(5).

The $CD4^-CD8\alpha^{high}CD205^+CD11b^-$ DCs are lymphoid in origin, and they constitute 20% of the DCs in spleens. They are present in the T cell areas of spleens and are also found at moderate levels in lymphnodes. They constitute a dominant subset amongst thymic DCs (10-12). They express equal B7-1(CD80), B7-2(CD86), and CD40 compared to $CD4^+CD8\alpha^-CD205^-CD11b^+$ DCs. They exhibit higher Toll-like receptor 3 (TLR3) expression, interleukin-12 (IL-12) production, MHC class I presentation, and cross-presentation activities than the other splenic DC subsets (13). Freshly isolated $CD4^-CD8\alpha^{high}CD205^+CD11b^-$ lymphoid DCs have a regulatory effect on T cells, in which they activate both $CD4^+$ and $CD8^+$T cells. However, they can induce apoptosis in $CD4^+$T cells and a limited $CD8^+$T cell responses as they produce reduced level of IL-2 (14). Moreover, they are responsible for maintaining peripheral tolerance under steady-state conditions through induction of cross-tolerance (15). These DCs may function to maintain T-cell tolerance in lymphoid organs in the absence of infection. In presence of danger signals, $CD4^-CD8\alpha^{high}CD205^+CD11b^-$ lymphoid DCs can not only activate $CD8^+$T cells, but also cross present for the stimulation of cytotoxic T cells (16). $CD4^-CD8\alpha^{high}CD205^+CD11b^-$ lymphoid DCs can trigger the development of Th1 cells in vivo. Th1 response is shown to be dependent on interleukin-12 production by these DCs (17), and they

induced IgG2a antibody responses (18). Some studies have shown that disruption of CD40-CD154 interactions inhibited the induction of the Th1 response by $CD8^+$ DCs in vivo. CD40-CD154 interactions were not required for the proliferation of antigen specific naive T-helper cells stimulated by either DC subset, but were indispensable in the production of IL-12 from $CD8^+$ DCs and their induction of Th1 cells in vitro (18)

The $CD4^+CD8\alpha^-CD205^-CD11b^+$ DCs are myeloid in origin, and they constitute 40% of the DCs in spleen. $CD4^+CD8\alpha^-CD205^-CD11b^+$ DCs express B7-1(CD80), B7-2(CD86), and CD40 (10). They show stronger MHC II presentation activity compared to that of other DC subsets (13). These $CD4^+CD8\alpha^-CD205^-CD11b^+$DCs stimulate both $CD4^+$ and $CD8^+$ T cells in in vitro conditions, but in in vivo they are known to stimulate Th2 responses and IgE antibody responses (18). Inhibition of CD40-CD40L interactions will not affect the T cell response stimulated by this DC subset (18).

In addition, $CD4^-CD8\alpha^-CD205^-CD11b^+$ DCs are double negative (CD4 and CD8) DCs and are myeloid in origin; they constitute 15% of total DCs in the spleen. Similar to $CD4^+$ DCs, these DCs also express B7-1(CD80), B7-2(CD86), CD40. These DCs are located in marginal zone in between white and red pulp; upon stimulation, they will move to a T-cell area to activate T cells (10). In in vitro condition they are efficient stimulators of both $CD4^+$ and $CD8^+$ T cells. In in vivo conditions they are shown to stimulate Th2 responses inducing IgE antibody responses (18) or immune tolerance responses (19).

$CD4^-CD8\alpha^-CD205^+CD11b^+$ and $CD4^-CD8\alpha^{low}CD205^+CD11b^-$ DCs both are myeloid in origin and normally found in lymph nodes and not normally found in spleens. They are also named as interstitial DCs. They both showed a relatively low expression of CD8, but a moderate or high expression of DEC-205. Both appeared among the DCs migrating out of skin into lymphnode, but $CD4^-CD8\alpha^{low}CD205^+CD11b^-$ DC was restricted to skin draining lymphnodes and was identified as the mature form of epidermal Langerhans cells. $CD4^-CD8\alpha^-CD205^+CD11b^+$ DCs may induce a Th1 or a Th2 kind of response. They are large in size and have high levels of MHC class II, DEC-205, CD40 and express many myeloid surface markers (20). Langerhans cell migration experiments indicate that Langerhans cells upregulated CD8 and LFA-1 upon stimulation and migration to the lymphnodes (11, 21). They express chemokine receptors CCR6 and CCR7, facilitating their recruitment in the periphery and their

migration to the T-cell zones of secondary lymphoid organs. Some studies have also shown that Langerhans cells in lymphnodes produce MDC and TARC chemokines involved in T-cell attraction. The expression of CCR7 and T cell attracting chemokines by Langerhans cells may explain their exclusive localization in T cell areas of lymphnodes. Langerhans cells may play a fundamental role in the induction of immunity by priming Th1 responses (21). When there is migration of DCs from peripheral tissues in absence of any danger signals, which occurs in steady state conditions, they will help in maintaining peripheral tolerance by inducing T cell anergy (22).

Plasmacytoid DCs are a cell type, displaying a unique set of surface antigens. Having been identified from their expression of lymphoid or myeloid related antigens these obscure cells were named plasmacytoid T cells or plasmacytoid monocytes (23). In 1997, Grouard et al. (24) and Olweus et al. (25) reported finding plasmacytoid T cells/monocytes with characteristics of precursor DCs; later, they were therefore renamed plasmacytoid DCs. They contribute 25% of total spleen DCs. Plasmacytoid DCs characteristics were different from other conventional DCs. Plasmacytoid DCs enter lymphnodes from blood through high endothelial venules with CD62L, whereas conventional DCs gain access from peripheral tissues (26). Plasmacytoid DCs reside in bone marrow, blood, thymus, and T cell rich areas of lymphoid organs in a steady-state condition and can localize to skin and other tissue areas in inflammation and autoimmunity. Freshly isolated plasmacytoid DCs display typical morphology of large round cells with diffused nucleus, few dendrites; they express Gr-1, B220, CD8, CD11c, CD205 and MHC class I, and lack co-stimulatory molecules (5). Some recent evidence has shown that they express some plasmacytoid DC markers, such as mPDCA-1, 120G8, 440c. Plasmacytoid DCs are specifically responsible for IFN-α production, which is dependent on toll like receptors TLR7 and TLR9 (27, 28). TLR ligation in plasmacytoid DCs activates IkB kinase-α leading to IFN-α production (29). Because of their specific role in IFN-α production they may play important role in viral infections. Plasmacytoid DCs play a regulatory role in peripheral tolerance in steady state condition and they are poor stimulators of T cells (30). In vitro activation of plasmacytoid DCs moderately up regulates their expression of CD8, MHC class II and co-stimulatory molecules. Plasmacytoid DC derived IFN-α induces IFN-γ production by NK cells and improves their cytotoxicity. In co-operation with IL-12, plasmacytoid DC derived IFN-α also induces Th1 differentiation and cytotoxic T cell production (31).

For efficient stimulation of naive T cells, they should interact with mature DCs. DCs must mature by the time they arrive at lymphnodes after seeing the antigen. It is well known that DC maturation is mediated by pro-inflammatory cytokines such as TNF-α, TLR stimulation, and CD40L co-stimulation signals (32). Mature DCs are DCs which should efficiently activate T cells through signals 1, 2, and 3. Signal 1 is delivered through the T-cell receptor (TCR) engagement with the MHC antigenic peptide complex (pMHC). Signal 2 is referred to as co-stimulation through CD80, CD86, and CD40L. Signal 3 is conducted through various cytokines produced by DCs, which will dictate the fate of T cell differentiation (7). For example, IL-12 is one of the signal 3 mediators. IL-12 in cooperation with IFN-α, induces naive T-cell differentiation into Th1 cells and induces cytotoxic activity and IFN-γ production by NK cells and cytotoxic T lymphocytes (33). Another example is the expression of notch family member Delta-1 on DCs, which induces differentiation of Th1 (34). Expression of jagged-2 initiates Th2 differentiation (34). Molecules like T-bet and semaphorin 4A are also involved in Th1 differentiation (35). Some studies have shown that regulatory T cells (Treg) are essential for the maintenance of peripheral immune tolerance. DC-derived cytokines TGF-β and IL-2 induce Treg differentiation, and Treg differentiation is suppressed in the presence of IL-6 (36).

1.3 $CD4^+$ T-helper cells

Immune response is the body's ability to defend against invading pathogens or pathogenic self antigens; it differs with different invaders or antigens with its specificity, memory, versatility and tolerance. Basically immune response is divided into two types, namely cellular immune response and humoral immune response. There are various cell types involved in maintaining the immune system's complexity in protecting specificity, versatility, memory, and tolerance to antigens. In T cells grossly, there are two types of cells classified according to their surface markers, CD4 and CD8 expression. $CD4^+$ T-cells are also known as T-helper cells as they have their function in regulation of both cellular and humoral immune responses. T-helper cells are classified into different subtypes based on various cytokines and signalling pathways controlling their differentiation and development. Mosmann and Coffman had proposed a model with two types of T-helper cells; of late there are number of other cell types added to the list of T-helper cells (37).

T-helper cells, as named, are shown to help in primary and secondary cellular, and humoral immune responses. The Mosmann and Coffman model had two types of T-helper cells, there are other cell types added to the list of T-helper cells (38). Naive $CD4^+$ T cells are maintained in pluripotent state and are quiescent in their effector function. They continuously circulate through blood and lymphoid organs surveying for specific MHC-peptide complexes on antigen presenting cells. Through activation by antigen presenting cells, in the presence of different cytokine milieu, they differentiate into different effector $CD4^+$ T cell subsets. To confer immunity to various kinds of antigens, the immune system has evolved different cell types. There are different terminally differentiated helper cells such as Th1, Th2, TFh, Th17, Treg, Th9 and Th22 cells tailored to combat different pathogens (Figure 1.1). Production of any subtype of cells depends on the type of antigen, dose of antigen, mode of entry and cytokine milieu at the microenvironment. They tailor their response to the character of the threat encountered, providing B cells and cytotoxic T cell stimulation, and activating immune cells of innate immune system. Different subsets of $CD4^+$ T cells are characterized by different sets of cytokines. These specific cytokine secretions are co-related with epigenetic changes in the cytokine genes, reflecting their competent or silent state (39). These $CD4^+$ T cells' importance is very much evident with the range of infections in HIV infected persons due to loss of $CD4^+$ T cells (40). The production of different subtypes will lead to antigen specific solid immunity (in various infections and cancer) or might lead to immunopathology (viz; autoimmunity, asthma, allergy) (40). Involvement of $CD4^+$ T cells in cancer is considered in chapter 2 and their involvement in autoimmunity in chapter 3.

1.3.1 $CD4^+$ T-helper-1 (Th1) cells

The evidence shows that Th1 cells participate in cell mediated immunity. They are essential for controlling intracellular pathogens such as viruses, certain bacteria, (e.g., Listeria and Mycobacterium tuberculosis), and anti-cancer immunity. They provide cytokine mediated help to cytotoxic T lymphocytes (CTL). Th1 cells are characterized by cell specific cytokine IFN-γ and transcription factor T-bet. Th1 cells are differentiated from naive $CD4^+$ T cells in presence of cytokines IL-2, IL-12 and anti-IL-4 antibody. They secrete high levels of IFN-γ (Figure 1.1). Th1 cells induce the production of IgG2a antibodies in mice and IgM, IgA, IgG1,

IgG2 and IgG3 antibodies in humans. IL-12 is a key cytokine for Th1 development. IL-12 and IFN-α produced by dendritic cells, stimulate Th1 development. Upon a danger signal received by dendritic cells, they produce IL-12, which activates STAT 1 in naive $CD4^+$ T cells. Activated STAT1 up regulates T-bet, which in turn induces IFN-γ production and IL-12Rβ2 expression. Those IL-12Rβ2 expressing T cells can directly respond to IL-12 in inducing IFN-γ production through STAT4 activation (41). It was also evident as knockdown of STAT4 expression resulted in reduced IL-12Rβ2 expression, leading to reduced Th1 development. T bet is the key regulator of Th1 development as T-bet deficient mice have shown impaired production of IFN-γ producing Th1 cells (42). T-bet appears to directly activate IFN-γ gene by binding to several of its regulatory elements, by inducing chromatin remodelling of the IFN-γ locus, and the expression of Hlx, a downstream target of T-bet required for stabilization of the Th1 phenotype (43). Upon IL-12 and STAT4 stimulation, IL-18Rα expression increases, leading to a further increase in IFN-γ production (44). Th1 cells preferentially express chemokine receptors CXCR3, CCR5, and CCR7 (45). Th1 cells play major roles in cell-mediated immunity, inducing immune-responses in intra-cytoplasmic parasitic infestations, viral infections and tumor immunity. Historically, Th1-associated autoimmune diseases, experimental autoimmune encephalitis (EAE) and collagen induced arthritis (CIA) were ablated with neutralizing antibodies to IL-12p40 subunit, which is also shared with newly discovered cytokine IL-23 (46, 47). Later, it was shown that IL-23 deficient mice ablated autoimmune diseases, but not the IL-12 deficient, showing that Th17 is main factor responsible for autoimmunity (48, 49). Th1 cells are shown to play a major pathogenic role in type-1 autoimmune diabetes (50, 51). In support of the very new novel concept of T-APC, Dr. Jim Xiang's lab has demonstrated that Th1 cells acquire pMHC I complexes and co-stimulatory molecules from DC_{OVA} upon DC_{OVA} activation, and become capable of stimulating OVA-specific $CD8^+$ CTL responses via IL-2 and pMHC I signaling and induce efficient antitumor immunity (52, 53)

1.3.2 $CD4^+$ T-helper-2 (Th2) cells

Th2 cells are characterized with production of cytokines IL-4, IL-5, IL-13 and IL-10 and transcription factor GATA binding protein-3 (GATA-3) (Figure 1.1). Th2 cells lead to tissue damage and fibrosis in parasitic infections like helmenths (54), and the persistence of Th2 cells

leads to allergic disorders (55, 56). Th2 cells play a major role in humoral immunity in modulating antibody responses, Th2 cytokines IL-4 and IL-13 induce IgE class switching in B cells (57). IL-5 influence the eosinophil function with increased production and release of eosinophils (58). Th2 cells induce IgG1 and IgE antibodies in mice whereas IgM, IgG4 and IgE are induced in humans. IL-4 (endogenous or exogenous) interacts with its receptor and induces the activation of STAT6, which in turn up-regulates the expression of GATA-3 and c-maf (59). GATA-3 is known to auto-stimulate itself, but GATA-3 alone is not sufficient to induce IL-4. IL-2 mediated stimulation of STAT5 is required for IL-4 production and maintenance (60). Th2 cells preferentially express chemokine factors CCR3, CCR4, and CCR8 (45). $CD8^-$ DCs were shown to induce Th2 response, leading to IgE antibody production in mice (61). IL-25 cytokine, which is structurally related to IL-17 is shown to induce Th2 cells (62). IL-25 is also shown to enhance allergic airway inflammation by inducing a Th2 cell dependent pathway (63) but absence of IL-25 has not impaired the ability of T cells to differentiate into Th1 and Th2 cells. IL-1α and IL-1β are shown to regulate the Th2 response in nematode infections (64). Th1 and Th2 cells mutually regulate one another through their cytokines and transcription factors.

Moreover, Th2 cells play major roles in allergic diseases. (65). In the sensitization phase of allergic diseases there will be differentiation and clonal expansion of allergen specific Th2 cells. Th2 produced IL-4 and IL-13 leads to the induction of B cell class switch to the ε-immunoglobulin heavy chain and to the production of allergen-specific IgE Ab. Allergen specific IgE binds to the high affinity FcεRI on the surface of mast cells and basophils, thus leading to the patient's sensitization to an allergen. During this phase, a pool of memory allergen specific T and B cells will be generated. In the effector phase, when there is a new encounter with the allergen, it will lead to cross linking of the IgE-FcRI complexes on sensitized basophils and mast cells, thus triggering their activation and subsequent release of anaphylactogenic mediators responsible for the classical symptoms of the immediate phase (type 1 hypersensitivity). In the later phase, when there is continuous presence of an allergen, it leads to T cell activation. Activated allergen-specific Th2 cells produce IL-4, IL-5, IL-10 and IL-13, which play a key role in the maintenance of allergen-specific IgE levels, eosinophilia, recruitment of inflammatory cells to inflamed tissues, production of mucus, and decreased threshold of contraction of smooth muscles (66). As a consequence of these events, the more severe clinical manifestations of allergy, such as chronic persistent asthma, allergic rhinitis,

atopic dermatitis, and in extreme cases, systemic anaphylactic reactions appear. To alleviate these pathogenic effects of Th2 cells, it is important to understand the biology of Th2 cells (chapter 4).

There are various studies showing the counter balance of Th1 and Th2 cells through their cytokines (67-71). An increase in activation induced cell death of Th1 cells in atopic diseases contributes to the predominant Th2 cells (72). In shistasomiasis Th2 cells and Tregs inhibit Th1 response through inhibition of IL-12 produced by dendritic cells. This inhibitory effect is mediated through IL-10 produced by them (73). When T-cells were co-stimulated by CD3/CD28 under Th1 and Th2 conditions they show high polarized secretion of cytokines. Th2-cells co-stimulation induces Th2 cytokines leading to abrogation of Th1 mediated bone marrow transplant rejection (74). Dendritic cells secreting Th1 cytokines like IL-12, IFN-γ along with expression of peptide specific MHC and intracellular adhesion molecule-1 (ICAM-1), converts established Th2 polarized $CD4^+$ T cells to Th1 in recall response (75). $CD8^-$ DCs were shown to induce Th2 response leading to IgE antibody production in vivo even in the presence of a pre-existing antigen specific Th1 environment (61) Th2 clones specific to alloantigen regulate alloimmune response are shown to promote allograft survival (38), but the mechanism of this response is not shown. On the contrary, some studies also show that antitumor CTL activity is not affected by the presence of Th2 cells (76, 77). Antigen-specific Th2 cells eradicated an established visceral and lung metastasis of a CTL-resistant melanoma (78). Similarly, injection of OVA-specific $CD4^+$ T cells expressing either Th1 or Th2 phenotype cleared an established A20-OVA B-cell lymphoma (79). IL-4 is shown to induce infiltration of macrophages, eosinophils and in some cases neutrophils and lymphocytes to a tumor site, leading to tumor clearance (80-82). Similar to IL-4, IL-13 over-expression also showed antitumor immune response through recruitment of neutrophils and macrophages (83, 84). Treg cells are shown to directly inhibit the activation of allergen specific Th2 cells, thus minimizing the production of IL-4, IL-5, IL-13 and IL-10, which are essential cytokines during the effector phase of allergic reactions (85).

1.3.3 $CD4^+$ T-Follecular helper (TFh) cell

T follicular helper (TFh) cells were first identified in humans, possessing a cytokine profile different from Th1 and Th2 cells. TFh cells have emerged as a subset of T-helper cells with unique transcriptional and cytokine production properties, providing help to B cells in maintaining a long-lived antibody response. They provide help to B cells in eliciting a long-lived antibody response in secondary lymphoid organs. Antigen-specific T cells interact with B cells at the border of the T-B cell area in the secondary lymphoid organs. At that stage, isotype switching will be initiated, and B cells migrate to the germinal centre. In the germinal centre, rare TFh cells help mutated B cells to differentiate into high affinity plasmacytes or memory B cells, which produce long lasting high affinity antibodies (86). Defining the TFh cell has become difficult because of the heterogeneity of T cells present in B cell follicles and the heterogeneity of various known markers for identifying these cells (87). TFh cells are characterized by transcription factor Bcl-6 and chemokine receptor CXCR5. CXCR5 is required for TFh migration into lymphoid follicles, which are attracted by chemokine receptor ligand CXCL13 expressed at B cell follicles (88). $CXCR5^{+}CCR7^{low}$ cells migrate into the germinal centres after exposure to antigen (89). CXCR5 is most widely used as a marker for TFh cells. TFh cells also express CXCR4, PD-1, and ICOS. There are studies showing the importance of Bcl6 in inducing CXCR5 expression (90, 91). Over expression of Bcl6 up-regulates the expression of CXCR5, CXCR4, PD-1, ICOS, IL-21R, and IL-6R, and down-regulates CCR7 both in vitro and in vivo (90-92). Over expression of Blimp-1, transcriptional repressor of Bcl6 will down-regulate these molecules (92). TFh cells produce high levels of IL-21 and low moderate levels of IL-4 and low levels of IFN-γ and IL-17 (93, 94). IL-21 has an autocrine effect on FTh cells and acts as a germinal centre B cell survival and differentiation factor (93).

1.3.4 $CD4^{+}$ T-helper-17 (Th17) cell

Th17 cells are the new subset of T-helper cells added in 2005, through the cell-specific cytokine IL-17, which was identified a decade back. Th17 cells produce IL-17 and express transcription factor RORγt through activation of STAT3 by IL-6 and IL-23 in mice (Figure 1.1) (95). STAT3 regulates IL-6 induced expression of RORγt and IL-17 production (96). IL-6

activates both STAT3 and STAT1. STAT1 being inhibitory on Th17 cells, SATA1 will be inhibited by Th17 cells whereas STAT3 expression is maintained (97). IL-27 and IFN-γ inhibits the development of Th17 cells through a STAT1 dependent mechanism (98). There is a small difference between mouse and human Th17 cells with regard to their origin. Human Th17 cells require cytokines IL-1β and IL-23 for their differentiation. Meanwhile the involvement of TGF-β in human Th17 cell differentiation is controversial (99). It has been recently shown that TGF-β is not essential for Th17 development in mice and humans and it has been demonstrated that TGF-β plays an indirect role in inhibiting Th1 and Th2 development (100, 101). Prostaglandin E2 is shown to play an important role in the development of human Th17 cells. Though Th17 cells secrete small amount of IL-2, cytokine IL-2 has been shown to constrain Th17 generation from $CD4^+$ T cell precursors via STAT5 (102, 103). Transcription factors interferon regulatory factor 4 (IRF-4) and T-bet are shown to inhibit Th17 cell differentiation (104, 105). Aryl hydrocarbon receptor (Ahr) promotes Th17 development through STAT1 inhibition and STAT5 activation (106).

Additional research demonstrates that Th17 cytokines are strongly pro-inflammatory, and Th17 cells have been shown to play critical role in immune-mediated inflammation (107). Historically, Th1 cells were associated with autoimmune diseases, experimental autoimmune encephalitis (EAE) and collagen induced arthritis (CIA), and they were ablated with neutralizing antibodies to the IL12p40 subunit, which is also shared with newly discovered cytokine IL-23 (46, 47). Later it was shown that IL-23 deficient mice ablated autoimmune diseases but not the IL-12 deficient showing that Th17 is a main pathogenic cell responsible for autoimmunity (48, 49). There is not much difference between Th17 and Th1 cells compared to Th17 and Th2 cells (108). Th1 cell cytokines IL-12 and IFN-γ are shown to inhibit Th17 cell differentiation (108). Th17 cells resemble Th1 cells in most of the surface marker expressions compared to Th2 cells, but Th17 cells are differentiated with Th1 cells by their expression of IL-17, TNF, and absence of transcription factor T-bet expression (108). With the invention of Th17 cells as a new subset of $CD4^+$ T helper cells, Th17 cell has replaced Th1 cell in autoimmune and allergic disorders as a pathogenic subset of T-helper cells. Th1 cytokine IFN-γ knockout is shown to have an increased susceptibility to EAE (109), and EAE is suppressed in mice deficient in IL-23, a Th17 expansion cytokine (48), suggesting that the Th1 cell type is replaced with the Th17 cell as a pathogenic cell type in EAE. This was also proved by adoptive transfer of Th17 cells, showing a

more efficient induction of EAE than Th1 cells (110). Absence of P19 receptor subunit which is specific to IL-23 makes mice resistant to EAE with a defect in Th17 production (48, 110). Recently, both Th17 and $CD8^+$ T cells have been identified in active lesions in brains of multiple sclerosis (MS) patients (111).

With Th-17 cytokines being strongly pro-inflammatory, Th17 cells have been shown to play a critical role in immune-mediated inflammation (95, 107, 112). Involvement of Th17 cells in antitumor immunity has recently been reported. Th17 cells and IL-17 expression have been found in various human tumors (113, 114). Transgenic IL-17 expression either induced tumor regression through enhanced antitumor immunity in immune competent mice (115, 116) or promoted tumor progression through an increase in inflammatory angiogenesis in immune deficient mice (117, 118). It has been demonstrated that Th17 cells secreting both IL-17 and IL-21 were indirectly linked to antitumor immunity(119). In addition, autoimmunity-inducing Th17 cells were found to eradicate established prostate tumors (120). More recently, tumor growth and lung metastasis were enhanced in IL-17 deficient mice associated with decreased IFN-γ^+ NK and T cells (121), and tumor-specific Th17-polarized cells were found to inhibit growth of well-established melanoma via INF-γ production (53). Th17 cell cytokines recruit various inflammatory cells. Pathogen specific cells produced during mycobacterial infection induced expression of chemokine ligands CXCL9, CXCL10 and CXCL11, which attract Th17 cells to the lung, helping in the control of infection (122).

1.3.5 $CD4^+$ T-regulatory (Treg) cell

In maintaining the immune homeostasis, immune tolerance is very important to keep hyperimmune-mediated damage at bay. Treg cells play a major role in maintaining tolerance, preventing autoimmune diseases and limiting chronic inflammatory diseases. Apart from that, Tregs are shown to have an immune suppressor mechanism with a suppressor effect on T cell responses in tumors, helping tumors evade the antitumor immune responses (123, 124). Some experiments have shown that depletion of Tregs has led to effective antitumor immune responses with reduced tumor growth in mice (125, 126). Moreover, a number of studies demonstrate that Tregs control self-reactive Th1, Th2, and Th17 cells (127, 128). Tregs are $CD4^+$ T cells with

high expression of CD25 and transcription factor Foxp3 (forkhead/winged helix transcription factor) (129). There are two categories of Treg cells. One category naturally occurring in the thymus is identified as (natural Treg) nTreg and the other, which differentiates in the periphery, is inducible by some cytokines and identified as iTreg. Tregs are characterized by the expression of forkhead transcription factor Foxp3 and cytokines TGF-β, IL-10, and IL-35 secretion (Figure 1.1) (130). The nTregs are generated during the early stages of foetal and neonatal T cell development (131). These cells are generated in the thymus and then they are exported to peripheral tissues, where they normally function. The thymus-induced regulatory T cells (nTregs) are $CD4^+$, and they typically express high levels of CD25 as well as the co-stimulatory molecule cytotoxic T-lymphocyte antigen 4 (CTLA-4), the tumor-necrosis factor (TNF) superfamily member GITR (glucocorticoid-induced TNF receptor family related protein, TNFRSF18), and the Foxp3 (129, 132). Foxp3 has been demonstrated to be an essential factor for the suppressive phenotype of nTregs, as mutations in the Foxp3 gene led to autoimmune disease (133). In addition, iTregs are generated from naive $CD4^+$ T cell populations under certain conditions of antigenic stimulation and they can be induced ex vivo by culturing naive $CD4^+$ T cells with an antigen or polyclonal activators in the presence of immunosuppressive cytokines (Figure 1.1) (134) Tr1 cells are part of iTregs producing IL-10, differentiated in the presence of TGF-β and IL-10 (135) (chapter 4). Studies from Dr. Jim Xiang`s laboratory had shown that double negative $CD4^-8^-$ DC subset induce IL-10 secreting Tr1 cells (19). Studies in mice and humans have shown the developmental link between Treg and Th17 cells. TGF-β is shown to be important for the production of Th17 cells, as TGF-β is essential for inducing Rorγt, a Th17 cell specific transcription factor (136, 137). Interestingly TGF- β is also shown to induce the Treg-specific transcription factor Foxp3 (138).

1.3.6 $CD4^+$ T-helper-9 (Th9) cells

Th9 is a very recently added new $CD4^+$ effector T cell subset which is characterized by IL-9 production (139). Earlier, IL-9 was known to be a Th2-derived cytokine (140). IL-9 was found to increase in an allergen challenge and was important in inducing the mucus hypersecretion in asthmatic subjects (141, 142). IL-9 also contributes to the development of

tuberculosis by reducing IFN-γ production in peripheral blood mononuclear cells stimulated with M. tuberculosis antigens (143). Murine Th2 cells cultured in the presence of IL-4 and TGF-β lost the capacity to produce IL-4, IL-5 and IL-13, but they maintained the ability to produce IL-9 in addition to IL-10 (144). Th9 does not express the transcription factors T-bet, GATA-3, RORγt, and FoxP3 which are specific to Th1, Th2, Th17, and Tregs, respectively, but they express the transcription factor PU.1 in both human and mouse T cells (139, 144, 145). The IL-9^{+}IL-10^{+} T cells demonstrated no immune regulatory properties despite producing abundant IL-10. On the other hand, their adoptive transfer into recombination-activating gene 1-deficient mice (RAG-1) induced colitis and peripheral neuritis. This novel Th subset therefore lacks the suppressive function and constitutes a distinct population of effector T cells that promote tissue inflammation (139). More recently, it was found that IL-9 is produced in high amounts by Th17 apart from Th2 and Th9 cells (146). IL-9 synergized with TGF-β to differentiate naïve $CD4^{+}$ T cells into Th17 cells, while IL-9 secretion by Th17 cells was regulated by IL-23. IL-9 enhanced the suppressive function of Tregs in vitro, and the absence of IL-9 signalling weakened the suppressive activity of Tregs in vivo, leading to an increase in effector cells and a worsening of EAE. These findings suggest a novel role of IL-9 as a regulator of pathogenic and protective mechanisms of immune responses (147).

1.3.7 $CD4^{+}$T-helper-22 (Th22) cells

IL-22 was originally described in mice and humans as a cytokine produced by mature Th17 cells (148). IL-22 belongs to IL-10 cytokine family with an overall sequence identity of 22% in mice and 25% in humans with IL-10 gene (149). Very recently, distinct subsets of human skin homing memory T cells have been shown to produce IL-22, IL-26 and IL-13, but they do not secrete IL-17, and/or IFN-γ (150, 151). IL-22 is also produced by Th1, Th17, NK and NKT cells, where in IL-22 is shown to play a protective role in infections or pathogenic role in chronic inflammatory conditions (152). Th22 cells are characterized with the expression of chemokine receptors CCR6, CCR4, CCR10 and the transcription factor aryl hydrocarbon receptor (Ahr). Cytokines IL-23 and IL-6 can directly induce the production of IL-22 from naive T cells both in mice and humans. Ahr agonists also induce the production of IL-22 both in mice and humans (153, 154). Differentiation of Th22 could be promoted by stimulation of naive T cells in the

presence of IL-6 and TNF-α or by the presence of plasmacytoid dendritic cells; it appears to be independent of Rorc but dependent upon the Ahr (151). Dermal DCs and Langerhans cells were shown to be very efficient in inducing the generation of Th22 cells. On the other hand, monocyte-derived DCs were shown to induce the development of Th17 cells that produced both IL-17 and IL-22 together (155). The human Th22 cell population co-expresses the chemokine receptor CCR6 and the skin-homing receptors CCR4 and CCR10, which led to hypotheses that these cells may be important in skin homeostasis and pathology (150, 151). Th22, along with Th17 and Th1 cells are increased in peripheral blood of psoriatic patients (156). Levels of IL-22 co-related with the disease severity in psoriatic patients (157). In rheumatoid arthritis, some studies have shown the possibility of Th22 playing the pathogenic role. IL-22 is shown to increase the proliferation of synovial fibroblasts and CCL-2 production by them (158). There was an increase in IL-22 and collagen-specific antibodies in the serum of collagen induced arthritis mice (159).

1.4 Plasticity of $CD4^+$ T cells

For decades, researchers have been looking for different regulators of immune responses in order to understand the immune system. In 1986, Robert Coffman and Timothy Mossman showed that the immune system is regulated by different kinds of T-helper cells. Later, there were various effector T-helper cells added to the list, based on their cytokine secretion. The cytokine milieu is very important in deciding the fate of an undifferentiated T-helper cell. Historically, T helper cells are deemed terminally differentiated cell lineages committed to their path. With the discovery of various new T-helper cells, there is also evidence that some T-helper cells are plastic in nature; a mature effector cell will be converted into a different effector T-helper cell in certain microenvironments. Most in vitro differentiation models suggest that such commitment of T-helper cells provide simplified experimental models that allow us to understand how they are regulated. Recent emerging evidence suggests that under certain conditions, seemingly committed T cells possess plasticity and may convert into other types of effector cells. However, how $CD4^+$ T-helper cells achieve such plasticity is not fully understood, but it is very well understood that under different microenvironments, even well-differentiated T-helper cells are ready to re-differentiate into different T helper subsets. However, the biological

significance of this plasticity remains unclear. It would be fruitful to harvest this phenomenon to treat immune-mediated disease conditions by designing effective immune-balancing strategies.

Various models of the plasticity of T cell subsets have been described. Long-lived Th1 effector/memory cells are able to turn off IFN-γ expression in vivo, appearing to be ready to re-differentiate (160). Th1 cell promoting lymphocytic choriomeningitis virus (LCMV) converted stably committed Th2 cells into Th2/Th1 phenotypes with GATA3 and T-bet expression through concerted action of TCR, interferon I and II, interleukin-12, and T-bet (161). However, the plasticity of Th1/Th2 cells seems to depend on their differentiation state (162). In addition, it is difficult to redirect Th1 or Th2 cells to become either Th17 or Tr cells, consistent with suppressive genomic modification at Rorc and Foxp3 loci in Th1 and Th2 cells (163). It is shown that in vitro generated Th17 cells are not stable in maintaining their cytokine expression capacities in vivo and can be converted into Th1 cells in lymphopenic environments (164, 165). However, they maintained their cytokine expression in normal mice and tumor-bearing mice (166, 167). The research shows that Treg cells known for their suppressor function are shown to become immune-stimulatory T-helper cells (168). Reduced expression of Foxp3 in Tr cells by genetic means resulted in the acquisition of Th2 phenotype (169). Tr cells can also be self-induced to become IL-17-producing cells in the absence of TGF-β when IL-6 is present (170), and the fully differentiated Tr cells can reverse into Th17 cells (171). Transferring Tr cells into lymphopenic mice also resulted in down-regulation and up-regulation of Foxp3 and IL-17, respectively (172). In addition, the existence of Th17 cells producing Foxp3 and IL-17/IFN-γ $CD4^+$ T cells has been shown (173, 174). Th17 cells have also been shown to convert into Th1 cells in the absence of TGF-β (50, 164, 165).

There are various mechanisms underlying this plasticity associated with $CD4^+$ T cells. These mechanisms could be divided into two categories. 1) Microenvironment: including cytokines and co-stimulation, with these two being the primary factors in differentiation, they also play roles in the plasticity associated with T-helper cells. Different antigen-presenting cells are shown to induce different kinds of T-helper subsets. For example, neutrophils are shown to promote Th17 cell response (175). NK cells are shown to promote Th1 response through IFN-γ secretion (176). As explained earlier, even well differentiated T-helper cells re-differentiate in different microenvironments. 2) Transcription factors: interaction between different cell specific

transcription factors is important in driving different T-helper subsets. For example mutual transcriptional repression of Th1 and Th2 cell specific transcription factors T-bet and GATA-3 plays a major role in deciding about the lineage (177). Treg specific transcription factor Foxp3 can inhibit the transcriptional activity of Th17 cell specific transcription factor Rorγt (178). These studies indicate that understanding the regulation of T-helper subsets by transcription factors has to be looked upon as a gradient of their expression rather than just as absence or presence, as they are shown to change during the course of infection.

1.5 Trogocytosis

In maintaining immune homeostasis and eliciting effective immune responses against any foreign antigens there should be a concerted activation of the immune system, where cellular communications mediated by either soluble or cell surface molecules amongst immune cells is certainly essential. The advent of the latest analytical and imaging tools has allowed researchers to enhance their understanding of the cellular communication through the intercellular exchanges of molecules. To explain transfer of membrane patches in intercellular communication the term trogocytosis was coined by Hudrisier in 2003; the term is derived from the ancient Greek word *trogo*, meaning gnaw or nibble. Trogocytosis is a phenomenon characterized by a transfer of membrane molecules from cell to cell (179). Trogocytosis is a common phenomenon affecting different stages of immune responses involving different immune cells (180). T cells have been shown to acquire MHC class I and class II proteins (102, 181), co-stimulatory molecules (182) from APCs and other proteins from endothelial cells (183). Dr. Jim Xiang's lab has shown bidirectional membrane molecule transfer between dendritic and T cells in murine system (184). NK cells are shown to capture the target cell-MHC class I protein both in vitro and in vivo (185) as well as the virus receptor (CD155) (186) and the membrane fragments (187). B cells, which are part of humoral immunity, are also shown to capture membrane-associated antigens from target cells, and the amount of antigen captured correlates with the affinity of the B-cell receptor for the antigen (188). In Burkitt's lymphoma, γδ T cells have been shown to capture the membrane fragments from the tumor cells (189).

Proteins are tagged to the cell surface by hydrophobic interactions, and the disruption of this hydrophobic bond is necessary to initiate the intercellular transfer of proteins (180). Trogocytosis of various immune-stimulatory or suppressor molecules will happen through various mechanisms, viz., internalization and recycling of membrane molecules, Dissociation-associated, Exosome uptake pathways or membrane nanotube formation (190).

Internalization and recycling: effective T cell responses are elicited by TCR recognition of peptide-MHC (pMHC) on APCs along with co-stimulatory and cytokine signalling (191). When there is a specific interaction of T cells with APCs, within minutes TCR and MHC molecules are assembled at the centre to form supramolecular activation clusters at the site of T cell contact or synapse (192, 193). Subsequent to synapse formation, TCR down regulation occurs and T cell-APC interactions cause transfer of APC derived surface molecules to the surface of T cells (194). These transferred clusters are internalized through TCR-mediated endocytosis and localized in endosomes and lysosomes, followed by recycling and expression of these molecules on T cell surfaces within 30 minutes (181). There are various studies endorsing this phenomenon wherein T cells acquire both MHC class I and class II proteins from APCs (195, 196).

Dissociation associated pathway: This phenomenon of torgocytosis was first demonstrated by Wetzel et al by using live cell imaging technology (197). They observed that when T cells come off from immune synapse from APCs they directly capture peptide-MHC complexes. Another study in macrophages showed repeated association and dissociation of $CD4^+$ T cells with macrophages (198). By using in vitro imaging, dendritic cells were shown to possess dissociation-association with $CD4^+$ T cells in three-dimensional collagen matrix (199).

Exosome uptake pathway: Exsosomes are membrane bodies or vesicles measuring approximately 50-90 nm, released by variety of cells. This phenomenon of vesicle secretion acts to lose potentially harmful components, as shown in case of the recovery of human neutrophils from complement attack by shedding membrane attack complex (200). Exosome-mediated intercellular membrane transfer is mediated by the secretion and uptake of exosome by a different cell. Various studies have shown that APCs shed MHC class II glycoproteins which are acquired by T cells (201, 202).

Membrane nanotubes: Advances in cell imaging technology have led to demonstrating the intercellular exchange of proteins through membrane tubes, long membrane tethers between cells. Nanotubes are reported to connect a wide range of immune cells, such as T cells, B cells, NK cells, and monocytes (203, 204). Nanotubes were also observed between B cells and NK cells (205). There is a study demonstrating the T cell with T cell nanotube formation which might have important consequences in allowing rapid spread of HIV-1(206).

Intercellular communication through trogocytosis is very important in maintaining the homeostasis of the complex immune system in order to elicit effective immune responses. When we look at the functional consequence of trogocytosis, these acquired molecules appear to have immune stimulatory or suppressive impact. In our study, we speculate that DC molecules are acquired by T cells (chapters 2&3).

1.6 Cytokines

Cytokines are small proteins/peptides/glycoproteins released by cells which have specific roles in the immune system by autocrine and paracrine mechanisms. Lymphocytes being the primary source of cytokines, other cells also produce cytokines. Cytokines include a number of groups: interleukins, lymphokines, and cell signal molecules, such as the tumor necrosis factor and the interferons. Cytokines are usually pleotrophic with diverse activity on different cells. They act through binding specific receptors. They are shown to regulate cell activation, hematopoiesis, apoptosis, cell migration, and cell proliferation. With various functions, they are involved in both innate and adaptive immune responses. There are various cytokines and cytokine families, of which I will be discussing those cytokines relevant to our study.

1.6.1 Interleukin-4 (IL-4)

IL-4 is multifunctional pleiotorpic cytokine that plays a critical role in Th2-mediated immune regulation. It is produced by Th2 cells, basophils and mast cells (207). IL-4 was also

produced by NK cells upon in vivo challenge with anti-CD3 (208). Upon Th2 pathogen stimulation, γδ T cells are shown to produce IL-4 (209). Clones of $CD8^+$ T cells produce IL-4-helping B cells (210). Addition of IL-4 in the secondary culture period of DCs induces strong antigen presenting capacity (211). IL-4 binds to IL-4Rα and recruits a common gamma chain (γc) for its downstream effects, this heterodimerization is necessary for physiological function (212). IL-4 promotes Th2 responses through GATA3. IL-4 upregulates the GATA3 expression which is expressed in low levels in naïve T cells. GATA3 is upregulated through STAT6 activation by IL-4 cytokine (213). The IL-4-mediated STAT6 signalling pathway leads to silencing of IFN-γ expression and, inhibition of a Th1 kind of response (214). On the contrary, Th1 cytokine, IL-12 inhibits Th2 cells through suppression of GATA3 (215). Th1 cytokine IFN-γ acts as a key activator of IL-12 and IL-12R, further suppressing a Th2 response (41). IFN-γ is also shown to directly suppress the IL-4 gene through interferon regulatory factors IRF1 and IRF2 (216). IL-4 plays an important physiological function through immunoglobulin class switching. In mice, IL-4 induces the secretion of IgE and IgG1 from B cells (217, 218) and in humans, induces IgE and IgG4 secretion (219). These antibody-switching functions of IL-4 demonstrate its role in allergic conditions. Through Th2 responses, IL-4 promotes protective immunity in helminth and extracellular parasitic infestation. IL-4 is shown to act as a co-mitogen along with IL-2 in B cells (220). Apart from that, it increases the expression of MHC class II molecules in B cells (221). IL-4 as well as TNF are shown to induce the expression of vascular cell adhesion molecule-1 (VCAM-1) in vascular endothelial cells along with down-regulation of E-selectin (222, 223). This shift in adhesion molecules will help in recruiting T cells and eosinophils than granulocytes to the site of inflammation.

1.6.2 Interleukin-6 (IL-6)

IL-6 is a pleiotropic cytokine with its receptors in various cell types, showing a wide range of biological activities, immune regulation, haematopoiesis, inflammation, and oncogenesis (224). IL-6 was first identified as B-cell stimulating factor-2, and later named as IL-6 (225). Many cell types produce this cytokine, including T cells, dendritic cells, macrophages, fibroblasts, synovial cells, endothelial cells, glial cells, and keratinocytes (224). IL-6 being a pro-inflammatory cytokine, overproduction of IL-6 has been associated with various inflammatory

diseases. Synovial cells from rheumatoid arthritis patients showed increased production of IL-6 (226). Physiologically, this cytokine functions through IL-6R, and the binding of this receptor also induces the interaction of gp130 (227, 228). The cell surface polypeptide gp130 is expressed in almost all tissues and cells, endorsing the pleiotropic nature of IL-6 (229). Several studies have shown that gp130 acts as a receptor component for several other cytokines such as CNTF in brain, LIF, oncostatin M, IL-11, IL-27, neuropoetin, and cardiotrophin (230). IL-6 is shown to be induced by various stimuli including bacterial and viral infections, microbial components like lipopolysaccharide, and by cytokines like IL-1, TNF-α and PDGF (224). IL-6 acts as an important link between innate and adaptive immune responses with its effects on T and B cells (231). Cytokine IL-6 acts through the JAK-STAT pathway, through STAT3 activation (232). STAT3 regulates IL-6-induced expression of RORγt and IL-17 production, indicating the primary role of IL-6 in Th17 cell differentiation (96). Through up-regulation of NFATc2 and c-maf, IL-6 influences T cell effector functions by promoting Th2 cell differentiation (233, 234). IL-6 is shown to be an important factor in antibody production because it induces plasma cell development from B cells (235). IL-6 deficient mice show reduced antigen specific IgG1, IgG2a, and IgG3 levels upon immunization with T cell-dependent antigen (236). Humanized antibody Tocilizumab is approved for clinical use in treating IL-6 induced pathologies (230, 237).

1.6.3 Interleukin-10 (IL-10)

IL-10 is an anti-inflammatory cytokine. It was initially identified as the cytokine synthesis inhibitory factor (CSIF) produced by Th2 cells in response to antigen presentation by APC, and it was shown to inhibit Th1 cells (238). IL-10 is produced by macrophages, dendritic cells, B cells, and various subsets of $CD4^+$ and $CD8^+$ T cells (239). IL-10 signals through IL-10R1 and IL-10R2 receptor complexes, inducing Jak1 and Tyk2 kinases, which phosphorylates SATA3 to drive STAT3 responsive genes (240). IL-10 being an anti-inflammatory cytokine will inhibit MHC class II and co-stimulatory molecule B7-1/B7-2 expression on macrophages and monocytes, and will also inhibit the production of pro-inflammatory cytokines and chemokines (241). In dendritic cells, autocrine signalling has led to inhibited IL-12 local production in mycobacterial infection (242). IL-10 also induced the differentiation of regulatory DCs secreting IL-10, thus leading to stimulation of Tr1 cells (243). Moreover, IL-10 is shown to play role in B

cell survival through Bcl2 up-regulation, showing its importance role in humoral response (244). Endorsing the anti-inflammatory role of IL-10, IL-10 deficient mice showed spontaneous enterocolitis (245). IL-10 deficient mice also showed increased Th1 responses with protection from granuloma formation with Chlamydia trachomatis lung infection (246).

1.6.4 Interleukin-17 (IL-17)

IL-17 plays host-defensive role in many infectious diseases, but promotes host-destructive inflammatory pathology in autoimmune disorders. IL-17 is essential for host defence against many microbes such as bacteria and fungi (247). Originally, IL-17 was thought to be produced only by T cells, but is currently shown to be produced by various other cells: dendritic cells, macrophages, smooth-muscle cells, NK cells, NKT cells, and γδ T cells are some known sources (248). The IL-17 family of cytokines are composed of IL-17A, IL-17B, IL-17C, IL-17D, IL-17E (IL-25), and IL-17F (249). IL-17 has five receptors IL-17RA, IL-17RB/IL-25R, IL-17RC, IL-17RD and IL-17RE (250). IL-17 receptors are expressed ubiquitously by most of the cell types, hence most of them can respond to IL-17 (251). IL-17 cytokines are strongly pro-inflammatory, and they induce the expression of several chemokines such as CCL2, CCL7 and CCL20, as well as pro-inflammatory cytokines like IL-6 and TNF-α (249, 252). Transgenic over-expression of IL-17A in the lungs provokes pro-inflammatory gene expression and tissue infiltration of leukocytes. In contrast, inhibition of IL-17A expression impairs host defenses against bacterial infection (253) and resistance to autoimmune diseases (110, 171). IL-17E (IL-25) induces Th2 type of cytokines and chemokines RANTES and Eotoxin-1, and it plays role in allergic responses (249). IL-17 acts as an angiogenic factor stimulating migration and chord formation of vascular endothelial cells in vitro and also leads to vessel formation in vivo (118, 254). IL-17 plays distinct roles in lymphopenic and immune-competent mice with reference to tumor immunity. Transgenic expression of IL-17 either induced tumor regression through enhanced antitumor immunity in immune-competent mice (115, 116) or promoted tumor progression through an increase in inflammatory angiogenesis in immune-deficient mice.

1.7 T-APC model

Antigen presenting cells (APCs) play critical roles in eliciting adaptive immune responses and keeping memory. APCs will acquire foreign protein or self-protein and process that antigen to present it on MHC molecules. T cells lack the ability to recognize the free antigen, where as they can recognize the antigen presented on MHC molecules through specific TCRs present on T cells. APCs are classified into professional APCs and non-professional APCs. APCs which have MHC class II on them are termed professional APCs. Expression of co-stimulatory molecules like CD80, CD86, CD40, etc. is a characteristic feature of professional APCs. Originally, dendritic cells, B cells, and macrophages fall under this category. There are various subsets of each cell type which are involved in immune responses. Recently, Dr. Jim Xiang's group has shown that $CD4^+$ T cells themselves act as APCs, inventing the new concept of a dynamic model of two cell interactions by $CD4^+$ Th-APC (255).

To generate effective CTL responses for minor histo-compatibility antigens and tumor antigens, which lack danger signals, DCs and $CD8^+$ T cells need help form $CD4^+$ T cells (256). There are three models explaining the help provided by $CD4^+$ T cells to $CD8^+$ T cells in generating effective CTL response. The first model is the passive model of three-cell interaction, wherein antigen-specific CD4 and $CD8^+$ T cells simultaneously come in contact with specific antigen-carrying APCs (257). One difficulty associated with this model is the chance of all these cells coming together at the same time. In the sequential two-cell interaction model, APCs activate $CD4^+$ T cells and gets reciprocally activated through CD40-CD40L signalling. Reciprocally activated APC will then directly stimulate $CD8^+$ T cells (258). There was a caveat in this model because of the temporal nature of antigen presentation and CTL production. To address the drawbacks associated with these two models, a novel concept of Th-APC was proposed by Dr. Jim Xian's group (255). Trogocytosis (intercellular transfer of membrane proteins) is a common phenomenon that occurs between immune cells, which play an important role in immune modulation (259). As a sequel to antigen-specific T cell-APC interactions an immunological synapse is formed, comprising a central cluster of TCR-MHC-peptide complexes and outer ring of CD28-CD80 interactions and other accessory molecular interactions (192, 193). When these cells come off the synapse, APCs-derived surface molecules are transferred to the T helper cells; later, they are recycled back to the surface by TCR internalization and recycling (181, 260). Dr. Jim Xinag's lab has shown that during the membrane molecule transfer from APCs to $CD4^+$ T cells by APC stimulation, $CD4^+$ T cells acquire the synapse-composed MHC

class II and co-stimulatory molecules (CD54 and CD80) along with the bystander peptide-MHC class I complexes. The $CD4^+$ T cells carrying acquired APC antigen-presenting machinery were shown to act as $CD4^+$ Th-APCs by stimulating in vitro and in vivo antigen-specific CTL responses (255).

Further endorsing the dynamic model of two cell interactions by $CD4^+$ Th-APC, by using confocal imaging technology, it was demonstrated that peptide-MHC class II and bystander peptide-MHC class I co-localize within the synapse formed between $CD4^+$ T cells and antigen-presenting DCs. Co-stimulatory molecules CD54, CD80, CD40, OX40L and 41BBL also co-localized in the same synapse. Recycling of acquired peptide-MHC class I molecules was also demonstrated (184, 261). It was also shown in human T cells that they acquire MHC molecules and co-stimulatory molecules from APCs and act as T-APCs to other T cells. These acquired molecules were also shown to persist on T cells for 72 hours (262). As a different mechanism of antigen presenting machinery transfer from APCs to T cells, exosomes containing peptide-MHC class I and co-stimulatory CD40 and CD80 molecules released by DCs were transferred to T cells, making them effective T-APCs in inducing antigen-specific CTL response and long term memory (263, 264). In vitro DC_{OVA} activated T-helper cells, acting as T-APCs, stimulated OVA-specific memory response with the involvement of IL-2 secreted by T-APCs (265). $CD4^+$ Th1 cells with acquired peptide-MHC I by DC_{OVA} stimulation were able to reduce the apoptosis and to prolong the survival of active $CD8^+$ Tc1 cells in vitro, and to promote $CD8^+$ Tc1 cell tumor localization and memory responses in vivo (266). DC-activated T-APCs were shown to elicit antigen specific CTL response in MHC class II knockout mice and were able to overcome the self tolerance in the transgenic RIPmOVA diabetic model (267, 268). Th-APC with acquired peptide MHC class I and II could also stimulate Th1 and central memory $CD8^+44^+CD62L^{high}IL\text{-}7R^+$ T cell responses, leading to effective antitumor immunity (52). As a part of this thesis, Th17 cells induced by DC activation were shown to behave as T-APCs to induce antigen-specific CTL responses in EAE and T1D autoimmune models (269). Th17 cells were also shown to induce both preventive and therapeutic antitumor immune responses through the acquired peptide MHC class I molecule from DCs (102).

1.8 Disease models

In our study, we have dealt with cancer immunotherapy and with the pathophysiology of autoimmune diseases with reference to T-helper cells. The autoimmune diseases addressed in our study are type 1 diabetis, experimental autoimmune encephalitis (animal model for human multiple sclerosis).

1.8.1 Cancer

Cancer is an uncontrolled growth of abnormal cells in the body. Cancer could be benign with localized growth or malignant with disseminating behaviour to other locations. Cancer cells are self cells, so there are only certain minimal differences between normal cells and cancerous cells. Because of this minor difference in antigen profile, cancer cells grow very well even in the presence of a strong intact immune system. Apart from that, tumors secrete immunosuppressive cytokines such as TGF-β and IL-10 that have negative effects on the immune system, letting tumors off the immune-scanner (270, 271). Another immunosuppressive molecule found in tumors is indoleamine 2, 3-dioxygenase, an inducible enzyme involved in tryptophan catabolism. Tryptophan depletion in tumors by this enzyme decreases the functionality of effector T cells and causes dendritic cells to become immunosuppressive (272). In addition, tumors often harbour immunosuppressive cells such as regulatory T cells, myeloid-derived suppressor cells, and immature dendritic cells, making the tumor microenvironment even more immunosuppressive (19). This phenomenon of evading immune response is referred to as immunoediting (273). To fight an immunosuppressive tumor environment, we can harness the immune system by stimulating the body's own immune system and/or by adaptive transfer of active humoral or cellular immune system. There are various studies showing the role of functionally differentiated T-helper cells in induction of antitumor immune response (274). Th1 cells are shown to counteract immune suppressive regulatory cells by stimulating efficient $CD8^+$ T cell and NK cell responses (264) and Th17 cells are shown to induce effective antitumor immune response through antigen-specific $CD8^+$ T cell induction (102).

1.8.2 Type 1 diabetes (T1D)

Type 1 diabetes (T1D) is a chronic autoimmune disease, wherein the pancreatic β-cells which secrete insulin are selectively destroyed. It is thought to be a Th1 cell-mediated disease that involves $CD8^+$ T cells and innate immune cells (275). $CD4^+$ and $CD8^+$ T cells, as well as macrophages, have been shown to have a role in β-cell death. The recurrence of T1D in recipients of segmental pancreas grafts from HLA-identical donors showed a clear role for T cells, particularly $CD8^+$ T cells and monocytes, with little evidence for a humoral immune response, in β-cell destruction (276). $CD8^+$ cytotoxic T cells could kill pancreatic β-cells through MHC class I-mediated cytotoxicity. As well, both $CD4^+$ Th1 and $CD8^+$ CTL cells produce cytokines, such as interferon-γ (IFN-γ) that induce expression of the death receptor FAS (CD95) and chemokine production by β-cells. Activation of FAS by FAS ligand (FASL)-expressing activated T cells could initiate β-cell apoptosis (275). IFN-γ can also activate macrophages and induce increased pro-inflammatory cytokine production, including IL-1β and tumor necrosis factor (TNF). In addition, IFN-γ, IL-1β, and TNF also induce the expression of reactive oxygen species (ROS), including nitric oxide by β-cells; ROS also have the potential to mediate apoptosis (275). Th17 cells are considered as contributing factor in the pathogenic process of T1D. For example, it has been found that IL-17 is expressed in the pancreas during the course of T1D in the mouse model (277); reducing the number of Th17 cells with induction of IFN- γ inhibited IL-17 production and restored normoglycemia at the prediabetic stage (278). Dr. Jim Xiang's lab has shown that Th17-stimulated $CD8^+$ CTL, but not Th17 cells, play a major pathogenic role in the induction of diabetes in RIP-mOVA mice (chapter 3) (51).

Although $CD4^+$ Th1 and Th17 cells have a pathological role in T1D onset, there is also evidence supporting a role for T cells in the prevention of β-cell destruction. Patients with IPEX (immunodysregulation, polyendocrinopathy, enteropathy, X-linked) syndrome have mutations in FoxP3 and can develop T1D, which highlights the importance of Treg cells in controlling the onset of this autoimmune disease (279). Studies in NOD mice have shown the importance of Treg cells in preventing T1D: CD28-deficient NOD mice, which lack Treg cells, develop accelerated disease (280). In addition, it is also evident from strategies such as injection of IL-2 to increase Treg cell numbers, is as potential therapeutic approach for T1D (281).

1.8.3 Multiple sclerosis (MS)

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease affecting the central nervous system. Both environmental and autoimmune causes have been attributed to MS. There is little evidence to support the complete environmental trigger for MS, whereas the autoimmune cause has been well established (282). Aberrantly high precursor frequencies of autoreactive T cells due to a failure of central (thymic) tolerance mechanisms or an aberrant activation or skewing of autoreactive T cells in the peripheral immune compartment by a failure of peripheral tolerance mechanisms, can potentially lead to T cell triggered autoimmune tissue inflammation. Myelin-specific T cells become activated in the peripheral immune system and overcome the blood brain barrier, causing disease. To understand the T cell biology in MS pathophysiology, mouse model of EAE has been used extensively.

In earlier studies, Th1 responses have been attributed for organ-specific autoimmunity including MS; they found IFN-γ in MS lesions, which peaked at the peak of the disease and declined with recovery (283). This concept of Th1 cell involvement was seriously challenged, when mice which were genetically deficient for IFN-γ showed serious disease than protection (283). IL-23 deficient mice were totally protected from EAE, endorsing the importance of IL-23 in EAE pathology (48). IL-23 is a member of the IL-12 family of heterodimeric cytokines and shares the p40 subunit with IL-12, but has a unique p19 subunit. IL-23 KO mice, which are completely resistant to EAE, were found to be devoid of a particular subset of $CD4^+$ T cells that produced IL-17 (110). Whereas IL-12 is an essential differentiation factor for Th1 cells, IL-23 is shown to be an important cytokine of Th17 cells. It has been shown that adoptive transfer of sensitized $CD4^+$ Th17 cells can induce EAE in wild-type C57BL/6 mice (284) (chapter 3). Not surprisingly, Th17 cells are associated with a series of autoimmune or chronic inflammatory disorders such as MS (285), rheumatoid arthritis (286), psoriaisis (287), and inflammatory bowel disease (288). Some studies have argued that both Th1 and Th17 cells are involved in CNS autoimmunity (289).

1.8.4 Experimental Autoimmune Encephalitis (EAE)

EAE is a demyelinating disease, a rodent model that has been valuable for characterization of the immunopathogenic process of human multiple sclerosis (MS). EAE is

induced in susceptible animals by immunizing them with one of the various number of myelin-specific antigens emulsified in complete freund's adjuvant (CFA) along with intra-peritoneal pertusis toxin (290, 291). Attention has originally been focused on the role of $CD4^+$ T cells in the induction of EAE because susceptibility to MS is associated with MHC class II genes (292). Earlier, it was canonically accepted that Th1 cells are pathogenic in EAE and Th2 are protective (293). With the invention of Th17 cells, there are various studies showing the role of both Th1 and Th17 cells in the pathogenesis of EAE (283, 294). Though there were differences in relative proportion of Th1 and Th17 cells in different mice models of EAE, both cell types were present in EAE lesions (110, 295). It has also been shown that MOG-specific $CD8^+$ T cell responses are involved prior to and after the onset of EAE (296); the adoptive transfer of MOG-specific $CD8^+$ T cells is also shown to induce EAE (291, 297) (chapter 3). As various studies have shown, the importance of both $CD4^+$ and $CD8^+$ T cells, it is important to understand the relationship of those cells.

1.9 General hypothesis and objectives

Specific hypothesis and objectives are described in introduction to each chapter. Hypothesis and objectives for each chapter were designed in order to understand different aspects of $CD4^+$ T cell biology.

First hypothesis: there were studies suggesting the involment of Th17 cells in antitumor immunity. However, the mechanism of antitumor immunity and $CD8^+$ T cell activation by Th17 cells was still elusive. To understand the mechanism we hypothesized that Th17 cells can directly stimulate $CD8^+$ T cell response, themselves acting as antigen-presenting cells, inducing effective antitumor immune response. The objective of this hypothesis was to decipher the roles of molecular signals in preventive and therapeutic antitumor immunity (chapter 2).

Second hypothesis: it had been documented that both $CD4^+$ and $CD8^+$ T cells are involved in T1D and EAE, but the relationship of these two cells in the pathogenesis of these autoimmune diseases was not addressed. To understand the relationship and pathogenic involvement of $CD4^+$ and $CD8^+$ T cells, we hypothesized that (i) $CD4^+$ Th17 cells directly stimulate $CD8^+$ T cells. (ii) both Th17 and Th17-induced $CD8^+$ T cells differentially regulate

T1D and EAE. There were two main objectives for this hypothesis: (i) to demonstrate that $CD4^+$ Th17 cells act as antigen-presenting cells in inducing antigen-specific $CD8^+$ T cell response, and (ii) to show that $CD4^+$ and $CD8^+$ T cells play distinct roles in T1D and EAE pathogenesis (chapter 3).

Third hypothesis: there was evidence showing overlapping cytokine expression by $CD4^+$ T helper cells, indicating the plasticity of these cells; based on that evidence, we hypothesized that the deletion of cytokine genes would lead to functional conversion of these cells. There were two main objectives for this hypothesis: (i) to show that $CD4^+$ Th2 cells are not inhibitory on DC_{OVA} induced $CD8^+$ T cell response, and (ii) to document that single cytokine gene deleted naive $CD4^+$ T cells differentiated under Th2 culture conditions are functionally converted to a different subset of $CD4^+$ T helper cell (Chapter 4).

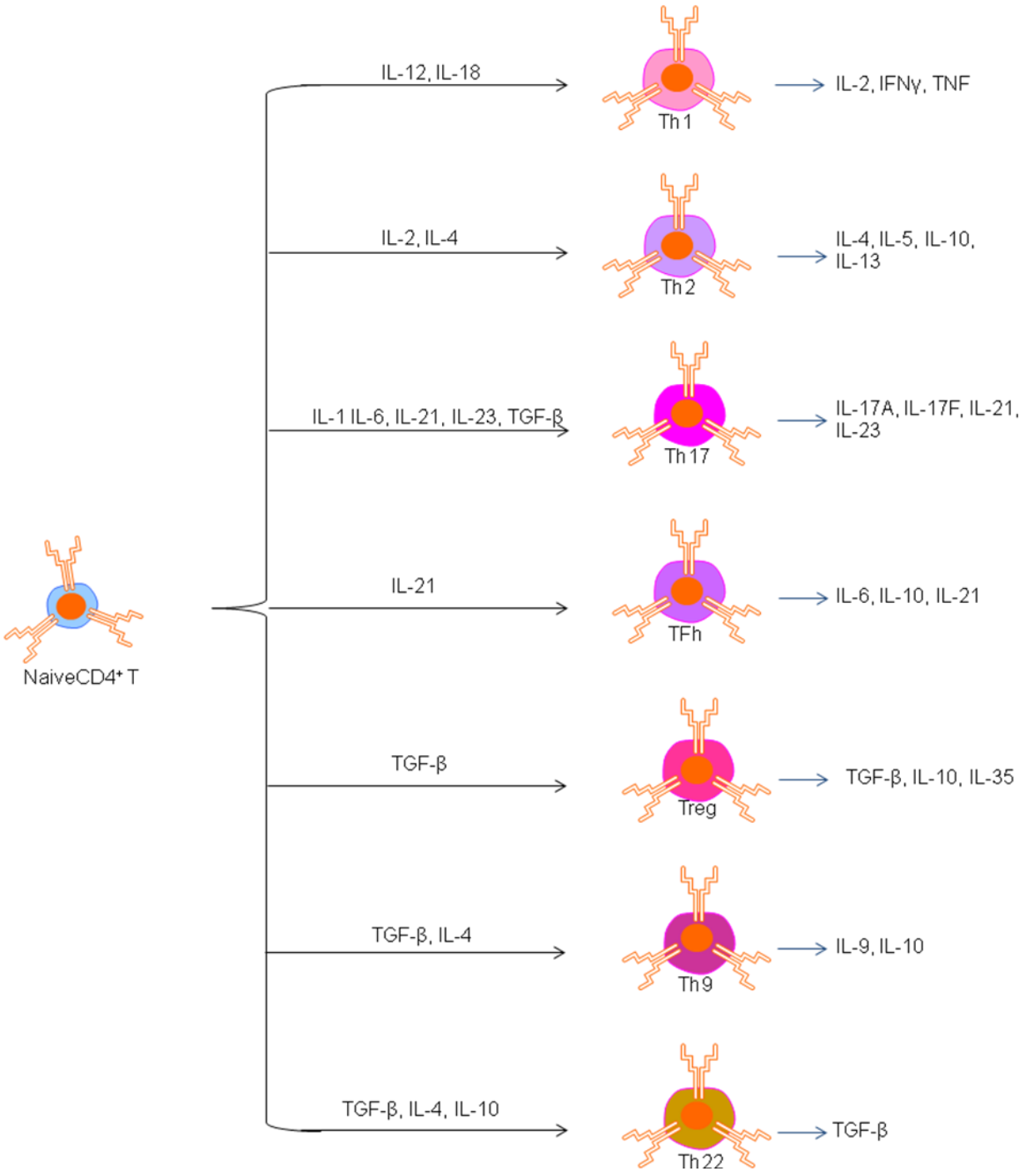

Figure 1.1 Differentiation of naive CD4$^+$ T cells into different CD4$^+$ T cell subsets. This figure illustrates the cytokines that play roles in differentiation and maintenance of different CD4$^+$ T cell subsets, and CD4$^+$ T cell subset-specific cytokines.

1.10 References

1. Liu, Y. J. 2001. Dendritic cell subsets and lineages, and their functions in innate and adaptive immunity. *Cell* 106:259-262.
2. Iwasaki, A., and R. Medzhitov. 2004. Toll-like receptor control of the adaptive immune responses. *Nat Immunol* 5:987-995.
3. Steinman, R. M., and Z. A. Cohn. 1973. Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. *J Exp Med* 137:1142-1162.
4. Hart, D. N. 1997. Dendritic cells: unique leukocyte populations which control the primary immune response. *Blood* 90:3245-3287.
5. Sato, K., and S. Fujita. 2007. Dendritic cells: nature and classification. *Allergol Int* 56:183-191.
6. Shortman, K., and Y. J. Liu. 2002. Mouse and human dendritic cell subtypes. *Nat Rev Immunol* 2:151-161.
7. Hao, S., J. Yuan, S. Xu, M. A. Munegowda, Y. Deng, J. Gordon, Z. Xing, and J. Xiang. 2008. Antigen specificity acquisition of adoptive CD4+ regulatory T cells via acquired peptide-MHC class I complexes. *J Immunol* 181:2428-2437.
8. Takeuchi, S., and M. Furue. 2007. Dendritic cells: ontogeny. *Allergol Int* 56:215-223.
9. Ohteki, T. 2007. The dynamics of dendritic cell: mediated innate immune regulation. *Allergol Int* 56:209-214.
10. Vremec, D., J. Pooley, H. Hochrein, L. Wu, and K. Shortman. 2000. CD4 and CD8 expression by dendritic cell subtypes in mouse thymus and spleen. *J Immunol* 164:2978-2986.
11. Anjuere, F., P. Martin, I. Ferrero, M. L. Fraga, G. M. del Hoyo, N. Wright, and C. Ardavin. 1999. Definition of dendritic cell subpopulations present in the spleen, Peyer's patches, lymph nodes, and skin of the mouse. *Blood* 93:590-598.
12. Vremec, D., and K. Shortman. 1997. Dendritic cell subtypes in mouse lymphoid organs: cross-correlation of surface markers, changes with incubation, and differences among thymus, spleen, and lymph nodes. *J Immunol* 159:565-573.
13. Watowich, S. S., and Y. J. Liu. 2010. Mechanisms regulating dendritic cell specification and development. *Immunol Rev* 238:76-92.

14. Kronin, V., D. Vremec, K. Winkel, B. J. Classon, R. G. Miller, T. W. Mak, K. Shortman, and G. Suss. 1997. Are CD8+ dendritic cells (DC) veto cells? The role of CD8 on DC in DC development and in the regulation of CD4 and CD8 T cell responses. *Int Immunol* 9:1061-1064.
15. Belz, G. T., G. M. Behrens, C. M. Smith, J. F. Miller, C. Jones, K. Lejon, C. G. Fathman, S. N. Mueller, K. Shortman, F. R. Carbone, and W. R. Heath. 2002. The CD8alpha(+) dendritic cell is responsible for inducing peripheral self-tolerance to tissue-associated antigens. *J Exp Med* 196:1099-1104.
16. den Haan, J. M., S. M. Lehar, and M. J. Bevan. 2000. CD8(+) but not CD8(-) dendritic cells cross-prime cytotoxic T cells in vivo. *J Exp Med* 192:1685-1696.
17. Maldonado-Lopez, R., T. De Smedt, P. Michel, J. Godfroid, B. Pajak, C. Heirman, K. Thielemans, O. Leo, J. Urbain, and M. Moser. 1999. CD8alpha+ and CD8alpha- subclasses of dendritic cells direct the development of distinct T helper cells in vivo. *J Exp Med* 189:587-592.
18. Yasumi, T., K. Katamura, T. Yoshioka, T. A. Meguro, R. Nishikomori, T. Heike, M. Inobe, S. Kon, T. Uede, and T. Nakahata. 2004. Differential requirement for the CD40-CD154 costimulatory pathway during Th cell priming by CD8 alpha+ and CD8 alpha- murine dendritic cell subsets. *J Immunol* 172:4826-4833.
19. Zhang, X., H. Huang, J. Yuan, D. Sun, W. S. Hou, J. Gordon, and J. Xiang. 2005. CD4-8- dendritic cells prime CD4+ T regulatory 1 cells to suppress antitumor immunity. *J Immunol* 175:2931-2937.
20. Henri, S., D. Vremec, A. Kamath, J. Waithman, S. Williams, C. Benoist, K. Burnham, S. Saeland, E. Handman, and K. Shortman. 2001. The dendritic cell populations of mouse lymph nodes. *J Immunol* 167:741-748.
21. Merad, M., L. Fong, J. Bogenberger, and E. G. Engleman. 2000. Differentiation of myeloid dendritic cells into CD8alpha-positive dendritic cells in vivo. *Blood* 96:1865-1872.
22. Lutz, M. B., and G. Schuler. 2002. Immature, semi-mature and fully mature dendritic cells: which signals induce tolerance or immunity? *Trends Immunol* 23:445-449.
23. Galibert, L., C. R. Maliszewski, and S. Vandenabeele. 2001. Plasmacytoid monocytes/T cells: a dendritic cell lineage? *Semin Immunol* 13:283-289.

24. Grouard, G., M. C. Rissoan, L. Filgueira, I. Durand, J. Banchereau, and Y. J. Liu. 1997. The enigmatic plasmacytoid T cells develop into dendritic cells with interleukin (IL)-3 and CD40-ligand. *J Exp Med* 185:1101-1111.

25. Olweus, J., A. BitMansour, R. Warnke, P. A. Thompson, J. Carballido, L. J. Picker, and F. Lund-Johansen. 1997. Dendritic cell ontogeny: a human dendritic cell lineage of myeloid origin. *Proc Natl Acad Sci U S A* 94:12551-12556.

26. Nakano, H., M. Yanagita, and M. D. Gunn. 2001. CD11c(+)B220(+)Gr-1(+) cells in mouse lymph nodes and spleen display characteristics of plasmacytoid dendritic cells. *J Exp Med* 194:1171-1178.

27. Heil, F., H. Hemmi, H. Hochrein, F. Ampenberger, C. Kirschning, S. Akira, G. Lipford, H. Wagner, and S. Bauer. 2004. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. *Science* 303:1526-1529.

28. Hemmi, H., O. Takeuchi, T. Kawai, T. Kaisho, S. Sato, H. Sanjo, M. Matsumoto, K. Hoshino, H. Wagner, K. Takeda, and S. Akira. 2000. A Toll-like receptor recognizes bacterial DNA. *Nature* 408:740-745.

29. Uematsu, S., S. Sato, M. Yamamoto, T. Hirotani, H. Kato, F. Takeshita, M. Matsuda, C. Coban, K. J. Ishii, T. Kawai, O. Takeuchi, and S. Akira. 2005. Interleukin-1 receptor-associated kinase-1 plays an essential role for Toll-like receptor (TLR)7- and TLR9-mediated interferon-{alpha} induction. *J Exp Med* 201:915-923.

30. Martin, P., G. M. Del Hoyo, F. Anjuere, C. F. Arias, H. H. Vargas, L. A. Fernandez, V. Parrillas, and C. Ardavin. 2002. Characterization of a new subpopulation of mouse CD8alpha+ B220+ dendritic cells endowed with type 1 interferon production capacity and tolerogenic potential. *Blood* 100:383-390.

31. Brawand, P., D. R. Fitzpatrick, B. W. Greenfield, K. Brasel, C. R. Maliszewski, and T. De Smedt. 2002. Murine plasmacytoid pre-dendritic cells generated from Flt3 ligand-supplemented bone marrow cultures are immature APCs. *J Immunol* 169:6711-6719.

32. Winzler, C., P. Rovere, M. Rescigno, F. Granucci, G. Penna, L. Adorini, V. S. Zimmermann, J. Davoust, and P. Ricciardi-Castagnoli. 1997. Maturation stages of mouse dendritic cells in growth factor-dependent long-term cultures. *J Exp Med* 185:317-328.

33. Reis e Sousa, C. 2006. Dendritic cells in a mature age. *Nat Rev Immunol* 6:476-483.

34. Amsen, D., J. M. Blander, G. R. Lee, K. Tanigaki, T. Honjo, and R. A. Flavell. 2004. Instruction of distinct CD4 T helper cell fates by different notch ligands on antigen-presenting cells. *Cell* 117:515-526.

35. Kumanogoh, A., T. Shikina, K. Suzuki, S. Uematsu, K. Yukawa, S. Kashiwamura, H. Tsutsui, M. Yamamoto, H. Takamatsu, E. P. Ko-Mitamura, N. Takegahara, S. Marukawa, I. Ishida, H. Morishita, D. V. Prasad, M. Tamura, M. Mizui, T. Toyofuku, S. Akira, K. Takeda, M. Okabe, and H. Kikutani. 2005. Nonredundant roles of Sema4A in the immune system: defective T cell priming and Th1/Th2 regulation in Sema4A-deficient mice. *Immunity* 22:305-316.

36. Wahl, S. M. 2007. Transforming growth factor-beta: innately bipolar. *Curr Opin Immunol* 19:55-62.

37. Mosmann, T. R., and R. L. Coffman. 1989. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol* 7:145-173.

38. Waaga, A. M., M. Gasser, J. E. Kist-van Holthe, N. Najafian, A. Muller, J. P. Vella, K. L. Womer, A. Chandraker, S. J. Khoury, and M. H. Sayegh. 2001. Regulatory functions of self-restricted MHC class II allopeptide-specific Th2 clones in vivo. *J Clin Invest* 107:909-916.

39. Ansel, K. M., D. U. Lee, and A. Rao. 2003. An epigenetic view of helper T cell differentiation. *Nat Immunol* 4:616-623.

40. O'Shea, J. J., and W. E. Paul. 2010. Mechanisms underlying lineage commitment and plasticity of helper CD4+ T cells. *Science* 327:1098-1102.

41. Szabo, S. J., A. S. Dighe, U. Gubler, and K. M. Murphy. 1997. Regulation of the interleukin (IL)-12R beta 2 subunit expression in developing T helper 1 (Th1) and Th2 cells. *J Exp Med* 185:817-824.

42. Szabo, S. J., B. M. Sullivan, C. Stemmann, A. R. Satoskar, B. P. Sleckman, and L. H. Glimcher. 2002. Distinct effects of T-bet in TH1 lineage commitment and IFN-gamma production in CD4 and CD8 T cells. *Science* 295:338-342.

43. Mullen, A. C., A. S. Hutchins, F. A. High, H. W. Lee, K. J. Sykes, L. A. Chodosh, and S. L. Reiner. 2002. Hlx is induced by and genetically interacts with T-bet to promote heritable T(H)1 gene induction. *Nat Immunol* 3:652-658.

44. Smeltz, R. B., J. Chen, R. Ehrhardt, and E. M. Shevach. 2002. Role of IFN-gamma in Th1 differentiation: IFN-gamma regulates IL-18R alpha expression by preventing the negative effects of IL-4 and by inducing/maintaining IL-12 receptor beta 2 expression. *J Immunol* 168:6165-6172.

45. Sallusto, F., D. Lenig, C. R. Mackay, and A. Lanzavecchia. 1998. Flexible programs of chemokine receptor expression on human polarized T helper 1 and 2 lymphocytes. *J Exp Med* 187:875-883.

46. Gran, B., G. X. Zhang, S. Yu, J. Li, X. H. Chen, E. S. Ventura, M. Kamoun, and A. Rostami. 2002. IL-12p35-deficient mice are susceptible to experimental autoimmune encephalomyelitis: evidence for redundancy in the IL-12 system in the induction of central nervous system autoimmune demyelination. *J Immunol* 169:7104-7110.

47. Constantinescu, C. S., M. Wysocka, B. Hilliard, E. S. Ventura, E. Lavi, G. Trinchieri, and A. Rostami. 1998. Antibodies against IL-12 prevent superantigen-induced and spontaneous relapses of experimental autoimmune encephalomyelitis. *J Immunol* 161:5097-5104.

48. Cua, D. J., J. Sherlock, Y. Chen, C. A. Murphy, B. Joyce, B. Seymour, L. Lucian, W. To, S. Kwan, T. Churakova, S. Zurawski, M. Wiekowski, S. A. Lira, D. Gorman, R. A. Kastelein, and J. D. Sedgwick. 2003. Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. *Nature* 421:744-748.

49. Murphy, C. A., C. L. Langrish, Y. Chen, W. Blumenschein, T. McClanahan, R. A. Kastelein, J. D. Sedgwick, and D. J. Cua. 2003. Divergent pro- and antiinflammatory roles for IL-23 and IL-12 in joint autoimmune inflammation. *J Exp Med* 198:1951-1957.

50. Bending, D., H. De la Pena, M. Veldhoen, J. M. Phillips, C. Uyttenhove, B. Stockinger, and A. Cooke. 2009. Highly purified Th17 cells from BDC2.5NOD mice convert into Th1-like cells in NOD/SCID recipient mice. *J Clin Invest* 119:565-572.

51. Ankathatti Munegowda, M., Y. Deng, R. Chibbar, Q. Xu, A. Freywald, S. J. Mulligan, S. van Drunen Littel-van den Hurk, D. Sun, S. Xiong, and J. Xiang. 2011. A Distinct Role of CD4(+) Th17- and Th17-Stimulated CD8(+) CTL in the Pathogenesis of Type 1 Diabetes and Experimental Autoimmune Encephalomyelitis. *J Clin Immunol* 31:811-826.

52. Umeshappa, C. S., H. Huang, Y. Xie, Y. Wei, S. J. Mulligan, Y. Deng, and J. Xiang. 2009. CD4+ Th-APC with acquired peptide/MHC class I and II complexes stimulate type 1 helper CD4+ and central memory CD8+ T cell responses. *J Immunol* 182:193-206.
53. Muranski, P., A. Boni, P. A. Antony, L. Cassard, K. R. Irvine, A. Kaiser, C. M. Paulos, D. C. Palmer, C. E. Touloukian, K. Ptak, L. Gattinoni, C. Wrzesinski, C. S. Hinrichs, K. W. Kerstann, L. Feigenbaum, C. C. Chan, and N. P. Restifo. 2008. Tumor-specific Th17-polarized cells eradicate large established melanoma. *Blood* 112:362-373.
54. Romagnani, S. 1995. Biology of human TH1 and TH2 cells. *J Clin Immunol* 15:121-129.
55. Fallon, P. G., and N. E. Mangan. 2007. Suppression of TH2-type allergic reactions by helminth infection. *Nat Rev Immunol* 7:220-230.
56. Gordon, J. R., F. Li, A. Nayyar, J. Xiang, and X. Zhang. 2005. CD8 alpha+, but not CD8 alpha-, dendritic cells tolerize Th2 responses via contact-dependent and -independent mechanisms, and reverse airway hyperresponsiveness, Th2, and eosinophil responses in a mouse model of asthma. *J Immunol* 175:1516-1522.
57. Kopf, M., G. Le Gros, M. Bachmann, M. C. Lamers, H. Bluethmann, and G. Kohler. 1993. Disruption of the murine IL-4 gene blocks Th2 cytokine responses. *Nature* 362:245-248.
58. Coffman, R. L., B. W. Seymour, S. Hudak, J. Jackson, and D. Rennick. 1989. Antibody to interleukin-5 inhibits helminth-induced eosinophilia in mice. *Science* 245:308-310.
59. Zhu, J., B. Min, J. Hu-Li, C. J. Watson, A. Grinberg, Q. Wang, N. Killeen, J. F. Urban, Jr., L. Guo, and W. E. Paul. 2004. Conditional deletion of Gata3 shows its essential function in T(H)1-T(H)2 responses. *Nat Immunol* 5:1157-1165.
60. Zhu, J., J. Cote-Sierra, L. Guo, and W. E. Paul. 2003. Stat5 activation plays a critical role in Th2 differentiation. *Immunity* 19:739-748.
61. Yasumi, T., K. Katamura, I. Okafuji, T. Yoshioka, T. A. Meguro, R. Nishikomori, T. Kusunoki, T. Heike, and T. Nakahata. 2005. Limited ability of antigen-specific Th1 responses to inhibit Th2 cell development in vivo. *J Immunol* 174:1325-1331.
62. Fort, M. M., J. Cheung, D. Yen, J. Li, S. M. Zurawski, S. Lo, S. Menon, T. Clifford, B. Hunte, R. Lesley, T. Muchamuel, S. D. Hurst, G. Zurawski, M. W. Leach, D. M. Gorman, and D. M. Rennick. 2001. IL-25 induces IL-4, IL-5, and IL-13 and Th2-associated pathologies in vivo. *Immunity* 15:985-995.

63. Tamachi, T., Y. Maezawa, K. Ikeda, S. Kagami, M. Hatano, Y. Seto, A. Suto, K. Suzuki, N. Watanabe, Y. Saito, T. Tokuhisa, I. Iwamoto, and H. Nakajima. 2006. IL-25 enhances allergic airway inflammation by amplifying a TH2 cell-dependent pathway in mice. *J Allergy Clin Immunol* 118:606-614.

64. Helmby, H., and R. K. Grencis. 2004. Interleukin 1 plays a major role in the development of Th2-mediated immunity. *Eur J Immunol* 34:3674-3681.

65. Larche, M., C. A. Akdis, and R. Valenta. 2006. Immunological mechanisms of allergen-specific immunotherapy. *Nat Rev Immunol* 6:761-771.

66. Akdis, M., and C. A. Akdis. 2009. Therapeutic manipulation of immune tolerance in allergic disease. *Nat Rev Drug Discov* 8:645-660.

67. Lukacs, N. W., and D. L. Boros. 1993. Lymphokine regulation of granuloma formation in murine schistosomiasis mansoni. *Clin Immunol Immunopathol* 68:57-63.

68. Oswald, I. P., P. Caspar, D. Jankovic, T. A. Wynn, E. J. Pearce, and A. Sher. 1994. IL-12 inhibits Th2 cytokine responses induced by eggs of Schistosoma mansoni. *J Immunol* 153:1707-1713.

69. Boros, D. L., and J. R. Whitfield. 1999. Enhanced Th1 and dampened Th2 responses synergize to inhibit acute granulomatous and fibrotic responses in murine schistosomiasis mansoni. *Infect Immun* 67:1187-1193.

70. Chensue, S. W., K. S. Warmington, J. Ruth, P. M. Lincoln, and S. L. Kunkel. 1994. Cross-regulatory role of interferon-gamma (IFN-gamma), IL-4 and IL-10 in schistosome egg granuloma formation: in vivo regulation of Th activity and inflammation. *Clin Exp Immunol* 98:395-400.

71. Sher, A., D. Fiorentino, P. Caspar, E. Pearce, and T. Mosmann. 1991. Production of IL-10 by CD4+ T lymphocytes correlates with down-regulation of Th1 cytokine synthesis in helminth infection. *J Immunol* 147:2713-2716.

72. Akkoc, T., P. J. de Koning, B. Ruckert, I. Barlan, M. Akdis, and C. A. Akdis. 2008. Increased activation-induced cell death of high IFN-gamma-producing T(H)1 cells as a mechanism of T(H)2 predominance in atopic diseases. *J Allergy Clin Immunol* 121:652-658 e651.

73. McKee, A. S., and E. J. Pearce. 2004. CD25+CD4+ cells contribute to Th2 polarization during helminth infection by suppressing Th1 response development. *J Immunol* 173:1224-1231.

74. Erdmann, A. A., U. Jung, J. E. Foley, Y. Toda, and D. H. Fowler. 2004. Co-stimulated/Tc2 cells abrogate murine marrow graft rejection. *Biol Blood Marrow Transplant* 10:604-613.

75. Radhakrishnan, S., K. R. Wiehagen, V. Pulko, V. Van Keulen, W. A. Faubion, K. L. Knutson, and L. R. Pease. 2007. Induction of a Th1 Response from Th2-Polarized T Cells by Activated Dendritic Cells: Dependence on TCR:Peptide-MHC Interaction, ICAM-1, IL-12, and IFN-{gamma}. *J Immunol* 178:3583-3592.

76. Fernando, G. J., T. J. Stewart, R. W. Tindle, and I. H. Frazer. 1998. Th2-type CD4+ cells neither enhance nor suppress antitumor CTL activity in a mouse tumor model. *J Immunol* 161:2421-2427.

77. Doan, T., K. A. Herd, P. F. Lambert, G. J. Fernando, M. D. Street, and R. W. Tindle. 2000. Peripheral tolerance to human papillomavirus E7 oncoprotein occurs by cross-tolerization, is largely Th-2-independent, and is broken by dendritic cell immunization. *Cancer Res* 60:2810-2815.

78. Mattes, J., M. Hulett, W. Xie, S. Hogan, M. E. Rothenberg, P. Foster, and C. Parish. 2003. Immunotherapy of cytotoxic T cell-resistant tumors by T helper 2 cells: an eotaxin and STAT6-dependent process. *J Exp Med* 197:387-393.

79. Nishimura, T., K. Iwakabe, M. Sekimoto, Y. Ohmi, T. Yahata, M. Nakui, T. Sato, S. Habu, H. Tashiro, M. Sato, and A. Ohta. 1999. Distinct role of antigen-specific T helper type 1 (Th1) and Th2 cells in tumor eradication in vivo. *J Exp Med* 190:617-627.

80. Modesti, A., L. Masuelli, A. Modica, G. D'Orazi, S. Scarpa, M. C. Bosco, and G. Forni. 1993. Ultrastructural evidence of the mechanisms responsible for interleukin-4-activated rejection of a spontaneous murine adenocarcinoma. *Int J Cancer* 53:988-993.

81. Tepper, R. I., R. L. Coffman, and P. Leder. 1992. An eosinophil-dependent mechanism for the antitumor effect of interleukin-4. *Science* 257:548-551.

82. Pericle, F., M. Giovarelli, M. P. Colombo, G. Ferrari, P. Musiani, A. Modesti, F. Cavallo, F. Di Pierro, F. Novelli, and G. Forni. 1994. An efficient Th2-type memory follows

CD8+ lymphocyte-driven and eosinophil-mediated rejection of a spontaneous mouse mammary adenocarcinoma engineered to release IL-4. *J Immunol* 153:5659-5673.

83. Lebel-Binay, S., B. Laguerre, F. Quintin-Colonna, H. Conjeaud, M. Magazin, B. Miloux, F. Pecceu, D. Caput, P. Ferrara, and D. Fradelizi. 1995. Experimental gene therapy of cancer using tumor cells engineered to secrete interleukin-13. *Eur J Immunol* 25:2340-2348.

84. Ma, H. L., M. J. Whitters, B. A. Jacobson, D. D. Donaldson, M. Collins, and K. Dunussi-Joannopoulos. 2004. Tumor cells secreting IL-13 but not IL-13Ralpha2 fusion protein have reduced tumorigenicity in vivo. *Int Immunol* 16:1009-1017.

85. Akdis, M., J. Verhagen, A. Taylor, F. Karamloo, C. Karagiannidis, R. Crameri, S. Thunberg, G. Deniz, R. Valenta, H. Fiebig, C. Kegel, R. Disch, C. B. Schmidt-Weber, K. Blaser, and C. A. Akdis. 2004. Immune responses in healthy and allergic individuals are characterized by a fine balance between allergen-specific T regulatory 1 and T helper 2 cells. *J Exp Med* 199:1567-1575.

86. Duffy, D., C. P. Yang, A. Heath, P. Garside, and E. B. Bell. 2006. Naive T-cell receptor transgenic T cells help memory B cells produce antibody. *Immunology* 119:376-384.

87. Yu, D., and C. G. Vinuesa. 2010. The elusive identity of T follicular helper cells. *Trends Immunol* 31:377-383.

88. Vinuesa, C. G., S. G. Tangye, B. Moser, and C. R. Mackay. 2005. Follicular B helper T cells in antibody responses and autoimmunity. *Nat Rev Immunol* 5:853-865.

89. Ansel, K. M., L. J. McHeyzer-Williams, V. N. Ngo, M. G. McHeyzer-Williams, and J. G. Cyster. 1999. In vivo-activated CD4 T cells upregulate CXC chemokine receptor 5 and reprogram their response to lymphoid chemokines. *J Exp Med* 190:1123-1134.

90. Nurieva, R. I., Y. Chung, G. J. Martinez, X. O. Yang, S. Tanaka, T. D. Matskevitch, Y. H. Wang, and C. Dong. 2009. Bcl6 mediates the development of T follicular helper cells. *Science* 325:1001-1005.

91. Yu, D., S. Rao, L. M. Tsai, S. K. Lee, Y. He, E. L. Sutcliffe, M. Srivastava, M. Linterman, L. Zheng, N. Simpson, J. I. Ellyard, I. A. Parish, C. S. Ma, Q. J. Li, C. R. Parish, C. R. Mackay, and C. G. Vinuesa. 2009. The transcriptional repressor Bcl-6 directs T follicular helper cell lineage commitment. *Immunity* 31:457-468.

92. Johnston, R. J., A. C. Poholek, D. DiToro, I. Yusuf, D. Eto, B. Barnett, A. L. Dent, J. Craft, and S. Crotty. 2009. Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation. *Science* 325:1006-1010.

93. Linterman, M. A., L. Beaton, D. Yu, R. R. Ramiscal, M. Srivastava, J. J. Hogan, N. K. Verma, M. J. Smyth, R. J. Rigby, and C. G. Vinuesa. 2010. IL-21 acts directly on B cells to regulate Bcl-6 expression and germinal center responses. *J Exp Med* 207:353-363.

94. Yusuf, I., R. Kageyama, L. Monticelli, R. J. Johnston, D. Ditoro, K. Hansen, B. Barnett, and S. Crotty. 2010. Germinal center T follicular helper cell IL-4 production is dependent on signaling lymphocytic activation molecule receptor (CD150). *J Immunol* 185:190-202.

95. Bettelli, E., M. Oukka, and V. K. Kuchroo. 2007. T(H)-17 cells in the circle of immunity and autoimmunity. *Nat Immunol* 8:345-350.

96. Mathur, A. N., H. C. Chang, D. G. Zisoulis, G. L. Stritesky, Q. Yu, J. T. O'Malley, R. Kapur, D. E. Levy, G. S. Kansas, and M. H. Kaplan. 2007. Stat3 and Stat4 direct development of IL-17-secreting Th cells. *J Immunol* 178:4901-4907.

97. Kimura, A., T. Naka, and T. Kishimoto. 2007. IL-6-dependent and -independent pathways in the development of interleukin 17-producing T helper cells. *Proc Natl Acad Sci U S A* 104:12099-12104.

98. Batten, M., J. Li, S. Yi, N. M. Kljavin, D. M. Danilenko, S. Lucas, J. Lee, F. J. de Sauvage, and N. Ghilardi. 2006. Interleukin 27 limits autoimmune encephalomyelitis by suppressing the development of interleukin 17-producing T cells. *Nat Immunol* 7:929-936.

99. Volpe, E., N. Servant, R. Zollinger, S. I. Bogiatzi, P. Hupe, E. Barillot, and V. Soumelis. 2008. A critical function for transforming growth factor-beta, interleukin 23 and proinflammatory cytokines in driving and modulating human T(H)-17 responses. *Nat Immunol* 9:650-657.

100. Santarlasci, V., L. Maggi, M. Capone, F. Frosali, V. Querci, R. De Palma, F. Liotta, L. Cosmi, E. Maggi, S. Romagnani, and F. Annunziato. 2009. TGF-beta indirectly favors the development of human Th17 cells by inhibiting Th1 cells. *Eur J Immunol* 39:207-215.

101. Das, J., G. Ren, L. Zhang, A. I. Roberts, X. Zhao, A. L. Bothwell, L. Van Kaer, Y. Shi, and G. Das. 2009. Transforming growth factor beta is dispensable for the molecular orchestration of Th17 cell differentiation. *J Exp Med* 206:2407-2416.

102. Ankathatti Munegowda, M., Y. Deng, S. J. Mulligan, and J. Xiang. 2011. Th17 and Th17-stimulated CD8(+) T cells play a distinct role in Th17-induced preventive and therapeutic antitumor immunity. *Cancer Immunol Immunother* 60:1473-1484.

103. Laurence, A., C. M. Tato, T. S. Davidson, Y. Kanno, Z. Chen, Z. Yao, R. B. Blank, F. Meylan, R. Siegel, L. Hennighausen, E. M. Shevach, and J. O'Shea J. 2007. Interleukin-2 signaling via STAT5 constrains T helper 17 cell generation. *Immunity* 26:371-381.

104. Brustle, A., S. Heink, M. Huber, C. Rosenplanter, C. Stadelmann, P. Yu, E. Arpaia, T. W. Mak, T. Kamradt, and M. Lohoff. 2007. The development of inflammatory T(H)-17 cells requires interferon-regulatory factor 4. *Nat Immunol* 8:958-966.

105. Rangachari, M., N. Mauermann, R. R. Marty, S. Dirnhofer, M. O. Kurrer, V. Komnenovic, J. M. Penninger, and U. Eriksson. 2006. T-bet negatively regulates autoimmune myocarditis by suppressing local production of interleukin 17. *J Exp Med* 203:2009-2019.

106. Kimura, A., T. Naka, K. Nohara, Y. Fujii-Kuriyama, and T. Kishimoto. 2008. Aryl hydrocarbon receptor regulates Stat1 activation and participates in the development of Th17 cells. *Proc Natl Acad Sci U S A* 105:9721-9726.

107. Weaver, C. T., R. D. Hatton, P. R. Mangan, and L. E. Harrington. 2007. IL-17 family cytokines and the expanding diversity of effector T cell lineages. *Annu Rev Immunol* 25:821-852.

108. Nakae, S., Y. Iwakura, H. Suto, and S. J. Galli. 2007. Phenotypic differences between Th1 and Th17 cells and negative regulation of Th1 cell differentiation by IL-17. *J Leukoc Biol* 81:1258-1268.

109. Krakowski, M., and T. Owens. 1996. Interferon-gamma confers resistance to experimental allergic encephalomyelitis. *Eur J Immunol* 26:1641-1646.

110. Langrish, C. L., Y. Chen, W. M. Blumenschein, J. Mattson, B. Basham, J. D. Sedgwick, T. McClanahan, R. A. Kastelein, and D. J. Cua. 2005. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J Exp Med* 201:233-240.

111. Tzartos, J. S., M. A. Friese, M. J. Craner, J. Palace, J. Newcombe, M. M. Esiri, and L. Fugger. 2008. Interleukin-17 production in central nervous system-infiltrating T cells and glial cells is associated with active disease in multiple sclerosis. *Am J Pathol* 172:146-155.

112. Park, H., Z. Li, X. O. Yang, S. H. Chang, R. Nurieva, Y. H. Wang, Y. Wang, L. Hood, Z. Zhu, Q. Tian, and C. Dong. 2005. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat Immunol* 6:1133-1141.

113. Zhang, B., G. Rong, H. Wei, M. Zhang, J. Bi, L. Ma, X. Xue, G. Wei, X. Liu, and G. Fang. 2008. The prevalence of Th17 cells in patients with gastric cancer. *Biochem Biophys Res Commun* 374:533-537.

114. Sfanos, K. S., T. C. Bruno, C. H. Maris, L. Xu, C. J. Thoburn, A. M. DeMarzo, A. K. Meeker, W. B. Isaacs, and C. G. Drake. 2008. Phenotypic analysis of prostate-infiltrating lymphocytes reveals TH17 and Treg skewing. *Clin Cancer Res* 14:3254-3261.

115. Benchetrit, F., A. Ciree, V. Vives, G. Warnier, A. Gey, C. Sautes-Fridman, F. Fossiez, N. Haicheur, W. H. Fridman, and E. Tartour. 2002. Interleukin-17 inhibits tumor cell growth by means of a T-cell-dependent mechanism. *Blood* 99:2114-2121.

116. Hirahara, N., Y. Nio, S. Sasaki, Y. Minari, M. Takamura, C. Iguchi, M. Dong, K. Yamasawa, and K. Tamura. 2001. Inoculation of human interleukin-17 gene-transfected Meth-A fibrosarcoma cells induces T cell-dependent tumor-specific immunity in mice. *Oncology* 61:79-89.

117. Numasaki, M., M. Watanabe, T. Suzuki, H. Takahashi, A. Nakamura, F. McAllister, T. Hishinuma, J. Goto, M. T. Lotze, J. K. Kolls, and H. Sasaki. 2005. IL-17 enhances the net angiogenic activity and in vivo growth of human non-small cell lung cancer in SCID mice through promoting CXCR-2-dependent angiogenesis. *J Immunol* 175:6177-6189.

118. Numasaki, M., J. Fukushi, M. Ono, S. K. Narula, P. J. Zavodny, T. Kudo, P. D. Robbins, H. Tahara, and M. T. Lotze. 2003. Interleukin-17 promotes angiogenesis and tumor growth. *Blood* 101:2620-2627.

119. Spolski, R., and W. J. Leonard. 2008. Interleukin-21: basic biology and implications for cancer and autoimmunity. *Annu Rev Immunol* 26:57-79.

120. Kottke, T., L. Sanchez-Perez, R. M. Diaz, J. Thompson, H. Chong, K. Harrington, S. K. Calderwood, J. Pulido, N. Georgopoulos, P. Selby, A. Melcher, and R. Vile. 2007.

Induction of hsp70-mediated Th17 autoimmunity can be exploited as immunotherapy for metastatic prostate cancer. *Cancer Res* 67:11970-11979.

121. Kryczek, I., S. Wei, W. Szeliga, L. Vatan, and W. Zou. 2009. Endogenous IL-17 contributes to reduced tumor growth and metastasis. *Blood* 114:357-359.
122. Khader, S. A., G. K. Bell, J. E. Pearl, J. J. Fountain, J. Rangel-Moreno, G. E. Cilley, F. Shen, S. M. Eaton, S. L. Gaffen, S. L. Swain, R. M. Locksley, L. Haynes, T. D. Randall, and A. M. Cooper. 2007. IL-23 and IL-17 in the establishment of protective pulmonary CD4+ T cell responses after vaccination and during Mycobacterium tuberculosis challenge. *Nat Immunol* 8:369-377.
123. Sakaguchi, S., T. Yamaguchi, T. Nomura, and M. Ono. 2008. Regulatory T cells and immune tolerance. *Cell* 133:775-787.
124. Yamaguchi, T., and S. Sakaguchi. 2006. Regulatory T cells in immune surveillance and treatment of cancer. *Semin Cancer Biol* 16:115-123.
125. Bennett, C. L., J. Christie, F. Ramsdell, M. E. Brunkow, P. J. Ferguson, L. Whitesell, T. E. Kelly, F. T. Saulsbury, P. F. Chance, and H. D. Ochs. 2001. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat Genet* 27:20-21.
126. Brunkow, M. E., E. W. Jeffery, K. A. Hjerrild, B. Paeper, L. B. Clark, S. A. Yasayko, J. E. Wilkinson, D. Galas, S. F. Ziegler, and F. Ramsdell. 2001. Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nat Genet* 27:68-73.
127. Sakaguchi, S., and F. Powrie. 2007. Emerging challenges in regulatory T cell function and biology. *Science* 317:627-629.
128. Chen, X., S. Vodanovic-Jankovic, B. Johnson, M. Keller, R. Komorowski, and W. R. Drobyski. 2007. Absence of regulatory T-cell control of TH1 and TH17 cells is responsible for the autoimmune-mediated pathology in chronic graft-versus-host disease. *Blood* 110:3804-3813.
129. Pacholczyk, R., H. Ignatowicz, P. Kraj, and L. Ignatowicz. 2006. Origin and T cell receptor diversity of Foxp3+CD4+CD25+ T cells. *Immunity* 25:249-259.

130. Zhou, J., T. Ding, W. Pan, L. Y. Zhu, L. Li, and L. Zheng. 2009. Increased intratumoral regulatory T cells are related to intratumoral macrophages and poor prognosis in hepatocellular carcinoma patients. *Int J Cancer* 125:1640-1648.

131. Sakaguchi, S., N. Sakaguchi, J. Shimizu, S. Yamazaki, T. Sakihama, M. Itoh, Y. Kuniyasu, T. Nomura, M. Toda, and T. Takahashi. 2001. Immunologic tolerance maintained by CD25+ CD4+ regulatory T cells: their common role in controlling autoimmunity, tumor immunity, and transplantation tolerance. *Immunol Rev* 182:18-32.

132. Ziegler, S. F. 2006. FOXP3: of mice and men. *Annu Rev Immunol* 24:209-226.

133. Lin, W., D. Haribhai, L. M. Relland, N. Truong, M. R. Carlson, C. B. Williams, and T. A. Chatila. 2007. Regulatory T cell development in the absence of functional Foxp3. *Nat Immunol* 8:359-368.

134. Feuerer, M., J. A. Hill, D. Mathis, and C. Benoist. 2009. Foxp3+ regulatory T cells: differentiation, specification, subphenotypes. *Nat Immunol* 10:689-695.

135. Gregori, S., D. Tomasoni, V. Pacciani, M. Scirpoli, M. Battaglia, C. F. Magnani, E. Hauben, and M. G. Roncarolo. 2010. Differentiation of type 1 T regulatory cells (Tr1) by tolerogenic DC-10 requires the IL-10-dependent ILT4/HLA-G pathway. *Blood* 116:935-944.

136. Ivanov, II, B. S. McKenzie, L. Zhou, C. E. Tadokoro, A. Lepelley, J. J. Lafaille, D. J. Cua, and D. R. Littman. 2006. The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. *Cell* 126:1121-1133.

137. Manel, N., D. Unutmaz, and D. R. Littman. 2008. The differentiation of human T(H)-17 cells requires transforming growth factor-beta and induction of the nuclear receptor RORgammat. *Nat Immunol* 9:641-649.

138. Hori, S., T. Nomura, and S. Sakaguchi. 2003. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 299:1057-1061.

139. Dardalhon, V., A. Awasthi, H. Kwon, G. Galileos, W. Gao, R. A. Sobel, M. Mitsdoerffer, T. B. Strom, W. Elyaman, I. C. Ho, S. Khoury, M. Oukka, and V. K. Kuchroo. 2008. IL-4 inhibits TGF-beta-induced Foxp3+ T cells and, together with TGF-beta, generates IL-9+ IL-10+ Foxp3(-) effector T cells. *Nat Immunol* 9:1347-1355.

140. Gessner, A., H. Blum, and M. Rollinghoff. 1993. Differential regulation of IL-9-expression after infection with Leishmania major in susceptible and resistant mice. *Immunobiology* 189:419-435.

141. Erpenbeck, V. J., J. M. Hohlfeld, M. Discher, H. Krentel, A. Hagenberg, A. Braun, and N. Krug. 2003. Increased expression of interleukin-9 messenger RNA after segmental allergen challenge in allergic asthmatics. *Chest* 123:370S.

142. Temann, U. A., G. P. Geba, J. A. Rankin, and R. A. Flavell. 1998. Expression of interleukin 9 in the lungs of transgenic mice causes airway inflammation, mast cell hyperplasia, and bronchial hyperresponsiveness. *J Exp Med* 188:1307-1320.

143. Wu, B., C. Huang, M. Kato-Maeda, P. C. Hopewell, C. L. Daley, A. M. Krensky, and C. Clayberger. 2008. IL-9 is associated with an impaired Th1 immune response in patients with tuberculosis. *Clin Immunol* 126:202-210.

144. Veldhoen, M., C. Uyttenhove, J. van Snick, H. Helmby, A. Westendorf, J. Buer, B. Martin, C. Wilhelm, and B. Stockinger. 2008. Transforming growth factor-beta 'reprograms' the differentiation of T helper 2 cells and promotes an interleukin 9-producing subset. *Nat Immunol* 9:1341-1346.

145. Chang, H. C., S. Sehra, R. Goswami, W. Yao, Q. Yu, G. L. Stritesky, R. Jabeen, C. McKinley, A. N. Ahyi, L. Han, E. T. Nguyen, M. J. Robertson, N. B. Perumal, R. S. Tepper, S. L. Nutt, and M. H. Kaplan. 2010. The transcription factor PU.1 is required for the development of IL-9-producing T cells and allergic inflammation. *Nat Immunol* 11:527-534.

146. Elyaman, W., E. M. Bradshaw, C. Uyttenhove, V. Dardalhon, A. Awasthi, J. Imitola, E. Bettelli, M. Oukka, J. van Snick, J. C. Renauld, V. K. Kuchroo, and S. J. Khoury. 2009. IL-9 induces differentiation of TH17 cells and enhances function of FoxP3+ natural regulatory T cells. *Proc Natl Acad Sci U S A* 106:12885-12890.

147. Nowak, E. C., C. T. Weaver, H. Turner, S. Begum-Haque, B. Becher, B. Schreiner, A. J. Coyle, L. H. Kasper, and R. J. Noelle. 2009. IL-9 as a mediator of Th17-driven inflammatory disease. *J Exp Med* 206:1653-1660.

148. Acosta-Rodriguez, E. V., L. Rivino, J. Geginat, D. Jarrossay, M. Gattorno, A. Lanzavecchia, F. Sallusto, and G. Napolitani. 2007. Surface phenotype and antigenic

specificity of human interleukin 17-producing T helper memory cells. *Nat Immunol* 8:639-646.

149. Dumoutier, L., E. Van Roost, G. Ameye, L. Michaux, and J. C. Renauld. 2000. IL-TIF/IL-22: genomic organization and mapping of the human and mouse genes. *Genes Immun* 1:488-494.

150. Trifari, S., C. D. Kaplan, E. H. Tran, N. K. Crellin, and H. Spits. 2009. Identification of a human helper T cell population that has abundant production of interleukin 22 and is distinct from T(H)-17, T(H)1 and T(H)2 cells. *Nat Immunol* 10:864-871.

151. Duhen, T., R. Geiger, D. Jarrossay, A. Lanzavecchia, and F. Sallusto. 2009. Production of interleukin 22 but not interleukin 17 by a subset of human skin-homing memory T cells. *Nat Immunol* 10:857-863.

152. Witte, E., K. Witte, K. Warszawska, R. Sabat, and K. Wolk. 2010. Interleukin-22: a cytokine produced by T, NK and NKT cell subsets, with importance in the innate immune defense and tissue protection. *Cytokine Growth Factor Rev* 21:365-379.

153. Veldhoen, M., K. Hirota, A. M. Westendorf, J. Buer, L. Dumoutier, J. C. Renauld, and B. Stockinger. 2008. The aryl hydrocarbon receptor links TH17-cell-mediated autoimmunity to environmental toxins. *Nature* 453:106-109.

154. Quintana, F. J., A. S. Basso, A. H. Iglesias, T. Korn, M. F. Farez, E. Bettelli, M. Caccamo, M. Oukka, and H. L. Weiner. 2008. Control of T(reg) and T(H)17 cell differentiation by the aryl hydrocarbon receptor. *Nature* 453:65-71.

155. Fujita, H., K. E. Nograles, T. Kikuchi, J. Gonzalez, J. A. Carucci, and J. G. Krueger. 2009. Human Langerhans cells induce distinct IL-22-producing CD4+ T cells lacking IL-17 production. *Proc Natl Acad Sci U S A* 106:21795-21800.

156. Kagami, S., H. L. Rizzo, J. J. Lee, Y. Koguchi, and A. Blauvelt. 2010. Circulating Th17, Th22, and Th1 cells are increased in psoriasis. *J Invest Dermatol* 130:1373-1383.

157. Wolk, K., E. Witte, E. Wallace, W. D. Docke, S. Kunz, K. Asadullah, H. D. Volk, W. Sterry, and R. Sabat. 2006. IL-22 regulates the expression of genes responsible for antimicrobial defense, cellular differentiation, and mobility in keratinocytes: a potential role in psoriasis. *Eur J Immunol* 36:1309-1323.

158. Ikeuchi, H., T. Kuroiwa, N. Hiramatsu, Y. Kaneko, K. Hiromura, K. Ueki, and Y. Nojima. 2005. Expression of interleukin-22 in rheumatoid arthritis: potential role as a proinflammatory cytokine. *Arthritis Rheum* 52:1037-1046.

159. Geboes, L., L. Dumoutier, H. Kelchtermans, E. Schurgers, T. Mitera, J. C. Renauld, and P. Matthys. 2009. Proinflammatory role of the Th17 cytokine interleukin-22 in collagen-induced arthritis in C57BL/6 mice. *Arthritis Rheum* 60:390-395.

160. Harrington, L. E., K. M. Janowski, J. R. Oliver, A. J. Zajac, and C. T. Weaver. 2008. Memory CD4 T cells emerge from effector T-cell progenitors. *Nature* 452:356-360.

161. Hegazy, A. N., M. Peine, C. Helmstetter, I. Panse, A. Frohlich, A. Bergthaler, L. Flatz, D. D. Pinschewer, A. Radbruch, and M. Lohning. 2010. Interferons direct Th2 cell reprogramming to generate a stable GATA-3(+)T-bet(+) cell subset with combined Th2 and Th1 cell functions. *Immunity* 32:116-128.

162. Murphy, E., K. Shibuya, N. Hosken, P. Openshaw, V. Maino, K. Davis, K. Murphy, and A. O'Garra. 1996. Reversibility of T helper 1 and 2 populations is lost after long-term stimulation. *J Exp Med* 183:901-913.

163. Wei, G., L. Wei, J. Zhu, C. Zang, J. Hu-Li, Z. Yao, K. Cui, Y. Kanno, T. Y. Roh, W. T. Watford, D. E. Schones, W. Peng, H. W. Sun, W. E. Paul, J. J. O'Shea, and K. Zhao. 2009. Global mapping of H3K4me3 and H3K27me3 reveals specificity and plasticity in lineage fate determination of differentiating CD4+ T cells. *Immunity* 30:155-167.

164. Lee, Y. K., H. Turner, C. L. Maynard, J. R. Oliver, D. Chen, C. O. Elson, and C. T. Weaver. 2009. Late developmental plasticity in the T helper 17 lineage. *Immunity* 30:92-107.

165. Martin-Orozco, N., Y. Chung, S. H. Chang, Y. H. Wang, and C. Dong. 2009. Th17 cells promote pancreatic inflammation but only induce diabetes efficiently in lymphopenic hosts after conversion into Th1 cells. *Eur J Immunol* 39:216-224.

166. Martin-Orozco, N., P. Muranski, Y. Chung, X. O. Yang, T. Yamazaki, S. Lu, P. Hwu, N. P. Restifo, W. W. Overwijk, and C. Dong. 2009. T helper 17 cells promote cytotoxic T cell activation in tumor immunity. *Immunity* 31:787-798.

167. Nurieva, R., X. O. Yang, Y. Chung, and C. Dong. 2009. Cutting edge: in vitro generated Th17 cells maintain their cytokine expression program in normal but not lymphopenic hosts. *J Immunol* 182:2565-2568.

168. Sharma, M. D., D. Y. Hou, Y. Liu, P. A. Koni, R. Metz, P. Chandler, A. L. Mellor, Y. He, and D. H. Munn. 2009. Indoleamine 2,3-dioxygenase controls conversion of Foxp3+ Tregs to TH17-like cells in tumor-draining lymph nodes. *Blood* 113:6102-6111.

169. Wan, Y. Y., and R. A. Flavell. 2007. Regulatory T-cell functions are subverted and converted owing to attenuated Foxp3 expression. *Nature* 445:766-770.

170. Xu, L., A. Kitani, I. Fuss, and W. Strober. 2007. Cutting edge: regulatory T cells induce CD4+CD25-Foxp3- T cells or are self-induced to become Th17 cells in the absence of exogenous TGF-beta. *J Immunol* 178:6725-6729.

171. Yang, X. O., R. Nurieva, G. J. Martinez, H. S. Kang, Y. Chung, B. P. Pappu, B. Shah, S. H. Chang, K. S. Schluns, S. S. Watowich, X. H. Feng, A. M. Jetten, and C. Dong. 2008. Molecular antagonism and plasticity of regulatory and inflammatory T cell programs. *Immunity* 29:44-56.

172. Komatsu, N., M. E. Mariotti-Ferrandiz, Y. Wang, B. Malissen, H. Waldmann, and S. Hori. 2009. Heterogeneity of natural Foxp3+ T cells: a committed regulatory T-cell lineage and an uncommitted minor population retaining plasticity. *Proc Natl Acad Sci U S A* 106:1903-1908.

173. Lochner, M., L. Peduto, M. Cherrier, S. Sawa, F. Langa, R. Varona, D. Riethmacher, M. Si-Tahar, J. P. Di Santo, and G. Eberl. 2008. In vivo equilibrium of proinflammatory IL-17+ and regulatory IL-10+ Foxp3+ RORgamma t+ T cells. *J Exp Med* 205:1381-1393.

174. Tartar, D. M., A. M. VanMorlan, X. Wan, F. B. Guloglu, R. Jain, C. L. Haymaker, J. S. Ellis, C. M. Hoeman, J. A. Cascio, M. Dhakal, M. Oukka, and H. Zaghouani. FoxP3+RORgammat+ T helper intermediates display suppressive function against autoimmune diabetes. *J Immunol* 184:3377-3385.

175. Torchinsky, M. B., J. Garaude, A. P. Martin, and J. M. Blander. 2009. Innate immune recognition of infected apoptotic cells directs T(H)17 cell differentiation. *Nature* 458:78-82.

176. Martin-Fontecha, A., L. L. Thomsen, S. Brett, C. Gerard, M. Lipp, A. Lanzavecchia, and F. Sallusto. 2004. Induced recruitment of NK cells to lymph nodes provides IFN-gamma for T(H)1 priming. *Nat Immunol* 5:1260-1265.

177. Hwang, E. S., S. J. Szabo, P. L. Schwartzberg, and L. H. Glimcher. 2005. T helper cell fate specified by kinase-mediated interaction of T-bet with GATA-3. *Science* 307:430-433.

178. Zhou, L., J. E. Lopes, M. M. Chong, Ivanov, II, R. Min, G. D. Victora, Y. Shen, J. Du, Y. P. Rubtsov, A. Y. Rudensky, S. F. Ziegler, and D. R. Littman. 2008. TGF-beta-induced Foxp3 inhibits T(H)17 cell differentiation by antagonizing RORgammat function. *Nature* 453:236-240.

179. Joly, E., and D. Hudrisier. 2003. What is trogocytosis and what is its purpose? *Nat Immunol* 4:815.

180. Davis, D. M. 2007. Intercellular transfer of cell-surface proteins is common and can affect many stages of an immune response. *Nat Rev Immunol* 7:238-243.

181. Huang, J. F., Y. Yang, H. Sepulveda, W. Shi, I. Hwang, P. A. Peterson, M. R. Jackson, J. Sprent, and Z. Cai. 1999. TCR-Mediated internalization of peptide-MHC complexes acquired by T cells. *Science* 286:952-954.

182. Tatari-Calderone, Z., R. T. Semnani, T. B. Nutman, J. Schlom, and H. Sabzevari. 2002. Acquisition of CD80 by human T cells at early stages of activation: functional involvement of CD80 acquisition in T cell to T cell interaction. *J Immunol* 169:6162-6169.

183. Brezinschek, R. I., N. Oppenheimer-Marks, and P. E. Lipsky. 1999. Activated T cells acquire endothelial cell surface determinants during transendothelial migration. *J Immunol* 162:1677-1684.

184. He, T., C. Tang, Y. Liu, Z. Ye, X. Wu, Y. Wei, T. Moyana, and J. Xiang. 2007. Bidirectional membrane molecule transfer between dendritic and T cells. *Biochem Biophys Res Commun* 359:202-208.

185. Sjostrom, A., M. Eriksson, C. Cerboni, M. H. Johansson, C. L. Sentman, K. Karre, and P. Hoglund. 2001. Acquisition of external major histocompatibility complex class I molecules by natural killer cells expressing inhibitory Ly49 receptors. *J Exp Med* 194:1519-1530.

186. Fuchs, A., M. Cella, E. Giurisato, A. S. Shaw, and M. Colonna. 2004. Cutting edge: CD96 (tactile) promotes NK cell-target cell adhesion by interacting with the poliovirus receptor (CD155). *J Immunol* 172:3994-3998.

187. Tabiasco, J., E. Espinosa, D. Hudrisier, E. Joly, J. J. Fournie, and A. Vercellone. 2002. Active trans-synaptic capture of membrane fragments by natural killer cells. *Eur J Immunol* 32:1502-1508.

188. Batista, F. D., D. Iber, and M. S. Neuberger. 2001. B cells acquire antigen from target cells after synapse formation. *Nature* 411:489-494.

189. Espinosa, E., J. Tabiasco, D. Hudrisier, and J. J. Fournie. 2002. Synaptic transfer by human gamma delta T cells stimulated with soluble or cellular antigens. *J Immunol* 168:6336-6343.

190. Ahmed, K. A., M. A. Munegowda, Y. Xie, and J. Xiang. 2008. Intercellular trogocytosis plays an important role in modulation of immune responses. *Cell Mol Immunol* 5:261-269.

191. Davis, M. M., J. J. Boniface, Z. Reich, D. Lyons, J. Hampl, B. Arden, and Y. Chien. 1998. Ligand recognition by alpha beta T cell receptors. *Annu Rev Immunol* 16:523-544.

192. Grakoui, A., S. K. Bromley, C. Sumen, M. M. Davis, A. S. Shaw, P. M. Allen, and M. L. Dustin. 1999. The immunological synapse: a molecular machine controlling T cell activation. *Science* 285:221-227.

193. Viola, A., S. Schroeder, Y. Sakakibara, and A. Lanzavecchia. 1999. T lymphocyte costimulation mediated by reorganization of membrane microdomains. *Science* 283:680-682.

194. Cai, Z., H. Kishimoto, A. Brunmark, M. R. Jackson, P. A. Peterson, and J. Sprent. 1997. Requirements for peptide-induced T cell receptor downregulation on naive CD8+ T cells. *J Exp Med* 185:641-651.

195. Puaux, A. L., J. Campanaud, A. Salles, X. Preville, B. Timmerman, E. Joly, and D. Hudrisier. 2006. A very rapid and simple assay based on trogocytosis to detect and measure specific T and B cell reactivity by flow cytometry. *Eur J Immunol* 36:779-788.

196. Patel, D. M., and M. D. Mannie. 2001. Intercellular exchange of class II major histocompatibility complex/peptide complexes is a conserved process that requires activation of T cells but is constitutive in other types of antigen presenting cell. *Cell Immunol* 214:165-172.

197. Wetzel, S. A., T. W. McKeithan, and D. C. Parker. 2005. Peptide-specific intercellular transfer of MHC class II to CD4+ T cells directly from the immunological synapse upon cellular dissociation. *J Immunol* 174:80-89.

198. Underhill, D. M., M. Bassetti, A. Rudensky, and A. Aderem. 1999. Dynamic interactions of macrophages with T cells during antigen presentation. *J Exp Med* 190:1909-1914.

199. Gunzer, M., A. Schafer, S. Borgmann, S. Grabbe, K. S. Zanker, E. B. Brocker, E. Kampgen, and P. Friedl. 2000. Antigen presentation in extracellular matrix: interactions of T cells with dendritic cells are dynamic, short lived, and sequential. *Immunity* 13:323-332.

200. Morgan, B. P., J. R. Dankert, and A. F. Esser. 1987. Recovery of human neutrophils from complement attack: removal of the membrane attack complex by endocytosis and exocytosis. *J Immunol* 138:246-253.

201. Lorber, M. I., M. R. Loken, A. M. Stall, and F. W. Fitch. 1982. I-A antigens on cloned alloreactive murine T lymphocytes are acquired passively. *J Immunol* 128:2798-2803.

202. Yu, D. T., J. M. McCune, S. M. Fu, R. J. Winchester, and H. G. Kunkel. 1980. Two types of Ia-positive T cells. Synthesis and exchange of Ia antigens. *J Exp Med* 152:89s-98s.

203. Stinchcombe, J. C., G. Bossi, S. Booth, and G. M. Griffiths. 2001. The immunological synapse of CTL contains a secretory domain and membrane bridges. *Immunity* 15:751-761.

204. Onfelt, B., S. Nedvetzki, R. K. Benninger, M. A. Purbhoo, S. Sowinski, A. N. Hume, M. C. Seabra, M. A. Neil, P. M. French, and D. M. Davis. 2006. Structurally distinct membrane nanotubes between human macrophages support long-distance vesicular traffic or surfing of bacteria. *J Immunol* 177:8476-8483.

205. Onfelt, B., S. Nedvetzki, K. Yanagi, and D. M. Davis. 2004. Cutting edge: Membrane nanotubes connect immune cells. *J Immunol* 173:1511-1513.

206. Sowinski, S., C. Jolly, O. Berninghausen, M. A. Purbhoo, A. Chauveau, K. Kohler, S. Oddos, P. Eissmann, F. M. Brodsky, C. Hopkins, B. Onfelt, Q. Sattentau, and D. M. Davis. 2008. Membrane nanotubes physically connect T cells over long distances presenting a novel route for HIV-1 transmission. *Nat Cell Biol* 10:211-219.

207. Seder, R. A., and W. E. Paul. 1994. Acquisition of lymphokine-producing phenotype by CD4+ T cells. *Annu Rev Immunol* 12:635-673.

208. Yoshimoto, T., and W. E. Paul. 1994. CD4pos, NK1.1pos T cells promptly produce interleukin 4 in response to in vivo challenge with anti-CD3. *J Exp Med* 179:1285-1295.

209. Ferrick, D. A., M. D. Schrenzel, T. Mulvania, B. Hsieh, W. G. Ferlin, and H. Lepper. 1995. Differential production of interferon-gamma and interleukin-4 in response to Th1- and Th2-stimulating pathogens by gamma delta T cells in vivo. *Nature* 373:255-257.

210. Cronin, D. C., 2nd, R. Stack, and F. W. Fitch. 1995. IL-4-producing CD8+ T cell clones can provide B cell help. *J Immunol* 154:3118-3127.

211. Lardon, F., H. W. Snoeck, Z. N. Berneman, V. F. Van Tendeloo, G. Nijs, M. Lenjou, E. Henckaerts, C. J. Boeckxtaens, P. Vandenabeele, L. L. Kestens, D. R. Van Bockstaele, and G. L. Vanham. 1997. Generation of dendritic cells from bone marrow progenitors using GM-CSF, TNF-alpha, and additional cytokines: antagonistic effects of IL-4 and IFN-gamma and selective involvement of TNF-alpha receptor-1. *Immunology* 91:553-559.

212. Russell, S. M., A. D. Keegan, N. Harada, Y. Nakamura, M. Noguchi, P. Leland, M. C. Friedmann, A. Miyajima, R. K. Puri, W. E. Paul, and et al. 1993. Interleukin-2 receptor gamma chain: a functional component of the interleukin-4 receptor. *Science* 262:1880-1883.

213. Zheng, W., and R. A. Flavell. 1997. The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. *Cell* 89:587-596.

214. Goenka, S., J. Youn, L. M. Dzurek, U. Schindler, L. Y. Yu-Lee, and M. Boothby. 1999. Paired Stat6 C-terminal transcription activation domains required both for inhibition of an IFN-responsive promoter and trans-activation. *J Immunol* 163:4663-4672.

215. Ouyang, W., S. H. Ranganath, K. Weindel, D. Bhattacharya, T. L. Murphy, W. C. Sha, and K. M. Murphy. 1998. Inhibition of Th1 development mediated by GATA-3 through an IL-4-independent mechanism. *Immunity* 9:745-755.

216. Elser, B., M. Lohoff, S. Kock, M. Giaisi, S. Kirchhoff, P. H. Krammer, and M. Li-Weber. 2002. IFN-gamma represses IL-4 expression via IRF-1 and IRF-2. *Immunity* 17:703-712.

217. Coffman, R. L., J. Ohara, M. W. Bond, J. Carty, A. Zlotnik, and W. E. Paul. 1986. B cell stimulatory factor-1 enhances the IgE response of lipopolysaccharide-activated B cells. *J Immunol* 136:4538-4541.

218. Vitetta, E. S., J. Ohara, C. D. Myers, J. E. Layton, P. H. Krammer, and W. E. Paul. 1985. Serological, biochemical, and functional identity of B cell-stimulatory factor 1 and B cell differentiation factor for IgG1. *J Exp Med* 162:1726-1731.

219. Gascan, H., J. F. Gauchat, M. G. Roncarolo, H. Yssel, H. Spits, and J. E. de Vries. 1991. Human B cell clones can be induced to proliferate and to switch to IgE and IgG4 synthesis by interleukin 4 and a signal provided by activated CD4+ T cell clones. *J Exp Med* 173:747-750.

220. Howard, M., J. Farrar, M. Hilfiker, B. Johnson, K. Takatsu, T. Hamaoka, and W. E. Paul. 1982. Identification of a T cell-derived b cell growth factor distinct from interleukin 2. *J Exp Med* 155:914-923.

221. Noelle, R., P. H. Krammer, J. Ohara, J. W. Uhr, and E. S. Vitetta. 1984. Increased expression of Ia antigens on resting B cells: an additional role for B-cell growth factor. *Proc Natl Acad Sci U S A* 81:6149-6153.

222. Thornhill, M. H., S. M. Wellicome, D. L. Mahiouz, J. S. Lanchbury, U. Kyan-Aung, and D. O. Haskard. 1991. Tumor necrosis factor combines with IL-4 or IFN-gamma to selectively enhance endothelial cell adhesiveness for T cells. The contribution of vascular cell adhesion molecule-1-dependent and -independent binding mechanisms. *J Immunol* 146:592-598.

223. Bennett, B. L., R. Cruz, R. G. Lacson, and A. M. Manning. 1997. Interleukin-4 suppression of tumor necrosis factor alpha-stimulated E-selectin gene transcription is mediated by STAT6 antagonism of NF-kappaB. *J Biol Chem* 272:10212-10219.

224. Kishimoto, T. 2005. Interleukin-6: from basic science to medicine--40 years in immunology. *Annu Rev Immunol* 23:1-21.

225. Hirano, T., K. Yasukawa, H. Harada, T. Taga, Y. Watanabe, T. Matsuda, S. Kashiwamura, K. Nakajima, K. Koyama, A. Iwamatsu, and et al. 1986. Complementary DNA for a novel human interleukin (BSF-2) that induces B lymphocytes to produce immunoglobulin. *Nature* 324:73-76.

226. Hirano, T., T. Matsuda, M. Turner, N. Miyasaka, G. Buchan, B. Tang, K. Sato, M. Shimizu, R. Maini, M. Feldmann, and et al. 1988. Excessive production of interleukin 6/B cell stimulatory factor-2 in rheumatoid arthritis. *Eur J Immunol* 18:1797-1801.

227. Hibi, M., M. Murakami, M. Saito, T. Hirano, T. Taga, and T. Kishimoto. 1990. Molecular cloning and expression of an IL-6 signal transducer, gp130. *Cell* 63:1149-1157.

228. Taga, T., M. Hibi, Y. Hirata, K. Yamasaki, K. Yasukawa, T. Matsuda, T. Hirano, and T. Kishimoto. 1989. Interleukin-6 triggers the association of its receptor with a possible signal transducer, gp130. *Cell* 58:573-581.

229. Taga, T., and T. Kishimoto. 1992. Cytokine receptors and signal transduction. *FASEB J* 6:3387-3396.

230. Kishimoto, T. 2010. IL-6: from its discovery to clinical applications. *Int Immunol* 22:347-352.

231. Jones, S. A. 2005. Directing transition from innate to acquired immunity: defining a role for IL-6. *J Immunol* 175:3463-3468.

232. Zhong, Z., Z. Wen, and J. E. Darnell, Jr. 1994. Stat3: a STAT family member activated by tyrosine phosphorylation in response to epidermal growth factor and interleukin-6. *Science* 264:95-98.

233. Yang, Y., J. Ochando, A. Yopp, J. S. Bromberg, and Y. Ding. 2005. IL-6 plays a unique role in initiating c-Maf expression during early stage of CD4 T cell activation. *J Immunol* 174:2720-2729.

234. Diehl, S., and M. Rincon. 2002. The two faces of IL-6 on Th1/Th2 differentiation. *Mol Immunol* 39:531-536.

235. Suematsu, S., T. Matsuda, K. Aozasa, S. Akira, N. Nakano, S. Ohno, J. Miyazaki, K. Yamamura, T. Hirano, and T. Kishimoto. 1989. IgG1 plasmacytosis in interleukin 6 transgenic mice. *Proc Natl Acad Sci U S A* 86:7547-7551.

236. Kopf, M., S. Herren, M. V. Wiles, M. B. Pepys, and M. H. Kosco-Vilbois. 1998. Interleukin 6 influences germinal center development and antibody production via a contribution of C3 complement component. *J Exp Med* 188:1895-1906.

237. Kimura, A., and T. Kishimoto. 2010. IL-6: regulator of Treg/Th17 balance. *Eur J Immunol* 40:1830-1835.

238. Fiorentino, D. F., M. W. Bond, and T. R. Mosmann. 1989. Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. *J Exp Med* 170:2081-2095.

239. Couper, K. N., D. G. Blount, and E. M. Riley. 2008. IL-10: the master regulator of immunity to infection. *J Immunol* 180:5771-5777.

240. Wehinger, J., F. Gouilleux, B. Groner, J. Finke, R. Mertelsmann, and R. M. Weber-Nordt. 1996. IL-10 induces DNA binding activity of three STAT proteins (Stat1, Stat3, and Stat5) and their distinct combinatorial assembly in the promoters of selected genes. *FEBS Lett* 394:365-370.

241. Moore, K. W., R. de Waal Malefyt, R. L. Coffman, and A. O'Garra. 2001. Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol* 19:683-765.

242. Demangel, C., P. Bertolino, and W. J. Britton. 2002. Autocrine IL-10 impairs dendritic cell (DC)-derived immune responses to mycobacterial infection by suppressing DC trafficking to draining lymph nodes and local IL-12 production. *Eur J Immunol* 32:994-1002.

243. Huang, H., W. Dawicki, X. Zhang, J. Town, and J. R. Gordon. 2010. Tolerogenic dendritic cells induce CD4+CD25hiFoxp3+ regulatory T cell differentiation from CD4+CD25-/loFoxp3- effector T cells. *J Immunol* 185:5003-5010.

244. Levy, Y., and J. C. Brouet. 1994. Interleukin-10 prevents spontaneous death of germinal center B cells by induction of the bcl-2 protein. *J Clin Invest* 93:424-428.

245. Kuhn, R., J. Lohler, D. Rennick, K. Rajewsky, and W. Muller. 1993. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell* 75:263-274.

246. Yang, X., J. Gartner, L. Zhu, S. Wang, and R. C. Brunham. 1999. IL-10 gene knockout mice show enhanced Th1-like protective immunity and absent granuloma formation following Chlamydia trachomatis lung infection. *J Immunol* 162:1010-1017.

247. O'Quinn, D. B., M. T. Palmer, Y. K. Lee, and C. T. Weaver. 2008. Emergence of the Th17 pathway and its role in host defense. *Adv Immunol* 99:115-163.

248. Onishi, R. M., and S. L. Gaffen. 2010. Interleukin-17 and its target genes: mechanisms of interleukin-17 function in disease. *Immunology* 129:311-321.

249. Kolls, J. K., and A. Linden. 2004. Interleukin-17 family members and inflammation. *Immunity* 21:467-476.

250. Gaffen, S. L. 2009. Structure and signalling in the IL-17 receptor family. *Nat Rev Immunol* 9:556-567.

251. Yao, Z., M. K. Spriggs, J. M. Derry, L. Strockbine, L. S. Park, T. VandenBos, J. D. Zappone, S. L. Painter, and R. J. Armitage. 1997. Molecular characterization of the human interleukin (IL)-17 receptor. *Cytokine* 9:794-800.

252. Moseley, T. A., D. R. Haudenschild, L. Rose, and A. H. Reddi. 2003. Interleukin-17 family and IL-17 receptors. *Cytokine Growth Factor Rev* 14:155-174.

253. Ye, P., P. B. Garvey, P. Zhang, S. Nelson, G. Bagby, W. R. Summer, P. Schwarzenberger, J. E. Shellito, and J. K. Kolls. 2001. Interleukin-17 and lung host defense against Klebsiella pneumoniae infection. *Am J Respir Cell Mol Biol* 25:335-340.

254. Takahashi, H., M. Numasaki, M. T. Lotze, and H. Sasaki. 2005. Interleukin-17 enhances bFGF-, HGF- and VEGF-induced growth of vascular endothelial cells. *Immunol Lett* 98:189-193.

255. Xiang, J., H. Huang, and Y. Liu. 2005. A new dynamic model of CD8+ T effector cell responses via CD4+ T helper-antigen-presenting cells. *J Immunol* 174:7497-7505.

256. Keene, J. A., and J. Forman. 1982. Helper activity is required for the in vivo generation of cytotoxic T lymphocytes. *J Exp Med* 155:768-782.

257. Bousso, P., and E. Robey. 2003. Dynamics of CD8+ T cell priming by dendritic cells in intact lymph nodes. *Nat Immunol* 4:579-585.

258. Ridge, J. P., F. Di Rosa, and P. Matzinger. 1998. A conditioned dendritic cell can be a temporal bridge between a CD4+ T-helper and a T-killer cell. *Nature* 393:474-478.

259. Ahmed, K. A., and J. Xiang. 2011. Mechanisms of cellular communication through intercellular protein transfer. *J Cell Mol Med* 15:1458-1473.

260. Hwang, I., J. F. Huang, H. Kishimoto, A. Brunmark, P. A. Peterson, M. R. Jackson, C. D. Surh, Z. Cai, and J. Sprent. 2000. T cells can use either T cell receptor or CD28 receptors to absorb and internalize cell surface molecules derived from antigen-presenting cells. *J Exp Med* 191:1137-1148.

261. He, T., S. Zong, X. Wu, Y. Wei, and J. Xiang. 2007. CD4+ T cell acquisition of the bystander pMHC I colocalizing in the same immunological synapse comprising pMHC II and costimulatory CD40, CD54, CD80, OX40L, and 41BBL. *Biochem Biophys Res Commun* 362:822-828.

262. Undale, A. H., P. J. van den Elsen, and E. Celis. 2004. Antigen-independent acquisition of MHC class II molecules by human T lymphocytes. *Int Immunol* 16:1523-1533.

263. Hao, S., J. Yuan, and J. Xiang. 2007. Nonspecific CD4(+) T cells with uptake of antigen-specific dendritic cell-released exosomes stimulate antigen-specific CD8(+) CTL responses and long-term T cell memory. *J Leukoc Biol* 82:829-838.

264. Hao, S., Y. Liu, J. Yuan, X. Zhang, T. He, X. Wu, Y. Wei, D. Sun, and J. Xiang. 2007. Novel exosome-targeted CD4+ T cell vaccine counteracting CD4+25+ regulatory T cell-mediated immune suppression and stimulating efficient central memory CD8+ CTL responses. *J Immunol* 179:2731-2740.

265. Shi, M., S. Hao, T. Chan, and J. Xiang. 2006. CD4(+) T cells stimulate memory CD8(+) T cell expansion via acquired pMHC I complexes and costimulatory molecules, and IL-2 secretion. *J Leukoc Biol* 80:1354-1363.

266. Huang, H., S. Hao, F. Li, Z. Ye, J. Yang, and J. Xiang. 2007. CD4+ Th1 cells promote CD8+ Tc1 cell survival, memory response, tumor localization and therapy by targeted delivery of interleukin 2 via acquired pMHC I complexes. *Immunology* 120:148-159.

267. Ye, Z., K. A. Ahmed, S. Hao, X. Zhang, Y. Xie, M. A. Munegowda, Q. Meng, R. Chibbar, and J. Xiang. 2008. Active CD4+ helper T cells directly stimulate CD8+ cytotoxic T lymphocyte responses in wild-type and MHC II gene knockout C57BL/6 mice and transgenic RIP-mOVA mice expressing islet beta-cell ovalbumin antigen leading to diabetes. *Autoimmunity* 41:501-511.

268. Ahmed, K. A., Y. Xie, X. Zhang, and J. Xiang. 2008. Acquired pMHC I complexes greatly enhance CD4(+) Th cell's stimulatory effect on CD8(+) T cell-mediated diabetes in transgenic RIP-mOVA mice. *Cell Mol Immunol* 5:407-415.

269. Ankathatti Munegowda, M., Y. Deng, R. Chibbar, Q. Xu, A. Freywald, S. J. Mulligan, S. van Drunen Littel-van den Hurk, D. Sun, S. Xiong, and J. Xiang. 2011. A distinct role of CD4+ Th17- and Th17-stimulated CD8+ CTL in the pathogenesis of type 1 diabetes and experimental autoimmune encephalomyelitis. *J Clin Immunol* 31:811-826.

270. Xie, Y., O. Bai, J. Yuan, R. Chibbar, K. Slattery, Y. Wei, Y. Deng, and J. Xiang. 2009. Tumor apoptotic bodies inhibit CTL responses and antitumor immunity via membrane-bound transforming growth factor-beta1 inducing CD8+ T-cell anergy and CD4+ Tr1 cell responses. *Cancer Res* 69:7756-7766.

271. Ye, Z., H. Huang, S. Hao, S. Xu, H. Yu, S. Van Den Hurk, and J. Xiang. 2007. IL-10 has a distinct immunoregulatory effect on naive and active T cell subsets. *J Interferon Cytokine Res* 27:1031-1038.

272. Soliman, H., M. Mediavilla-Varela, and S. Antonia. 2010. Indoleamine 2,3-dioxygenase: is it an immune suppressor? *Cancer J* 16:354-359.

273. Reiman, J. M., M. Kmieciak, M. H. Manjili, and K. L. Knutson. 2007. Tumor immunoediting and immunosculpting pathways to cancer progression. *Semin Cancer Biol* 17:275-287.

274. Zhang, X., M. A. Munegowda, J. Yuan, Y. Wei, and J. Xiang. 2010. Optimal TLR9 signal converts tolerogenic CD4-8- DCs into immunogenic ones capable of stimulating antitumor immunity via activating CD4+ Th1/Th17 and NK cell responses. *J Leukoc Biol* 88:393-403.

275. Lehuen, A., J. Diana, P. Zaccone, and A. Cooke. Immune cell crosstalk in type 1 diabetes. *Nat Rev Immunol* 10:501-513.

276. Sibley, R. K., D. E. Sutherland, F. Goetz, and A. F. Michael. 1985. Recurrent diabetes mellitus in the pancreas iso- and allograft. A light and electron microscopic and immunohistochemical analysis of four cases. *Lab Invest* 53:132-144.

277. Vukkadapu, S. S., J. M. Belli, K. Ishii, A. G. Jegga, J. J. Hutton, B. J. Aronow, and J. D. Katz. 2005. Dynamic interaction between T cell-mediated beta-cell damage and beta-cell repair in the run up to autoimmune diabetes of the NOD mouse. *Physiol Genomics* 21:201-211.

278. Jain, R., D. M. Tartar, R. K. Gregg, R. D. Divekar, J. J. Bell, H. H. Lee, P. Yu, J. S. Ellis, C. M. Hoeman, C. L. Franklin, and H. Zaghouani. 2008. Innocuous IFNgamma induced by adjuvant-free antigen restores normoglycemia in NOD mice through inhibition of IL-17 production. *J Exp Med* 205:207-218.

279. Wildin, R. S., and A. Freitas. 2005. IPEX and FOXP3: clinical and research perspectives. *J Autoimmun* 25 Suppl:56-62.

280. Salomon, B., D. J. Lenschow, L. Rhee, N. Ashourian, B. Singh, A. Sharpe, and J. A. Bluestone. 2000. B7/CD28 costimulation is essential for the homeostasis of the CD4+CD25+ immunoregulatory T cells that control autoimmune diabetes. *Immunity* 12:431-440.

281. Tang, Q., and J. A. Bluestone. 2008. The Foxp3+ regulatory T cell: a jack of all trades, master of regulation. *Nat Immunol* 9:239-244.

282. Korn, T. 2008. Pathophysiology of multiple sclerosis. *J Neurol* 255 Suppl 6:2-6.

283. Ferber, I. A., S. Brocke, C. Taylor-Edwards, W. Ridgway, C. Dinisco, L. Steinman, D. Dalton, and C. G. Fathman. 1996. Mice with a disrupted IFN-gamma gene are susceptible to the induction of experimental autoimmune encephalomyelitis (EAE). *J Immunol* 156:5-7.

284. Yang, Y., J. Weiner, Y. Liu, A. J. Smith, D. J. Huss, R. Winger, H. Peng, P. D. Cravens, M. K. Racke, and A. E. Lovett-Racke. 2009. T-bet is essential for encephalitogenicity of both Th1 and Th17 cells. *J Exp Med* 206:1549-1564.

285. Kebir, H., K. Kreymborg, I. Ifergan, A. Dodelet-Devillers, R. Cayrol, M. Bernard, F. Giuliani, N. Arbour, B. Becher, and A. Prat. 2007. Human TH17 lymphocytes promote blood-brain barrier disruption and central nervous system inflammation. *Nat Med* 13:1173-1175.

286. Hirota, K., H. Yoshitomi, M. Hashimoto, S. Maeda, S. Teradaira, N. Sugimoto, T. Yamaguchi, T. Nomura, H. Ito, T. Nakamura, N. Sakaguchi, and S. Sakaguchi. 2007. Preferential recruitment of CCR6-expressing Th17 cells to inflamed joints via CCL20 in rheumatoid arthritis and its animal model. *J Exp Med* 204:2803-2812.

287. Lowes, M. A., T. Kikuchi, J. Fuentes-Duculan, I. Cardinale, L. C. Zaba, A. S. Haider, E. P. Bowman, and J. G. Krueger. 2008. Psoriasis vulgaris lesions contain discrete populations of Th1 and Th17 T cells. *J Invest Dermatol* 128:1207-1211.

288. Fujino, S., A. Andoh, S. Bamba, A. Ogawa, K. Hata, Y. Araki, T. Bamba, and Y. Fujiyama. 2003. Increased expression of interleukin 17 in inflammatory bowel disease. *Gut* 52:65-70.

289. El-behi, M., A. Rostami, and B. Ciric. Current views on the roles of Th1 and Th17 cells in experimental autoimmune encephalomyelitis. *J Neuroimmune Pharmacol* 5:189-197.

290. Stromnes, I. M., and J. M. Goverman. 2006. Active induction of experimental allergic encephalomyelitis. *Nat Protoc* 1:1810-1819.

291. Sun, D., J. N. Whitaker, Z. Huang, D. Liu, C. Coleclough, H. Wekerle, and C. S. Raine. 2001. Myelin antigen-specific CD8+ T cells are encephalitogenic and produce severe disease in C57BL/6 mice. *J Immunol* 166:7579-7587.

292. Haines, J. L., M. Ter-Minassian, A. Bazyk, J. F. Gusella, D. J. Kim, H. Terwedow, M. A. Pericak-Vance, J. B. Rimmler, C. S. Haynes, A. D. Roses, A. Lee, B. Shaner, M. Menold, E. Seboun, R. P. Fitoussi, C. Gartioux, C. Reyes, F. Ribierre, G. Gyapay, J. Weissenbach, S. L. Hauser, D. E. Goodkin, R. Lincoln, K. Usuku, J. R. Oksenberg, and et al. 1996. A complete genomic screen for multiple sclerosis underscores a role for the major histocompatability complex. The Multiple Sclerosis Genetics Group. *Nat Genet* 13:469-471.

293. Kuchroo, V. K., A. C. Anderson, H. Waldner, M. Munder, E. Bettelli, and L. B. Nicholson. 2002. T cell response in experimental autoimmune encephalomyelitis (EAE): role of self and cross-reactive antigens in shaping, tuning, and regulating the autopathogenic T cell repertoire. *Annu Rev Immunol* 20:101-123.

294. Bettelli, E., B. Sullivan, S. J. Szabo, R. A. Sobel, L. H. Glimcher, and V. K. Kuchroo. 2004. Loss of T-bet, but not STAT1, prevents the development of experimental autoimmune encephalomyelitis. *J Exp Med* 200:79-87.

295. Korn, T., J. Reddy, W. Gao, E. Bettelli, A. Awasthi, T. R. Petersen, B. T. Backstrom, R. A. Sobel, K. W. Wucherpfennig, T. B. Strom, M. Oukka, and V. K. Kuchroo. 2007. Myelin-specific regulatory T cells accumulate in the CNS but fail to control autoimmune inflammation. *Nat Med* 13:423-431.

296. Ford, M. L., and B. D. Evavold. 2005. Specificity, magnitude, and kinetics of MOG-specific CD8+ T cell responses during experimental autoimmune encephalomyelitis. *Eur J Immunol* 35:76-85.

297. Sun, D., Y. Zhang, B. Wei, S. C. Peiper, H. Shao, and H. J. Kaplan. 2003. Encephalitogenic activity of truncated myelin oligodendrocyte glycoprotein (MOG) peptides and their recognition by CD8+ MOG-specific T cells on oligomeric MHC class I molecules. *Int Immunol* 15:261-268.

CHAPTER 2

2. Th17 and Th17-stimulated $CD8^+$ T cells play a distinct role in Th17-induced preventive and therapeutic antitumor immunity.

Brief introduction to chapter 2

This chapter addresses the mechanism of stimulatory effect of Th17 cells on antitumor specific $CD8^+$ CTL responses in preventive and therapeutic tumor models. It was demonstrated by various other studies that Th17 cells induce antitumor immunity leading to eradication of established tumors. However, the mechanism of $CD8^+$ CTL activation by Th17 cells, and the distinct role of Th17, and Th17 activated $CD8^+$ CTLs in antitumor immunity were still elusive. In this study, we have demonstrated that Th17 cells acquired major pMHC-I, and express RORγt, IL-17 and IL-2. Th17 cells did not show any direct in vitro tumor cell killing activity, whereas they were able to stimulate $CD8^+$ CTL responses via IL-2, and pMHC-I, but was not via IL-17 signalling. The therapeutic effect of Th17 cells was shown to be associated with IL-17, but not with IFN-γ, and was mediated by Th17 stimulated $CD8^+$ CTLs via the perforin pathway, which were recruited into B16 melanoma via Th17 stimulated CCL20 chemoattraction. Taken together, this study elucidates a distinct role of Th17 and Th17-stimulated $CD8^+$ CTLs in induction of preventive and therapeutic antitumor immunity, which may greatly impact the development of Th17-based cancer immunotherapy.

Reprinted with consent from Cancer Immunology Immunotherapy 2011 Oct;60(10):1473-84.

Cancer Immunol Immunother
DOI 10.1007/s00262-011-1054-y

ORIGINAL ARTICLE

Th17 and Th17-stimulated CD8$^+$ T cells play a distinct role in Th17-induced preventive and therapeutic antitumor immunity

Manjunatha Ankathatti Munegowda · Yulin Deng · Sean J. Mulligan · Jim Xiang

Received: 4 February 2011 / Accepted: 26 May 2011

Abstract CD4$^+$ Th17 cells induce antitumor immunity leading to the eradication of established tumors. However, the mechanism of antitumour immunity and CTL activation by Th17 cells and the distinct role of Th17 and Th17-activated CTLs in antitumor immunity are still elusive. In this study, we generated ovalbumin (OVA)-specific Th17 cells by cultivating OVA-pulsed dendritic cells with CD4$^+$ T cells derived from transgenic OTII mice in the presence of IL-6, IL-23, TGF-β, and anti-IFN-γ antibody. We demonstrated that Th17 cells acquired major histocompatibility complex/peptide (pMHC)-I and expressed RORγt, IL-17, and IL-2. Th17 cells did not have any direct in vitro tumor cell–killing activity. However, Th17 cells were able to stimulate CD8$^+$ CTL responses via IL-2 and pMHC I, but not IL-17 signaling, which play a major role in Th17-induced preventive immunity against OVA-expressing B16 melanoma. Th17 cells stimulated the expression of CCL2 and CCL20 in lung tumor microenvironments promoting the recruitment of various inflammatory leukocytes (DCs, CD4$^+$, and CD8$^+$ T cells) stimulating more pronounced therapeutic immunity for early-stage (5-day lung metastases or 3 mm, s.c.) tumor than for well-established (6 mm, s.c.) tumor. The therapeutic effect of Th17 cells is associated with IL-17 and is mediated by Th17-stimulated CD8$^+$ CTLs and other inflammatory leukocytes recruited into B16 melanoma via Th17-stimulated CCL20 chemoattraction. Taken together, our data elucidate a distinct role of Th17 and Th17-stimulated CD8$^+$ CTLs in the induction of preventive and therapeutic antitumor immunity, which may greatly impact the development of Th17-based cancer immunotherapy.

Keywords Th17 · pMHC I complexes · CD8$^+$ CTL · CCL2/20 · Antitumor immunity

M. Ankathatti Munegowda · Y. Deng · J. Xiang (✉)
Research Unit, Saskatchewan Cancer Agency,
Department of Oncology, University of Saskatchewan,
Saskatoon, SK S7N 4H4, Canada
e-mail: jim.xiang@saskcancer.ca

M. Ankathatti Munegowda · Y. Deng · J. Xiang
Research Unit, Saskatchewan Cancer Agency,
Department of Immunology Pathology,
University of Saskatchewan, Saskatoon, SK S7N 4H4, Canada

S. J. Mulligan
Research Unit, Saskatchewan Cancer Agency,
Department of Physiology, University of Saskatchewan,
Saskatoon, SK S7N 4H4, Canada

Introduction

Effector CD4$^+$ T cells are classically divided into two lineages based on distinct cytokine secretion profiles: the IFN-γ-producing Th1 lineage and IL-4/IL-13-producing Th2 lineage. Recently, a lineage of effector Th17 cells that produce IL-17A and IL-17F and express the transcription factor RORγt through activation of STAT3 by IL-6 and IL-23 have been identified [1]. IL-17 cytokines are strongly proinflammatory and induce the expression of several chemokines such as CCL2, CCL7, and CCL20. Transgenic overexpression of IL-17A in the lungs provokes proinflammatory gene expression and tissue infiltration of leukocytes [2]. In contrast, inhibition of IL-17A expression impairs host defense against bacterial infection [3] and resistance to autoimmune diseases [4, 5].

Th17 cells and IL-17 expression have been found in various human tumors [6–10]. However, the involvement of IL-17 and Th17 cells in antitumor immunity is still controversial. For example, transgenic IL-17 expression

Published online: 10 June 2011

either induced tumor regression through enhanced antitumor immunity in immune-competent mice [11, 12] or promoted tumor progression through an increase in inflammatory angiogenesis in immune-deficient mice [13]. It has been demonstrated that Th17 cells were indirectly linked to antitumor immunity [14]. Tumor-specific Th17 polarized cells were found to inhibit the growth of well-established melanoma via IFN-γ production [15]. However, the nature of Th17 cell's role in the context of antitumor immunity still remains largely unknown. Th17 cells have been shown to stimulate antitumor immunity in both prevention and therapeutic models by recruiting DCs, granulocytes, and $CD4^+$ and $CD8^+$ T cells [16]. However, (i) the molecular mechanism of $CD8^+$ CTL activation by Th17 cells, (ii) the precise role of Th17 secreted IL-17 and different types of recruited leukocytes in Th17-induced antitumor immunity, and (iii) the potentially distinct role of Th17 and Th17-stimulated $CD8^+$ CTLs in Th17-induced antitumor immunity are still unknown.

Intercellular membrane transfer through trogocytosis plays an important role in immune modulation [17]. We have recently demonstrated that $CD4^+$ T cells derived from ovalbumin (OVA)-specific T-cell receptor (TCR) transgenic OTII mice, which were activated in vitro with OVA-pulsed DCs (DC_{OVA}), differentiated into Th1 cells [17]. These Th1 cells acquired peptide major histocompatibility complexes I (pMHC I) and CD80 from DC_{OVA} by DCova activation and became capable of directly stimulating $CD8^+$ CTL responses via endogenous IL-2 and acquired pMHC I and CD80 signaling [18, 19]. However, whether Th17 cells with distinctive phenotype from Th1 cell have a similar stimulatory effect as Th1 cells on the stimulation of $CD8^+$ CTL responses is elusive.

In this study, we generated RORγt-expressing and IL-17-secreting OVA-specific Th17 cells by cultivation of OTII $CD4^+$ T cells with OVA-pulsed DC_{OVA} in the presence of IL-6, IL-23, TGF-β, and anti-IFN-γ antibody. We then immunized C57BL/6 mice with these Th17 cells to assess the potential stimulatory effect on $CD8^+$ T-cell responses and antitumor immunity in preventive and therapeutic models against OVA-expressing B16 melanoma ($BL6\text{-}10_{OVA}$).

Results

Th17 acquires pMHC I complexes by DC_{OVA} activation

Transgenic mouse OT II $CD4^+$ T cells activated with irradiated DC_{OVA} in the presence of IL-23/IL-6/TGF-β and anti-IFN-γ antibody expressed cell surface FasL, intranuclear RORγt, and intracellular perforin and IL-17 by flow cytometric and RT–PCR analysis (Fig. 1a, c). By using double staining for IL-17 and IFN-γ, polarized Th17 and Th1 cells expressed intracellular IL-17 and IFN-γ, respectively (Fig. 1b). These Th17 cells also secreted IL-2 (2.8 ng/ml), IL-6 (4.5 ng/ml), IL-17 (1.8 ng/ml), and TGF-β (0.2 ng/ml) by ELISA analysis, indicating that they are Th17 cells, which is consistent with a recent report showing that Th17 simultaneously expressed both IL-17 and IL-2 [20]. There was no $CD11c^+$ DC_{OVA} contamination in these purified Th17 population (Fig. 1d). In addition, these Th17 cells did display pMHC I (Fig. 1a), indicating that they may acquire pMHC I from DC_{OVA} upon DC_{OVA} activation. This was confirmed by evidence that $CD4^+$ T cells derived from pMHC I-negative ($K^{b-/-}$)DC_{OVA} activation did not express pMHC I (Fig. 1e).

Th17 stimulates in vitro $CD8^+$ T-cell proliferation via IL-2 and pMHC I, but not IL-17 signaling

We previously demonstrated that DCova-activated Th1 with acquired pMHC I stimulated CTL responses via IL-2 and pMHC I signaling [18, 19]. To assess Th17's stimulatory effect, we performed ^{3}H-thymidine incorporation assay. DCova-activated Th17 with acquired pMHC I also stimulated in vitro OTI $CD8^+$ T-cell proliferation in a dose-dependent fashion. Interestingly, ($K^{b-/-}$)Th17 without acquired pMHC I or Th17 in the presence of anti-IL-2 Ab, but not anti-IL-17 Ab, failed to stimulate $CD8^+$ T-cell proliferation (Fig. 2a), indicating that the in vitro Th17's stimulatory effect on CTLs is via IL-2 and pMHC I, but not via IL-17 signaling.

Th17-activated $CD8^+$ T, but not Th17, cells have in vitro cytotoxicity

Since Th17 expressed cytotoxic FasL and perforin, they may have killing activity to pMHC II-expressing target cells. To assess their killing effect, we performed a chromium release assay. We found that Th17-activated CTLs showed killing activity to OVA-expressing EG7 tumor cells, and the killing activity was significantly ($P < 0.01$) or slightly ($P > 0.05$) reduced when T cells were pre-incubated with CMA or emetin, indicating that $CD8^+$ T-cell-mediated killing activity is mainly via perforin pathway (Fig. 2b) [21]. In addition, we found that Th1 [19], but not Th17, killed OVAII peptide-pulsed LB27 cells (Fig. 2c), indicating that Th17 do not have any direct killing activity to tumor cells.

Th17 stimulates the host DC-independent $CD8^+$ CTL responses

To assess DCova-activated Th17's ability to induce in vivo $CD8^+$ T-cell proliferation, we i.v. transferred B6 mice with

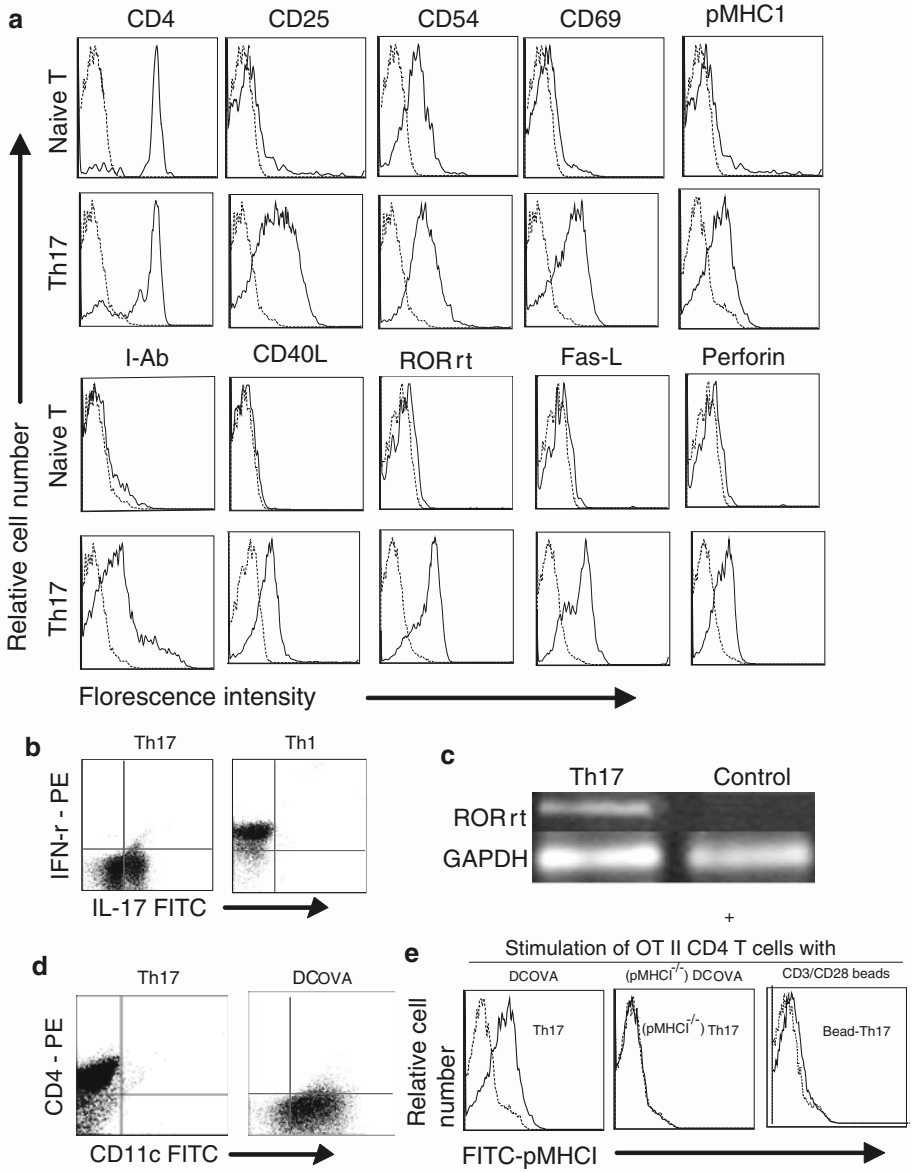

Fig. 1 Phenotypic analysis of OVA-specific Th17 cells. **a** Naïve $CD4^+$ T cells and in vitro DC_{OVA}-activated Th17 were stained with a panel of antibodies (*solid lines*) and analyzed by flow cytometry. Irrelevant isotype-matched biotin-conjugated antibodies were used as controls (*light dotted lines*). **b** In vitro DC_{OVA}-activated Th1 and Th17 cells were double stained with FITC-anti-IL-17 Ab and PE-anti-IFN-γ Ab and analyzed by flow cytometry. **c** RNA extracted from DC_{OVA}-activated Th17 and ConA-stimulated $CD4^+$ T (control) cells were analyzed by RT–PCR to assess the expression of ROR-γt. **d** DC_{OVA} and Th17 were stained with PE-antiCD4 and FITC-anti-CD11c Abs and analyzed by flow cytometry. **e** Th17, ($K^{b-/-}$), Th17 cells, and CD3/CD28 bead-activated Th17 (bead-Th17) cells were stained with FITC-anti-pMHC I antibody (*solid lines*), and irrelevant isotype-matched antibody was used as control (*dotted lines*). One representative experiment of two experiments is shown

DCova and Th17 and then performed an OVA-specific tetramer staining assay to detect OVA-specific $CD8^+$ T-cell proliferation [19]. As shown in Fig. 2d, DCova and Th17 stimulated OVA-specific $CD8^+$ T cells accounted for 1.43 and 0.98% of the total $CD8^+$ T-cell population, respectively. To assess whether the host DCs are involved in Th17-stimulated CTL responses by the uptake of antigen epitopes of Th17, we i.v. transferred the transgenic DTR-CD11c mice with DT treatment for complete depletion of endogenous DCs and macrophages (Clin Exp Immunol 141: 398, 2005) with Th17 and then performed an OVA-specific tetramer staining assay to detect OVA-specific $CD8^+$ T-cell proliferation. We found that Th17-stimulated OVA-specific CTL responses in PBS- and DT-treated DTR-CD11c mice with and without endogenous APCs were similar (Fig. 2d), indicating that Th17 stimulates the host DC-independent $CD8^+$ CTL responses.

Th17 stimulates CTL-mediated preventive antitumor immunity via IL-2 and pMHC I, but not via IL-17 signaling

To elucidate the molecular mechanism of Th17-stimulated CTL responses, DCova-activated Th17, ($IL\text{-}2^{-/-}$)Th17 with IL-2 deficiency, ($K^{b-/-}$)Th17 without acquired pMHC I, and CD3/CD28 bead-activated Th17 (bead-Th17) without pMHC I expression (Fig. 1e) were used in the in vivo proliferation and cytotoxicity experiments. To assess the involvement of IL-17, Th17 cell transferred mice were treated with anti-IL-17 Ab to block IL-17 effect. As shown

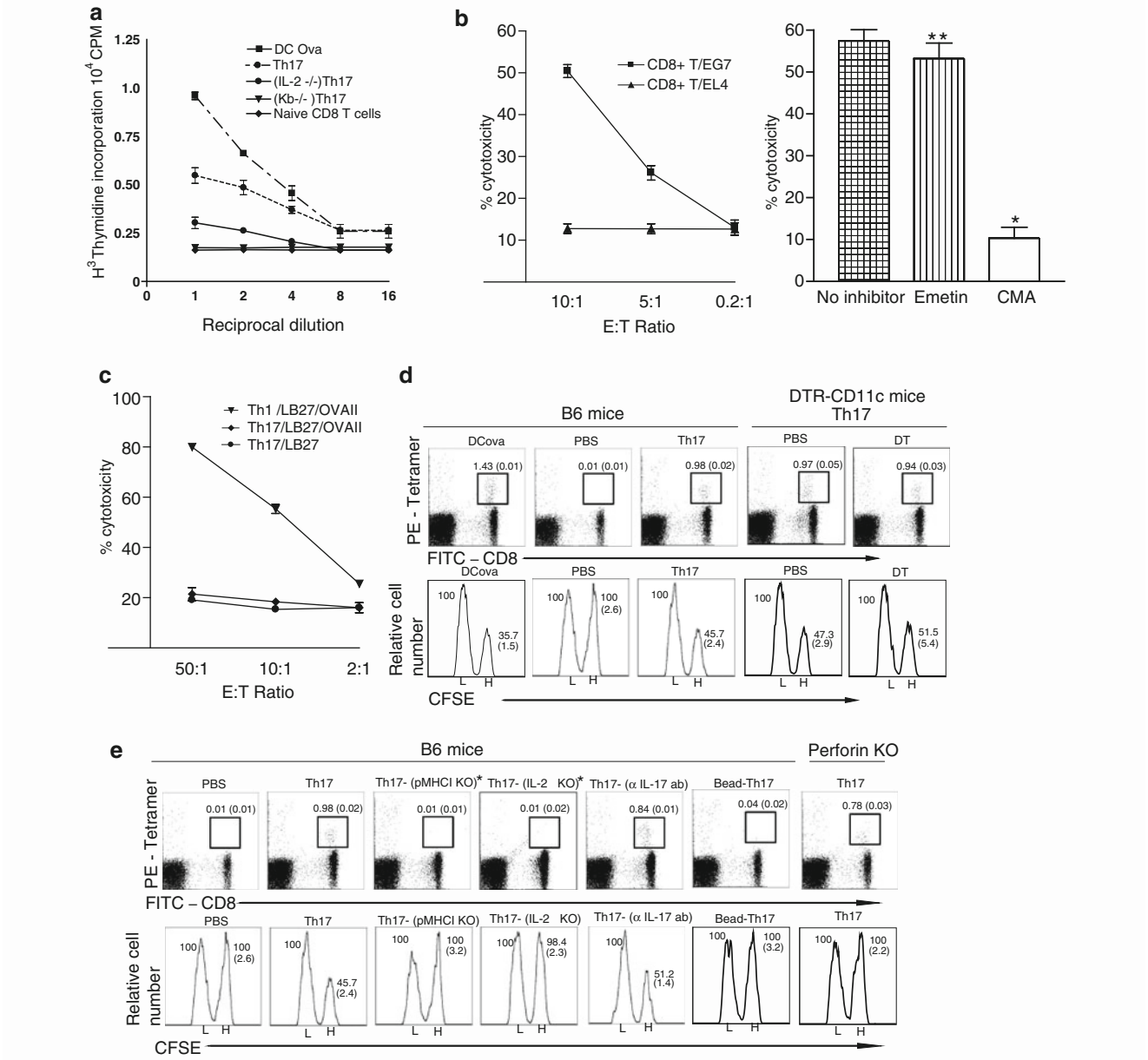

Fig. 2 Functional effect analysis of OVA-specific Th17 cells. **a** Irradiated DC_{OVA}, Th17, (IL-$2^{-/-}$)Th17 and ($K^{b-/-}$)Th17, and their 2-fold dilutions were co-cultured with OTI $CD8^+$ T cells. After 2 days, the proliferative responses of $CD8^+$ T cells were determined by overnight ^{3}H-thymidine uptake assay. **b** Th17-activated $CD8^+$ T cells with or without preincubation of concanamycin A (CMA, 1 μM) or emetin (5 μM) for 2 h were used as effector (E) cells, while ^{51}Cr-labeled EG7 and EL4 cells were used as target (T) cells. **c** DC_{OVA}-activated Th17 and Th1 were used as effector (E) cells, while ^{51}Cr-labeled OVAII-pulsed LB27 cells and LB27 cells were used as target (T) cells. $*P < 0.01$ and $**P > 0.05$ versus cohorts of 'no inhibitor' group (*Student's t test*). **d** In tetramer staining assay, the tail blood samples of wild-type B6 or DT-treated DTR-CD11c mice (6 per group) transferred with Th17 were stained with PE-H-$2K^b$/OVA I (PE-tetramer) and FITC-anti-CD8 Ab (FITC-CD8) and then analyzed by flow cytometry. **e** In tetramer staining assay, the tail blood samples from wild-type B6 mice (6 per group) transferred with DCova-activated Th17, Th17 with various KO and CD3/CD28 bead-activated Th17 (bead-Th17) or from anti-IL-17 Ab-treated B6 mice transferred with Th17 or perforin$^{-/-}$ mice were stained with PE-H-$2K^b$/OVA I (PE-tetramer) and FITC-anti-CD8 Ab (FITC-CD8) and then analyzed by flow cytometry. The value represents the percentage of tetramer-positive $CD8^+$ T cells in the total $CD8^+$ T-cell population with standard deviation in parenthesis. In in vivo cytotoxicity assay (in both panel d and e), the residual $CFSE^{high}$ (H) and $CFSE^{low}$ (L) target cells remaining in the recipients' spleens (6 per group) were analyzed by flow cytometry. The value in each panel represents the percentage of $CFSE^{high}$ vs $CFSE^{low}$ target cells remaining in the spleen. ($n = 6$, average ± SD). $*P < 0.05$ versus cohorts of mice immunized with Th17 (*Student's t test*). One representative experiment of two is shown

in Fig. 2e, the stimulatory effect was significantly reduced in mice transferred with ($K^{b-/-}$)Th17, bead-Th17, and (IL-$2^{-/-}$)Th17 ($P < 0.05$), but not in Th17-transferred mice with the treatment of anti-IL-17 Ab, indicating that endogenous IL-2 and acquired pMHC I, but not IL-17 signaling influence in vivo Th17's stimulatory effect. In in vivo cytotoxicity assay, we found that Th17-transferred mice showed substantial loss of OVA-specific $CFSE^{high}$ cells, indicating that Th17 can stimulate $CD8^+$ T-cell differentiation into effector CTLs with killing activity for OVA-specific target cells in vivo. To assess the pathway responsible for the killing activity of $CD4^+$ Th17-stimulated $CD8^+$ T cells in vivo, we also transferred $CD4^+$ Th17 cells into $perforin^{-/-}$ mice and repeated the above-stated tetramer staining and in vivo cytotoxicity assays. We found that OVA-specific $CD8^+$ T-cell in vivo proliferation in C57BL/6 and $perforin^{-/-}$ mice were similar (Fig. 2e). However, $CD8^+$ T-cell-induced killing activity to OVA-specific $CFSE^{high}$ target cells was lost in $perforin^{-/-}$ mice (Fig. 2e), indicating that the in vivo $CD4^+$ Th17-stimulated $CD8^+$ T-cell-induced killing activity to OVA-specific target cells is via perforin-dependent pathway. Interestingly, (IL-$2^{-/-}$)Th17-, ($K^{b-/-}$)Th17-, and bead-Th17-transferred mice maintained their OVA-specific $CFSE^{high}$ target cell numbers, indicating that in vivo stimulatory effect of Th17 is mediated by its IL-2 secretion and pMHC I targeting. To assess preventive antitumor immunity, we performed animal studies by i.v. injection of BL6-10_{OVA} cells into the above-transferred mice 6 days subsequent to transfer. We found that all mice (8/8) were free from metastasis, whereas all (8/8) Th17-transferred mice with treatment of anti-CD8 Ab completely lost their antitumor immunity (Exp I of Table 1), indicating that Th17-induced antitumor immunity is mainly mediated by $CD8^+$ T cells. We also found that all (8/8) (IL-$2^{-/-}$)Th17- and ($K^{b-/-}$)Th17-transferred, but not mice with treatment of anti-IL-17 Ab, lost their antitumor immunity (Exp I of Table 1), indicating that Th17's stimulatory effect on preventive antitumor immunity is also mediated by IL-2 (not IL-17) signaling and pMHC I targeting. To assess the long-term immunity, we also challenged Th17-transferred mice 60 days after the primary immunization. As shown in Exp II of Table 1, all transferred mice had a long-term protective antitumor immunity.

Table 1 Vaccination with Th17 protects against lung tumor metastases in mice

Immunization[a]	Tumor-bearing mice (%)
Experiment I	
Th17	0/8 (0)
Th17(IL-$2^{-/-}$)	8/8 (100)
Th17($K^{b-/-}$)	8/8 (100)
Th17 + anti-IL17 Ab	0/8 (0)
Th17 + anti-CD8 Ab	8/8 (100)
Th17 + control Ab	0/8 (0)
PBS	8/8 (100)
Experiment II	
Th17-immunized mice	0/8 (0)
PBS-treated mice	8/8 (100)

[a] In Experiment I, C57BL/6 mice (8 per group) were i.v. injected with Th17 or Th17 with gene deficiency or Th17 plus antibody. Six days after Th17 injection, mice were challenged with BL6-10_{OVA} cells. In Experiment II, C57BL/6 mice (8 per group) were i.v. injected with Th17. Sixty days after Th17 injection, mice were challenged with BL6-10_{OVA} cells. The mice were killed 3 weeks after tumor cell challenge, and lung metastatic tumor colonies were counted. One representative experiment of two is shown

IL-17 is associated with DCova-activated Th17-induced eradication of early-stage (5 day) lung tumor metastases

To assess Th17's therapeutic effect, we i.v. injected C57BL/6 mice with BL6-10_{OVA} tumor cells. Five days after tumor cell injection, mice were i.v. transferred with DCova-activated Th17 cells with pMHC I expression and CD3/CD28 bead-activated Th17 (bead-Th17) cells without pMHC I expression. Lung tumor colonies were numerated 10 days after transfer. Compared with untreated control mice, those mice transferred with DCova-activated Th17, but not with bead-Th17, had significantly fewer tumor foci ($P < 0.05$) (Fig. 3a), indicating that DCova-activated Th17 cells have efficient therapeutic effect on early-stage (5 day) tumor lung metastasis via acquired pMHC I signaling. To assess the role of Th17-secreted IL-17 in DCova-activated Th17-induced antitumor immunity, we treated immunized mice with anti-IL-17 Ab to block IL-17 signaling. We found that Th17-transferred mice with treatment of anti-IL-17 Ab had numerous tumor foci as the control mouse group (Fig. 3a), indicating that IL-17 is critically involved in DCova-activated Th17-induced therapeutic antitumor immunity.

Th17 induces recruitment of leukocytes into tumors via Th17-stimulated CCL2/CCL20 expression

To assess the potential Th17 stimulated CCL2/CCL20 expression, we first analyzed the expression of CCL2/CCL20 by lung cells using real-time PCR. We found that BL6-10 tumor cells and normal lungs did not express any CCL2/CCL20 chemokines (Fig. 3b). However, the expression of CCL2/CCL20 was greatly increased in lung cell fractionations containing both tumor and lung cells, indicating that Th17 cells stimulate expression of CCL2/

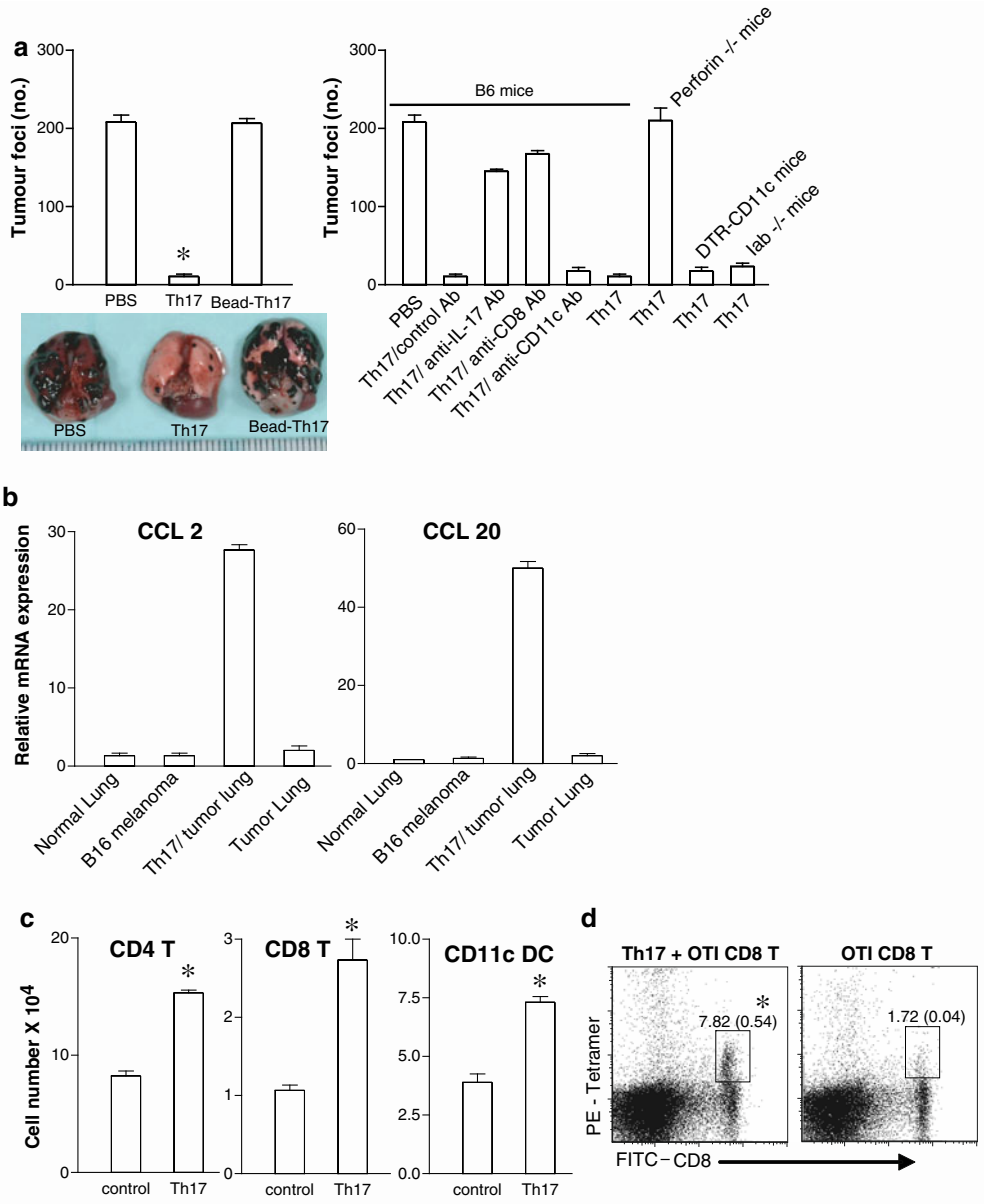

Fig. 3 Therapeutic effect of OVA-specific Th17 cells on eradication of established lung tumor metastasis. **a** C57BL/6 mice or $Ia^{b-/-}$ KO mice or toxin-treated DTR-CD11c mice or $perforin^{-/-}$ mice bearing 5-day lung B16 melanoma were i.v. immunized with DCova-activated Th17 with or without various Abs against CD8, CD11c, IL-17 for depletion of host $CD8^+$ T cells, DCs, and cytokine, respectively. C57BL/6 mice bearing 5-day lung B16 melanoma were also i.v. immunized with CD3/CD28 bead-activated Th17 (bead-Th17). Tumor colonies were counted on day 16 after tumor challenge ($n = 8$, average ± SD). *$P < 0.05$, versus cohorts of tumor foci in mice injected with control Ab (Student's t test). **b** mRNA gene expression analysis was assessed by RT–PCR. Data shown were normalized to the reference gene GAPDH. Graphs represent the average values of four mice after duplicate analysis per sample ($n = 4$, average ± SD). **c** Total numbers of leukocytes from cell fractions of tumor-bearing lungs were calculated from percentages of live cells gated on CD45.2 ($n = 4$, average ± SD). **d** 5-day lung tumor-bearing mice were transferred with 3×10^6 OTI $CD8^+$ T cells and after 3 days percentages of OTI $CD8^+$ T cells out of total $CD8^+$ T-cell population in lungs were measured by gating on FITC-CD8 and PE-tetramer-positive T cells ($n = 4$, average ± SD). One representative experiment of two is shown

CCL20. Further analysis of lung leukocyte fractionations by flow cytometry revealed that $CD11c^+$ DCs, $CD4^+$, and $CD8^+$ T cells were significantly increased in Th17-treated mouse lungs (Fig. 3c) ($P < 0.05$). To assess the potential recruitment of OVA-specific $CD8^+$ T cells, we transferred DC_{OVA}-activated OTI $CD8^+$ T cells into B6 mice bearing 5-day lung tumor metastasis and then numerated tumor-infiltrating $CD8^+$ T cells by flow cytometry. We found that

Th17 significantly promoted tumor infiltration of OVA-specific $CD8^+$ T cells indicating that Th17 cells promotes tumor infiltration of OVA-specific $CD8^+$ T cells via CCL20 chemoattraction.

Th17-activated $CD8^+$ T cells mediate therapeutic effect via perforin-dependent pathway on early-stage (5 day) lung tumor metastases

To assess the role of recruited DCs, $CD4^+$, and $CD8^+$ T cells, the above immunized C57BL/6 mice were treated with different antibodies to deplete $CD8^+$ T cells or DCs. Alternatively, DTR-CD11c transgenic mice with the treatment of toxin to deplete the host DCs were transferred with Th17. To assess the role of recruited $CD4^+$ T cells in Th17-induced therapeutic effect, we also i.v. transferred $Ia^{b-/-}$ KO mice with Th17, which lack the host $CD4^+$ T cells. We found that only the mice treated with anti CD8 Ab (host $CD8^+$ T cells depleted), but not mice with depletion of host DCs (anti CD11c Ab or DTR-CD11c mice treated with toxin) or lacking host $CD4^+$ T cells ($Ia^{b-/-}$ KO mice), showed numerous tumor foci (Fig. 3a), indicating that Th17-stimulated $CD8^+$ CTLs play an important role, but the host DCs and $CD4^+$ T cells recruited into tumor tissues are not involved in Th17-induced therapeutic effect. To assess the molecular mechanism of $CD8^+$ T-mediated killing, we repeated Th17 treatment in tumor-bearing perforin$^{-/-}$ mice, where Th17-stimulated host OVA-specific $CD8^+$ T cells are perforin deficient. We found that Th17-transferred perforin$^{-/-}$ mice showed numerous tumor foci (Fig. 3a), indicating that Th17-stimulated CTLs mediate therapeutic effect via perforin-dependent pathway.

Th17-activated $CD8^+$ T cells are potent in the eradication of early-stage (3 mm), but not well-established (6 mm), s.c. tumors

To determine whether Th17 protects against tumors in different tissues in addition to lung tissues, we s.c. inoculated B6 mice with BL6-10_{OVA} cells. We then treated mice bearing different sizes (early stage: 3 mm and well-established tumor: 6 mm) of B16 melanoma with Th17. We found that all tumor (3 mm)-bearing mice (8/8) died of tumor within 21 days without treatment, whereas all 8/8 tumor-bearing mice with treated with Th17 survived (Fig. 4a), indicating that DCova-activated Th17 cells have immunotherapeutic effect on early-stage tumors. To assess the role of IFN-γ expression, and host $CD4^+$ and $CD8^+$ T cells in the immunotherapeutic effect, we used IFN-$\gamma^{-/-}$, $Ia^{b-/-}$, and H-2 $K^{b-/-}$ KO mice in the above experiments. We found that DCova-activated Th17 cell-induced therapeutic effect was not affected in IFN-$\gamma^{-/-}$ and $Ia^{b-/-}$ KO mice (Fig. 4b), indicating that Th17-induced therapeutic effect is not mediated via host IFN-γ and $CD4^+$ T cells. However, its therapeutic effect was completely lost in H-2 $K^{b-/-}$ KO mice lacking host $CD8^+$ T cells (Fig. 4b), confirming that Th17-stimulated host $CD8^+$ CTLs play a major role in Th17-induced therapeutic antitumor immunity. To assess the potential immunotherapeutic effect on well-established tumors, we repeated experiments in mice bearing BL6-10_{OVA} tumors (6 mm). We found that none (0/8) of the treated mice survived though their survival was significantly prolonged ($P < 0.05$) (Fig. 4c), indicating that DCova-activated Th17 cells, though having therapeutic effect, are not potent enough in well-established tumors.

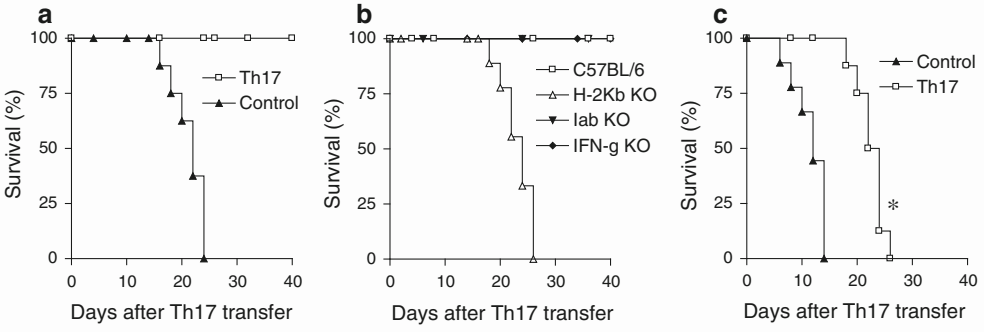

Fig. 4 Therapeutic effect of OVA-specific Th17 cells on the eradication of established s.c. tumors. **a** Wild-type C57BL/6 mice ($n = 8$) bearing early-stage (3 mm) B16 melanoma were i.v. immunized with DCova-activated Th17 cells or PBS as a control. **b** Wild-type C57BL/6, IFN-$\gamma^{-/-}$, H-2 $K^{b-/-}$, and $Ia^{b-/-}$ gene KO mice ($n = 8$) bearing early-stage (3 mm) B16 melanoma were i.v. immunized with DCoav-activated Th17 cells. Tumor regression or growth was monitored. The evolution of the tumors in individual mouse is depicted for their survival period. **c** Wild-type C57BL/6 mice ($n = 8$) bearing well-established (6 mm) B16 melanoma were i.v. immunized with Th17. Tumor size was measured daily using an engineering caliper. The evolution of the tumors in individual mouse is depicted for their survival period. *$P < 0.05$ versus cohorts of control group (*Log rank test*). One representative experiment of three is shown

Discussion

Th17 cells are an important inflammatory component and have been shown to promote inflammation in autoimmune diseases [22, 23]. Recent evidence suggests that Th17 cells are also involved in tumor immunology and may be a target for cancer immunotherapy [24]. We have previously demonstrated that Th1 cells acquired pMHC I complexes and co-stimulatory molecules from DC_{OVA} upon DC_{OVA} activation and became capable of stimulating OVA-specific $CD8^+$ CTL responses via IL-2 and pMHC I signaling and antitumor immunity [15, 19]. In our current study, we demonstrated for the first time that (i) in vitro DC_{OVA}-activated Th17 cells expressing RORγt, IL-17, and IL-2 also acquire pMHC I complexes upon activation by DC_{OVA} and (ii) Th17 with cytokine profile distinct from Th1 is also capable of stimulating $CD8^+$ CTL responses and long-term memory via IL-2 and pMHC I, but not IL-17 signaling. Endogenous IL-2 of Th17 cells is important in CTL induction, though Il-2 has been shown to constrain Th17 generation from $CD4^+$ T precursors via STAT5 [25]. In addition, we also demonstrated that Th17-induced preventive antitumor immunity is mainly mediated by Th17-stimulated CTLs.

In the therapeutic model for early-stage (5 day) lung tumor metastases, we found that (i) it is Th17-activated $CD8^+$ T cells that play a major role in the eradication of lung metastatic tumors and (ii) Th17 stimulated the expression of CCL2/20 in lung tumor microenvironments, which promoted the recruitment of various inflammatory leukocytes (DCs, $CD4^+$, and $CD8^+$ T cells) to induce therapeutic immunity. Our study elucidates the molecular mechanism of Th17's stimulatory effect on $CD8^+$ CTL responses and also demonstrated that (i) it is the Th17-stimulated CTLs, but not Th17 cells, that themselves have direct in vitro killing activity to tumor cells, (ii) Th17-secreted IL-17, but not the host IFN-γ, is associated with Th17-induced therapeutic effect, and (iii) although Th17 cells promote tumor infiltration of various inflammatory leukocytes, the tumor-specific $CD8^+$ T cells with killing activity via perforin pathway [26], but not DCs and $CD4^+$ T cells recruited via CCL20 chemoattraction, play a major role in Th17-induced therapeutic effect. To date, adoptive T-cell immunotherapy for cancer by using in vitro expanded tumor-infiltrating $CD8^+$ T cells has achieved some degree of success [27, 28]. However, one of the major obstacles in this therapy is only very limited number of transferred $CD8^+$ T cells that eventually infiltrate into tumors [29, 30], which greatly affects its therapeutic efficacy. Therefore, Th17 cells may be useful in enhancing adoptive $CD8^+$ T-cell immunotherapy for cancer.

Th17-polarized cells derived from TRP-1-specific TCR transgenic mice inhibited the growth of large s.c. established B16 melanoma ($\sim$0.6 cm^2, equal to $\sim$7–8 mm in diameter) after adoptive transfer of Th17 cells [15]. However, in our current study, we found that Th17 cells efficiently cured s.c B16 melanoma only in early stage (3 mm), but not well-established stage (6 mm), which is consistent with a previous report on the eradication of 6-day s.c. tumors [31]. The discrepancy in therapeutic efficiency may be due to the different treatment protocols in these reports. In our protocol, we simply i.v. transferred OVA-specific Th17 into B16 melanoma-bearing mice for direct assessment of Th17 therapeutic effect. In their protocol [15], they have combined adoptive transfer of Th17 with an extra-total body sublethal irradiation plus TRP-1 virus and IL-2 administration. Their complex protocol will definitely interfere with the assessment of Th17-mediated therapeutic effect. For example, sublethal irradiation will induce lymphopenia leading to proliferation and prolonged survival of transferred T cells [32, 33] and conversion of Th17 into Th1 [34, 35], whereas TRP-1 virus vaccine alone can activate both Th1 and CTL responses important for tumor rejection [36, 37].

Based upon previous reports and our own findings, we propose the following model for Th17-induced antitumor immunity: (i) Th17 directly stimulates tumor-specific $CD8^+$ T-cell responses via pMHC I and IL-2 signaling, (ii) homing molecule (CXCR4, CCR6, and CD161)-expressing Th17 migrates into tumors [20, 38] by tumor environmental RANTES and MCP-1 chemoattraction [39], (iii) tumor-infiltrating Th17 stimulates tumor tissues to express CCL20 for recruiting CCR6-expressing tumor-specific $CD8^+$ T cells into tumors via CCL20 chemoattraction, and (iv) tumor-specific $CD8^+$ T cells exert direct killing activity to tumor cells via perforin/granzyme B pathway (Fig. 5).

Taken together, our data demonstrate a distinct role of Th17 and Th17-stimulated $CD8^+$ T-cell responses in preventive and therapeutic antitumor immunity, which may greatly impact the development of Th17-based cancer immunotherapy.

Materials and methods

Reagents, antibodies, cell lines, and animals

The biotin-labeled anti-CD4 (GK1.5), CD11c (HL3), CD25 (7D4), CD40L (TRAP1), CD69 (H1.2F3), FasL (CD178), and major histocompatibility complex (MHC) class I (H-2 K^b) (AF6-88) antibodies (Abs) were purchased from BD Biosciences (San Diego, CA). The R-phycoerythrin (PE)-conjugated anti-mouse IFN-γ (XMG1.2), perforin (δG9), and FITC-conjugated IL-17 (TC11-18H10) Abs were obtained from Pharmingen Inc. (Mississauga, Ontario,

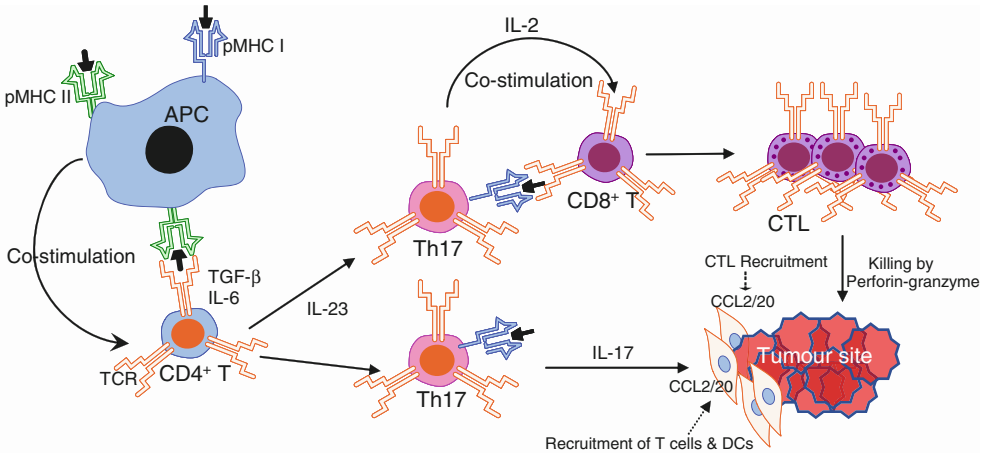

Fig. 5 Mechanism of Th17-mediated antitumor immune responses. Tumor-infiltrating Th17 cells stimulate tumor microenvironment to express CCL2/20 leading to the recruitment of inflammatory cells such as $CD8^+$ CTLs derived from direct Th17 stimulation via pMHC I and IL-2 signaling into the tumor site, which exert direct killing activity to tumor cells via perforin/granzyme B pathway

Canada). The PE-conjugated anti-mouse RORγt (RORg2) Abs were purchased from BioLegend (San Diego, CA). The FITC-conjugated avidin was obtained from Jackson Immuno Research Laboratory Inc. (West Grove, PN). Peptides OVAI ($OVA_{257–264}$) specific for H-$2K^b$, OVAII ($OVA_{323–339}$) specific for Ia^b, and 3LL lung carcinoma antigen (Ag) Mut1 peptide specific for H-$2K^b$ were synthesized by Multiple Peptide Systems (San Diego, CA). The FITC-labeled anti-CD8 Ab and PE-H-$2K^b$/OVAI tetramer were obtained from Beckman Coulter (Mississauga, Ontario, Canada). Recombinant cytokines were obtained from R&D Systems Inc. (Minneapolis, MN). Tumor cell lines including ovalbumin (OVA)-expressing thymoma (EG7) and BL6-10_{OVA} and Ia^b-expressing LB27 were available in our laboratory [40]. Wild-type C57BL/6, IL-$2^{+/-}$, perforin$^{-/-}$, $Ia^{b-/-}$ and H-$2K^{b-/-}$ knockout (KO), diphtheria toxin receptor (DTR)-CD11c transgenic mice [41], and OVA-specific T-cell receptor (TCR) transgenic OTI and OTII mice, which express TCR specific for OVAI and OVAII, respectively, were all purchased from the Jackson Laboratory (Bar Harbor, MA). Homozygous OTII/H-$2K^{b-/-}$ and OTII/IL-$2^{-/-}$ mice were generated by backcrossing the designated gene KO mice onto OTII background. All mice were treated according to Animal Care Committee guidelines of University of Saskatchewan.

Preparation of dendritic cells

Bone marrow–derived dendritic cells (DCs) of C57BL/6 mice were generated in the presence of GM-CSF (20 ng/ml) and IL-4 (20 ng/ml) as described previously [18, 19]. These DCs expressed MHC II, CD40, and CD80, indicating that they were mature DCs. They were then pulsed with OVA (0.3 mg/ml) overnight at 37°C and termed as DC_{OVA}. OVA-pulsed DCs generated from H-$2K^b$ gene KO mice were referred to as ($K^{b-/-}$)DC_{OVA}.

Preparation of OVA-specific T cells

To generate OVA-specific Th17 cells, naïve $CD4^+$ T cells (2×10^5 cells/ml) from OT II mice were stimulated for 3 days with irradiated (4,000 rads) DC_{OVA} (1×10^5 cells/ml) in the presence of IL-6 (10 ng/ml), IL-23 (10 ng/ml), TGF-β (10 ng/ml), and anti-IFN-γ antibody (20 μg/ml). These DCova-activated Th17 cells were purified by positive selection using CD4 microbeads (Miltenyi Biotech, Auburn, CA). ($K^{b-/-}$)DC_{OVA}-activated $CD4^+$ T cells derived from OTII mice were termed ($K^{b-/-}$)Th17, whereas DC_{OVA}-activated $CD4^+$ T cells derived from OTII/IL-$2^{-/-}$ mice were termed (IL-$2^{-/-}$)Th17. Alternatively, naïve $CD4^+$ T cells (2×10^5 cells/ml) from OT II mice were stimulated with CD3/CD28 T-cell expander Dynabeads (Invitrogen) at a ratio of 1:1 (bead:cell) for 4–5 days in the presence of IL-6 (10 ng/ml), IL-23 (10 ng/ml), and TGF-β (10 ng/ml) [42]. The cytokine profiles of the above various gene KO Th17 or CD3/CD28-bead-activated Th17 (bead-Th17) were similar to the cytokine profile of DCova-activated Th17 cells except for the specific molecule deficiency (data not shown). Preparation of DC_{OVA}-activated OVA-specific Th1 cells expressing IL-2, IFN-γ, FasL, and perforin, but not IL-4 and OVA-specific $CD8^+$ T cells, were previously described [19, 43].

Phenotypic characterization of OVA-specific Th17 cells

The above Th17 cells and Th17 cells with various gene KO were stained with antibodies and analyzed by flow cytometry. To measure intracellular expression of cytokines, Th17 were processed using a intracellular staining commercial kit (BD Biosciences, San Diego, CA) and stained with anti-perforin and RORγt Ab or double stained with FITC-conjugated anti-IL-17 Ab and PE-conjugated anti-IFN-γ Ab. Culture supernatants of Th17 re-stimulated

with irradiated (4,000 rads) OVAII peptide-pulsed LB27 cells [18, 19] were analyzed for cytokine expression using ELISA kits (Endogen, Cambridge, MA).

$CD8^+$ T-cell proliferation assays

To assess the functional effect of OVA-specific Th17, we performed in vitro $CD8^+$ T-cell proliferation assay. Two-fold serially diluted irradiated (4,000 rads) DC_{OVA}, Th17, (IL-$2^{-/-}$),Th17, and ($K^{b-/-}$)Th17 cells (0.4×10^5 cells/well) were co-cultured with a constant number of naive OT I $CD8^+$ T cells (1×10^5 cells/well). After culturing for 48 h, overnight thymidine incorporation was quantified by liquid scintillation counting. In in vivo proliferation assay, C57BL/6 or perforin$^{-/-}$ mice (6 per group), or transgenic DTR-CD11c mice (6 per group) with a single dose (4 ng/g mouse body weight) of i.v. diphtheria toxin (DT) treatment 1 day before Th17 transfer were i.v. transferred with DC_{OVA} (1×10^6 cells), DCova-activated Th17 (3×10^6 cells) or Th17 (3×10^6 cells) with various gene KO or CD3/CD28 bead-activated Th17 (bead-Th17) (3×10^6 cells). Six days subsequent to transfer, tail blood samples were stained with FITC-anti-CD8 Ab and PE-H-2 K^b/OVAI tetramer and analyzed by flow cytometry.

Cytotoxicity assays

In in vitro cytotoxicity assay, DC_{OVA}-activated OTII Th17 and Th1 were used as effector (E) cells, while ^{51}Cr-labeled OVAII-pulsed LB27 cells were used as target (T) cells. In another experiment, Th17-activated OTI $CD8^+$ cytotoxic (Tc) cells were used as effector (E) cells, while ^{51}Cr-labeled EG7 were used as target (T) cells in a chromium release assay. CMA and emetin inhibitors were used in Th17-activated OTI $CD8^+$ cytotoxic (Tc) cell cytotoxicity assay to inhibit perforin and FAS ligand–mediated cytotoxicity, respectively [19]. In in vivo cytotoxicity assay, 6 days following Th17 transfer, the Th-transferred C57BL/6 mice (6 per group) were i.v. injected with a 1:1 (OVA-specific $CFSE^{high}$:nonspecific control $CFSE^{low}$) mixture of splenocyte targets. Sixteen hours subsequent to target cell delivery, the proportion of $CFSE^{high}$ and $CFSE^{low}$ target cells remaining in the spleens was analyzed by flow cytometry [21].

Real-time RT–PCR

Total RNA was extracted from BL6-10_{OVA} cells, lung cell fraction from normal or tumor-bearing lungs with Qiagen RNeasy purification kit (Qiagen, Mississauga, Ontario, Canada) as per manufacturer's protocols. The primer sets for real-time PCR analysis of RORγt, CCL2, and CCL20 were designed as previously described [16]. Qiagen quantitative reverse transcription kit (Qiagen) was used to synthesize cDNA, which was then analyzed by real-time quantitative PCR in triplicates by using SYBR Green PCR mastermix (Applied Biosystems, Foster City, CA, USA) in the Stepone Real-time PCR system (Applied Biosystems). Each gene expression was normalized to GAPDH expression level using comparative CT method.

Lung fractionation and cell analysis

Mouse lungs were digested with collagenase D (1 mg/ml, Worthington Biochemical, Freehold, NJ) at 37°C for 30 min and 5 min with 0.01 M EDTA for prevention of DC–T-cell aggregate formation [44]. The cells were separated using Histopaque (Sigma, St. Louis, MO). The middle section of the gradient, which was enriched with leukocytes, was counted and analyzed by flow cytometry, whereas the bottom fraction that was enriched with tumor cells and lung cells was used for RNA extraction.

Animal studies

In preventive immunity model, wild-type C57BL/6 mice (8 per group) were i.v. transferred with OVA-specific Th17 (3×10^6), Th17 (3×10^6) with various gene KO and Th17 ($3X10^6$) with various antibodies. Six days subsequent to transfer, mice were i.v. challenged with BL6-10_{OVA} cells (0.5×10^6). Mice were killed after 3 weeks, and the numbers of metastatic lung tumor colonies were counted [18]. In the lung tumor metastasis therapeutic model, C57BL/6 mice (8 per group) were i.v. injected with BL6-10_{OVA} tumor cells (0.5×10^6). After 5 days of tumor cell injection (5-day lung tumor metastasis), mice were i.v. injected with Th17 or CD3/CD28 bead-activated Th17 (bead-Th17) (3×10^6 cells/mouse) cells. To deplete $CD8^+$ T cells or DCs or to block IL-17, C57BL/6 mice were i.p. injected with anti-CD8 or anti-CD11c or anti-IL-17 Ab (each, 0.5 mg/mouse) 1 day before Th17 transfer and followed by another two injections (once every 3 days). To assess the involvement of host $CD4^+$ T cells or DCs or host $CD8^+$ T-cell's perforin in therapeutic effect, $Ia^{b-/-}$ KO mice (lacking $CD4^+$ T cells) or DTR-CD11c transgenic mice (8 per group) with diphtheria toxin (DT) treatment (a single dose of 4 ng/g body weight of mouse; i.p.) to deplete host $CD11c^+$ DCs or perforin$^{-/-}$ mice were used [45]. Mice were killed on day 16 after i.v. injection of tumor cells. The metastatic lung tumor colonies were counted. In the s.c. tumor therapeutic model, C57BL/6 or $K^{b-/-}$ or $Ia^{b-/-}$ or IFN-$\gamma^{-/-}$ KO mice (8 per group) were s.c. injected with BL6-10_{OVA} tumor cells (0.5×10^6). When s.c. tumors reached 3 or 6 mm in diameter, these mice were i.v. injected with Th17 cells (3×10^6 cells). Tumor growth

was monitored by measuring tumor diameter using caliper; for ethical reasons, all mice with tumors that achieved a size of 1.5 cm in diameter were killed.

Statistical analysis

Mouse survival was analyzed using *log rank* test [46, 47], and all other experiments were tested for statistical differences using unpaired, two-tailed, *Student's t* test. Differences were considered significant if $P < 0.05$.

Acknowledgments This study was supported by a grant of Canadian Institute of Health Research (MOP 79415). Manjunatha Ankathatti Munegowda was supported by Dean's Scholarship of University of Saskatchewan.

References

1. Weaver CT, Hatton RD, Mangan PR, Harrington LE (2007) IL-17 family cytokines and the expanding diversity of effector T cell lineages. Annu Rev Immunol 25:821–852
2. Park H, Li Z, Yang XO, Chang SH, Nurieva R, Wang YH, Wang Y, Hood L, Zhu Z, Tian Q, Dong C (2005) A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. Nat Immunol 6:1133–1141
3. Ye P, Garvey PB, Zhang P, Nelson S, Bagby G, Summer WR, Schwarzenberger P, Shellito JE, Kolls JK (2001) Interleukin-17 and lung host defense against Klebsiella pneumoniae infection. Am J Respir Cell Mol Biol 25:335–340
4. Langrish CL, Chen Y, Blumenschein WM, Mattson J, Basham B, Sedgwick JD, McClanahan T, Kastelein RA, Cua DJ (2005) IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. J Exp Med 201:233–240
5. Yang XO, Nurieva R, Martinez GJ, Kang HS, Chung Y, Pappu BP, Shah B, Chang SH, Schluns KS, Watowich SS, Feng XH, Jetten AM, Dong C (2008) Molecular antagonism and plasticity of regulatory and inflammatory T cell programs. Immunity 29:44–56
6. Langowski JL, Zhang X, Wu L, Mattson JD, Chen T, Smith K, Basham B, McClanahan T, Kastelein RA, Oft M (2006) IL-23 promotes tumour incidence and growth. Nature 442:461–465
7. Kryczek I, Wei S, Zou L, Altuwaijri S, Szeliga W, Kolls J, Chang A, Zou W (2007) Cutting edge: Th17 and regulatory T cell dynamics and the regulation by IL-2 in the tumor microenvironment. J Immunol 178:6730–6733
8. Miyahara Y, Odunsi K, Chen W, Peng G, Matsuzaki J, Wang RF (2008) Generation and regulation of human CD4+ IL-17-producing T cells in ovarian cancer. Proc Natl Acad Sci USA 105:15505–15510
9. Sfanos KS, Bruno TC, Maris CH, Xu L, Thoburn CJ, DeMarzo AM, Meeker AK, Isaacs WB, Drake CG (2008) Phenotypic analysis of prostate-infiltrating lymphocytes reveals TH17 and Treg skewing. Clin Cancer Res 14:3254–3261
10. Zhang B, Rong G, Wei H, Zhang M, Bi J, Ma L, Xue X, Wei G, Liu X, Fang G (2008) The prevalence of Th17 cells in patients with gastric cancer. Biochem Biophys Res Commun 374:533–537
11. Benchetrit F, Ciree A, Vives V, Warnier G, Gey A, Sautes-Fridman C, Fossiez F, Haicheur N, Fridman WH, Tartour E (2002) Interleukin-17 inhibits tumor cell growth by means of a T-cell-dependent mechanism. Blood 99:2114–2121
12. Hirahara N, Nio Y, Sasaki S, Minari Y, Takamura M, Iguchi C, Dong M, Yamasawa K, Tamura K (2001) Inoculation of human interleukin-17 gene-transfected Meth-A fibrosarcoma cells induces T cell-dependent tumor-specific immunity in mice. Oncology 61:79–89
13. Numasaki M, Watanabe M, Suzuki T, Takahashi H, Nakamura A, McAllister F, Hishinuma T, Goto J, Lotze MT, Kolls JK, Sasaki H (2005) IL-17 enhances the net angiogenic activity and in vivo growth of human non-small cell lung cancer in SCID mice through promoting CXCR-2-dependent angiogenesis. J Immunol 175:6177–6189
14. Spolski R, Leonard WJ (2008) Interleukin-21: basic biology and implications for cancer and autoimmunity. Annu Rev Immunol 26:57–79
15. Muranski P, Boni A, Antony PA, Cassard L, Irvine KR, Kaiser A, Paulos CM, Palmer DC, Touloukian CE, Ptak K, Gattinoni L, Wrzesinski C, Hinrichs CS, Kerstann KW, Feigenbaum L, Chan CC, Restifo NP (2008) Tumor-specific Th17-polarized cells eradicate large established melanoma. Blood 112:362–373
16. Martin-Orozco N, Muranski P, Chung Y, Yang XO, Yamazaki T, Lu S, Hwu P, Restifo NP, Overwijk WW, Dong C (2009) T helper 17 cells promote cytotoxic T cell activation in tumor immunity. Immunity 31:787–798
17. Ahmed KA, Munegowda MA, Xie Y, Xiang J (2008) Intercellular trogocytosis plays an important role in modulation of immune responses. Cell Mol Immunol 5:261–269
18. Xiang J, Huang H, Liu Y (2005) A new dynamic model of CD8+ T effector cell responses via CD4+ T helper-antigen-presenting cells. J Immunol 174:7497–7505
19. Umeshappa CS, Huang H, Xie Y, Wei Y, Mulligan SJ, Deng Y, Xiang J (2009) CD4+ Th-APC with acquired peptide/MHC class I and II complexes stimulate type 1 helper CD4+ and central memory CD8+ T cell responses. J Immunol 182:193–206
20. Kryczek I, Banerjee M, Cheng P, Vatan L, Szeliga W, Wei S, Huang E, Finlayson E, Simeone D, Welling TH, Chang A, Coukos G, Liu R, Zou W (2009) Phenotype, distribution, generation, and functional and clinical relevance of Th17 cells in the human tumor environments. Blood 114:1141–1149
21. Huang H, Bi XG, Yuan JY, Xu SL, Guo XL, Xiang J (2005) Combined CD4+ Th1 effect and lymphotactin transgene expression enhance CD8+ Tc1 tumor localization and therapy. Gene Ther 12:999–1010
22. Sutton C, Brereton C, Keogh B, Mills KH, Lavelle EC (2006) A crucial role for interleukin (IL)-1 in the induction of IL-17-producing T cells that mediate autoimmune encephalomyelitis. J Exp Med 203:1685–1691
23. Komiyama Y, Nakae S, Matsuki T, Nambu A, Ishigame H, Kakuta S, Sudo K, Iwakura Y (2006) IL-17 plays an important role in the development of experimental autoimmune encephalomyelitis. J Immunol 177:566–573
24. Zou W, Restifo NP (2010) T(H)17 cells in tumour immunity and immunotherapy. Nat Rev Immunol 10:248–256
25. Laurence A, Tato CM, Davidson TS, Kanno Y, Chen Z, Yao Z, Blank RB, Meylan F, Siegel R, Hennighausen L, Shevach EM, O'Shea JJ (2007) Interleukin-2 signaling via STAT5 constrains T helper 17 cell generation. Immunity 26:371–381
26. Bolitho P, Voskoboinik I, Trapani JA, Smyth MJ (2007) Apoptosis induced by the lymphocyte effector molecule perforin. Curr Opin Immunol 19:339–347
27. Rosenberg SA, Dudley ME (2004) Cancer regression in patients with metastatic melanoma after the transfer of autologous antitumor lymphocytes. Proc Natl Acad Sci USA 101(Suppl 2):14639–14645
28. Dudley ME, Rosenberg SA (2007) Adoptive cell transfer therapy. Semin Oncol 34:524–531

29. Ogawa M, Umehara K, Yu WG, Uekusa Y, Nakajima C, Tsujimura T, Kubo T, Fujiwara H, Hamaoka T (1999) A critical role for a peritumoral stromal reaction in the induction of T-cell migration responsible for interleukin-12-induced tumor regression. Cancer Res 59:1531–1538
30. Mukai S, Kjaergaard J, Shu S, Plautz GE (1999) Infiltration of tumors by systemically transferred tumor-reactive T lymphocytes is required for antitumor efficacy. Cancer Res 59:5245–5249
31. Kottke T, Sanchez-Perez L, Diaz RM, Thompson J, Chong H, Harrington K, Calderwood SK, Pulido J, Georgopoulos N, Selby P, Melcher A, Vile R (2007) Induction of hsp70-mediated Th17 autoimmunity can be exploited as immunotherapy for metastatic prostate cancer. Cancer Res 67:11970–11979
32. Brown IE, Blank C, Kline J, Kacha AK, Gajewski TF (2006) Homeostatic proliferation as an isolated variable reverses CD8+ T cell anergy and promotes tumor rejection. J Immunol 177:4521–4529
33. Kline J, Brown IE, Zha YY, Blank C, Strickler J, Wouters H, Zhang L, Gajewski TF (2008) Homeostatic proliferation plus regulatory T-cell depletion promotes potent rejection of B16 melanoma. Clin Cancer Res 14:3156–3167
34. Nurieva R, Yang XO, Chung Y, Dong C (2009) Cutting edge: in vitro generated Th17 cells maintain their cytokine expression program in normal but not lymphopenic hosts. J Immunol 182:2565–2568
35. Martin-Orozco N, Chung Y, Chang SH, Wang YH, Dong C (2009) Th17 cells promote pancreatic inflammation but only induce diabetes efficiently in lymphopenic hosts after conversion into Th1 cells. Eur J Immunol 39:216–224
36. Zhang S, Bernard D, Khan WI, Kaplan MH, Bramson JL, Wan Y (2009) CD4+ T-cell-mediated anti-tumor immunity can be uncoupled from autoimmunity via the STAT4/STAT6 signaling axis. Eur J Immunol 39:1252–1259
37. Overwijk WW, Lee DS, Surman DR, Irvine KR, Touloukian CE, Chan CC, Carroll MW, Moss B, Rosenberg SA, Restifo NP (1999) Vaccination with a recombinant vaccinia virus encoding a "self" antigen induces autoimmune vitiligo and tumor cell destruction in mice: requirement for CD4(+) T lymphocytes. Proc Natl Acad Sci USA 96:2982–2987
38. Kryczek I, Bruce AT, Gudjonsson JE, Johnston A, Aphale A, Vatan L, Szeliga W, Wang Y, Liu Y, Welling TH, Elder JT, Zou W (2008) Induction of IL-17+ T cell trafficking and development by IFN-gamma: mechanism and pathological relevance in psoriasis. J Immunol 181:4733–4741
39. Su X, Ye J, Hsueh EC, Zhang Y, Hoft DF, Peng G (2010) Tumor microenvironments direct the recruitment and expansion of human Th17 cells. J Immunol 184:1630–1641
40. He T, Zong S, Wu X, Wei Y, Xiang J (2007) CD4+ T cell acquisition of the bystander pMHC I colocalizing in the same immunological synapse comprising pMHC II and costimulatory CD40, CD54, CD80, OX40L, and 41BBL. Biochem Biophys Res Commun 362:822–828
41. Jung S, Unutmaz D, Wong P, Sano G, De los Santos K, Sparwasser T, Wu S, Vuthoori S, Ko K, Zavala F, Pamer EG, Littman DR, Lang RA (2002) In vivo depletion of CD11c(+) dendritic cells abrogates priming of CD8(+) T cells by exogenous cell-associated antigens. Immunity 17:211–220
42. Purvis HA, Stoop JN, Mann J, Woods S, Kozijn AE, Hambleton S, Robinson JH, Isaacs JD, Anderson AE, Hilkens CM (2010) Low-strength T-cell activation promotes Th17 responses. Blood 116:4829–37
43. Xia D, Hao S, Xiang J (2006) CD8+ cytotoxic T-APC stimulate central memory CD8+ T cell responses via acquired peptide-MHC class I complexes and CD80 costimulation, and IL-2 secretion. J Immunol 177:2976–2984
44. Vremec D, Pooley J, Hochrein H, Wu L, Shortman K (2000) CD4 and CD8 expression by dendritic cell subtypes in mouse thymus and spleen. J Immunol 164:2978–2986
45. Prlic M, Hernandez-Hoyos G, Bevan MJ (2006) Duration of the initial TCR stimulus controls the magnitude but not functionality of the CD8+ T cell response. J Exp Med 203:2135–2143
46. Xiang J, Moyana T (1998) Cytotoxic CD4+ T cells associated with the expression of major histocompatibility complex class II antigen of mouse myeloma cells secreting interferon-gamma are cytolytic in vitro and tumoricidal in vivo. Cancer Gene Ther 5:313–320
47. Sas S, Chan T, Sami A, El-Gayed A, Xiang J (2008) Vaccination of fiber-modified adenovirus-transfected dendritic cells to express HER-2/neu stimulates efficient HER-2/neu-specific humoral and CTL responses and reduces breast carcinogenesis in transgenic mice. Cancer Gene Ther 15:655–666

CHAPTER 3

3. A Distinct Roles of $CD4^+$ Th17 and Th17-Stimulated $CD8^+$ CTL in the Pathogenesis of Type 1 Diabetes and Experimental Autoimmune Encephalomyelitis.

Brief introduction to chapter 3

This chapter addresses the mechanism of stimulatory effect of Th17 cells on self antigen specific $CD8^+$ CTL responses in EAE and T1D models. Earlier studies had shown that both $CD4^+$ Th17 cells and $CD8^+$ CTLs are involved in T1D and EAE. However, their relationship in pathogenesis was still elusive. In this study, we found that $CD4^+$ Th17 cells stimulate OVA- and MOG-specific $CD8^+$ CTL responses, respectively, in mice. When $CD4^+$ Th17 cells were transferred into (i) transgenic RIP-mOVA and wild-type C57BL/6 mice, where both $CD4^+$ Th17 cells and $CD4^+$ Th17-stimulated $CD8^+$ CTLs existed, (ii) RIP-mOVA mice treated with anti-CD8 antibody to eliminate Th17-stimulated $CD8^+$ T cells, and (iii) H-$2Kb^{-/-}$ mice, lacking the ability to generate Th17-stimulated $CD8^+$ T cells, we found that OVA specific $CD4^+$ Th17 cells stimulated $CD8^+$ CTLs, but not $CD4^+$ Th17 cells themselves, induce diabetes in RIP-mOVA, whereas MOG-specific $CD4^+$ Th17 lymphocytes, but not $CD4^+$ Th17-activated $CD8^+$ CTL induced EAE in C57BL/6 mice. This study demonstrated the distinct roles of $CD4^+$ Th17 and $CD4^+$ Th17 stimulated $CD8^+$ CTLs in the pathogenesis of autoimmune diseases, which may have great impact on the overall understanding of $CD4^+$ Th17 cells in the pathogenesis of autoimmune diseases.

Reprinted with consent from Journal of Clinical Immunology 2011;31(5):811-26.

J Clin Immunol
DOI 10.1007/s10875-011-9549-z

A Distinct Role of $CD4^+$ Th17- and Th17-Stimulated $CD8^+$ CTL in the Pathogenesis of Type 1 Diabetes and Experimental Autoimmune Encephalomyelitis

Manjunatha Ankathatti Munegowda · Yulin Deng · Rajni Chibbar · Qingyong Xu · Andrew Freywald · Sean J. Mulligan · Sylvia van Drunen Littel-van den Hurk · Deming Sun · Sidong Xiong · Jim Xiang

Received: 14 February 2011 / Accepted: 18 May 2011

Abstract Both $CD4^+$ Th17-cells and $CD8^+$ cytotoxic T lymphocytes (CTLs) are involved in type 1 diabetes and experimental autoimmune encephalomyelitis (EAE). However, their relationship in pathogenesis of these autoimmune diseases is still elusive. We generated ovalbumin (OVA)- or myelin oligodendrocyte glycoprotein (MOG)-specific Th17 cells expressing RORγt and IL-17 by in vitro co-culturing OVA-pulsed and MOG_{35-55} peptide-pulsed dendritic cells (DC_{OVA} and DC_{MOG}) with $CD4^+$ T cells derived from transgenic OTII and MOG-T cell receptor mice, respectively. We found that these Th17 cells when transferred into C57BL/6 mice stimulated OVA- and MOG-specific CTL responses, respectively. To assess the above question, we adoptively transferred OVA-specific Th17 cells into transgenic rat insulin promoter (RIP)-mOVA mice or RIP-mOVA mice treated with anti-CD8 antibody to deplete Th17-stimulated $CD8^+$ T cells. We demonstrated that OVA-specific Th17-stimulated CTLs, but not Th17 cells themselves, induced diabetes in RIP-mOVA. We also transferred MOG-specific Th17 cells into C57BL/6 mice and H-$2K^{b-/-}$ mice lacking of the ability to generate Th17-stimulated CTLs. We further found that MOG-specific Th17 cells, but not Th17-activated CTLs induced EAE in C57BL/6 mice. Taken together, our data indicate a distinct role of Th17 cells and Th17-stimulated CTLs in the pathogenesis of TID and EAE, which may have great impact on the overall understanding of Th17 cells in the pathogenesis of autoimmune diseases.

Keywords Th17 · pMHC I complex · $CD8^+$ CTL · type 1 diabetes · experimental autoimmune encephalitis

M. Ankathatti Munegowda · Y. Deng · J. Xiang (✉)
Research Unit, Saskatchewan Cancer Agency,
Department of Oncology, Saskatoon Cancer Center,
20 Campus Drive,
Saskatoon, Saskatchewan S7N 4H4, Canada
e-mail: jim.xiang@saskcancer.ca

M. Ankathatti Munegowda · R. Chibbar · Q. Xu · A. Freywald · J. Xiang
Department of Pathology, University of Saskatchewan,
Saskatoon, Saskatchewan, Canada

S. J. Mulligan
Department of Physiology, University of Saskatchewan,
Saskatoon, Saskatchewan, Canada

S. van Drunen Littel-van den Hurk
Vaccine and Infectious Disease Organization,
University of Saskatchewan,
Saskatoon, Saskatchewan, Canada

D. Sun
Doheny Eye Institute, Keck School of Medicine,
University of Southern California,
Los Angeles, CA, USA

S. Xiong
Institute of Medical Sciences, Soocow University,
Suzhou, China

Introduction

$CD4^+$ Th17 lymphocytes have unique cytokine expression profile, transcriptional regulation, and biological function,

Published online: 15 June 2011

and represent an independent lineage of $CD4^+$ Th cells [1, 2]. The discovery of $CD4^+$ Th17 subset not only changes the classical Th1/Th2 paradigm of Th differentiation, but also markedly facilitates our understanding of immune responses under both physiological and pathological conditions [3, 4]. The differentiation and regulation of Th17 cells have been extensively studied. Transcriptional factors RORγt and STAT3 are critical and are required for the development of Th17 cells [5, 6], and cytokine transforming growth factor (TGF)-β, IL-6, and IL-21 are critical for initiation and differentiation of Th17 cells [7–10].

Accumulated data suggest that Th17 cells play an important role in host defense against microbial infections and in the pathogenesis of autoimmune diseases such as type 1 diabetes (T1D) and experimental autoimmune encephalomyelitis (EAE) [11]. In T1D, the involvement of $CD8^+$ T cells in pathogenesis has been recognized. T1D is caused by autoimmune destruction of insulin-producing islet β cells of the pancreas [12]. Antigen-specific $CD8^+$ T cells have been found in the peripheral blood of T1D patients [13]. Studies in a nonobese diabetic (NOD) mouse model of T1D have indicated that $CD8^+$ T cells inflict damage to islet β cells both at the early stage in diabetes development and at the final effector phase of the disease [14–16]. On the contrary, there is some preliminary evidence showing that Th17 cells may be considered as a contributing factor in the pathogenic process of T1D. For example, it has been found that IL-17 is expressed in the pancreas of T1D mouse model [17], and the reduction of Th17 cells with the induction of IFN-γ inhibited IL-17 production and restored normoglycemia at the prediabetic stage [18]. However, the relative contribution of Th17 cells and CTLs in T1D has not been addressed.

EAE is a rodent model that has been valuable for the characterization of the immunopathogenic processes of human multiple sclerosis (MS). Attention has originally been focused on the role of $CD4^+$ T cells in the induction of EAE because susceptibility to MS is associated with MHC class II genes [19–21] and the critical role of $CD4^+$ Th17 cells in pathogenesis of EAE has, eventually, been demonstrated [22–26]. Recently, both $CD4^+$ Th17 and $CD8^+$ T cells have been identified in active lesions in brains of MS patients [27]. It has been shown that myelin oligodendrocyte glycoprotein (MOG)-specific $CD8^+$ T cell responses are involved prior to and after the onset of EAE [28, 29], and adoptive transfer of MOG-specific $CD8^+$ T cells can also induce EAE [30–32]. However, (a) the potential relationship between the pathogenic $CD4^+$ Th17 and $CD8^+$ T cells and (b) the extent of their relative involvement in the pathogenesis of T1D and EAE are still not very clear.

Intercellular membrane transfer through trogocytosis plays an important role in immune modulation [33]. We have recently demonstrated that ovalbumin (OVA)-specific T cell receptor (TCR) transgenic OT II mouse $CD4^+$ Th1 cells in vitro activated by OVA-specific dendritic cells (DC_{OVA}) acquired DC_{OVA}'s peptide-MHC (pMHC) I and co-stimulatory molecules and became capable of directly stimulating antigen-specific $CD8^+$ cytotoxic T lymphocyte (CTL) responses leading to antitumor immunity in wild-type C57BL/6 mice [34, 35] and diabetes in transgenic rat insulin promoter (RIP)-mOVA mice expressing islet β-cell antigen OVA [36, 37]. Based on the phenomenon of trogocytosis, we speculate that $CD4^+$ Th17 cells may similarly acquire pMHC I by APC activation and become capable of stimulating $CD8^+$ T cell responses in autoimmune diseases such as T1D and EAE.

In this study, we generated OVA-specific $CD4^+$ Th17 cells and MOG-specific $CD4^+$ MOG-TCR-Th17 cells by cultivating OVA-pulsed dendritic cells (DC_{OVA}) or MOG peptide-pulsed dendritic cells (DC_{MOG}) with $CD4^+$ T cells derived from T cell receptor transgenic OTII mice or MOG-TCR transgenic mice, respectively. We also generated MOG-specific $CD4^+$ Th17 by culturing MOG_{35-55} peptide-pulsed splenocytes with $CD4^+$ T cells purified from C57BL/6 mice with MOG_{35-55} immunization-induced EAE in presence of IL-6/IL-23/TGF-β. Through the utilization of this experimental model, we found that $CD4^+$ Th17 cells acquired pMHC I in the process of activation by dendritic cells (DCs) and became capable of stimulating OVA or MOG-specific $CD8^+$ CTL responses, when transferred into the mice. To assess the pathogenic behavior of $CD4^+$ Th17- and Th17-induced $CD8^+$ T cells in T1D and EAE, we transferred OVA- and MOG-specific $CD4^+$ Th17 cells into RIP-mOVA and C57BL/6 mice, where both $CD4^+$ Th17- and Th17-stimulated $CD8^+$ CTL populations co-existed. We also used anti-CD8 antibody treatment in RIP-mOVA mice to deplete Th17-stimulated $CD8^+$ T cells or employed H-$2K^{b-/-}$ mice lacking the endogenous $CD8^+$ population to independently assess the effect of $CD4^+$ Th17 cells or of Th17-stimulated $CD8^+$ CTLs in pathogenesis of T1D and EAE. These experiments have clearly showed a distinct role of $CD4^+$ Th17- and Th17-stimulated $CD8^+$ CTLs in pathogenesis of autoimmune diseases demonstrating that T1D is directly mediated by $CD8^+$ lymphocytes, whereas EAE appears to be induced by the $CD4^+$ Th17 cells.

Materials and Methods

Reagents, Antibodies, Cell Lines, and Animals

The biotin-labeled anti-CD4 (GK1.5), CD11c (HL3), CD25 (7D4), CD40L (TRAP1), CD69 (H1.2 F3), FasL (CD178), and major histocompatibility complex (MHC) class I (K^b) (AF6-88) and II (Ia^b) (KH74) antibodies (Abs)

were purchased from BD-Biosciences (San Diego, CA). Anti-mouse IFN-γ (XMG1.2), IL-17 (TC11-18H10), and perforin (δG9) Abs were obtained from Pharmingen Inc. (Mississauga, Ontario, Canada).The PE-conjugated anti-mouse T-bet (4B10) and RORγt (RORg2) Abs were purchased from BioLegend (San Diego, CA). The FITC-conjugated avidin was obtained from Jackson Immuno Research Laboratory Inc, West Grove, PN. Chicken OVA protein was purchased from Sigma, St. Louis, MO. Various peptides including OVAI ($OVA_{257-264}$, SIINFEKL) peptide specific for H-2K^b, OVAII ($OVA_{323-339}$, ISQAVHAA-HAAHAEINEAGR) peptide specific for Ia^b, 3LL lung carcinoma antigen Mut1 (FEQNTAQP) peptide specific for H-2K^b, MOG peptide (MOG_{35-55}, MEVGWYRSPFSRVVH-LYRNGK) specific for both H-2K^b and Ia^b, and MOGI (MOG_{41-50}, RSPFSRVVHL) peptide specific for H-2K^b were synthesized by Multiple Peptide Systems (San Diego, CA). The FITC-labeled anti-CD8 Ab and PE-H-2K^b/OVAI tetramer were obtained from Beckman Coulter, Mississauga, Ontario, Canada. PE-H-2D^b/MOGI pentamer was obtained from Proimmune, Oxford, UK. The recombinant granulocyte macrophage colony-stimulating factor (GM-CSF), IL-2, IL-4, IL-6, IL-23, TGF-β, and anti-IL-2 antibody were obtained from R&D Systems Inc, Minneapolis, MN. Thymoma cell lines EL4 and OVA-expressing EG7 were obtained from American type culture collection (ATCC, Rockville, MD). Wild-type C57BL/6 mice were obtained from Charles River Laboratories (St. Laurent, Quebec, Canada). The OVA-specific TCR transgenic OTII mice, and Ia$^{b-/-}$, H-2K$^{b-/-}$, and perforin$^{-/-}$ knockout (KO) mice were purchased from the Jackson Laboratory (Bar Harbor, MA). Homozygous OTII/H-2K$^{b-/-}$ mice were generated by backcrossing the designated H-2K$^{b-/-}$ KO mice onto OTII background for two generations. The homozygosity was confirmed through PCR analysis according to Jackson laboratory's protocols. Transgenic RIP-mOVA mice with C57BL/6 background were obtained from Dr. W. Heath, Walter and Eliza Hall Institute of Medical Research (Melbourne, Australia). These transgenic RIP-mOVA mice express OVA under the control of RIP. They are transgenic for truncated OVA gene that is expressed as a membrane bound molecule in pancreatic islets, kidney proximal tubules, and testis of male mice [38]. All mice were housed in the animal facility at the Saskatoon Cancer Center and treated according to Animal Care Committee guidelines of University of Saskatchewan.

Preparation of Dendritic Cell and OVA-Specific CD4$^+$ Th1 and CD8$^+$ Tc1 Cells

Bone marrow-derived DCs were generated in presence of GM-CSF (20 ng/ml) and IL-4 (20 ng/ml) as described previously [39]. DC derived from wild-type C57BL/6 mice were pulsed with OVA (0.3 mg/ml) overnight at 37°C, and termed as DC_{OVA}. DC derived from MOG-TCR transgenic mice were pulsed with MOG_{35-55} peptide (20 μg/ml) for 2 h at 37°C, and termed as DC_{MOG}. OVA-pulsed DC generated from H-2K$^{b-/-}$ was referred to as (K$^{b-/-}$)DC_{OVA}. The preparation of DC_{OVA}-activated OVA-specific OT II CD4$^+$ type 1 help T (Th1) and OT I CD8$^+$ type 1 cytotoxic T (Tc1) cells was previously described [35, 40].

OVA- and MOG-specific CD4$^+$ Th17 Cell Preparation

Spleens were removed from OT II, transgenic MOG-TCR, and EAE-induced mice, and were mechanically disrupted to obtain a single-cell suspension. The erythrocytes were lysed using 0.84% ammonium chloride. Naïve T cells were enriched by passing through nylon wool columns (C&A Scientific Inc, Mannose, VA). Naïve OVA-specific CD4$^+$ T cells were then purified by negative selection using anti-mouse CD8 (Ly2) paramagnetic beads (DYNAL Inc, Lake Success, NY). To generate OVA-specific CD4$^+$ Th17 cells, naïve CD4$^+$ T cells (2×10^5 cells/ml) from OT II mice were stimulated for 3 days with irradiated (4,000 rads) DC_{OVA} (1×10^5 cells/ml) in the presence of IL-6 (10 ng/ml), IL-23 (10 ng/ml), TGF-β (5 ng/ml), and anti-IFN-γ antibody (20 μg/ml). These in vitro-activated CD4$^+$ Th17 cells were separated by Ficoll-Paque (Sigma) density gradient centrifugation, further purified by positive selection using CD4-microbeads (Miltenyi Biotech, Auburn, CA). In vitro (K$^{b-/-}$)DC_{OVA}-activated CD4$^+$ T cells derived from OTII mice with K$^{b-/-}$ KO were termed CD4$^+$ (K$^{b-/-}$)Th17 cells. The cytokine profiles of the above (K$^{b-/-}$)Th17 were similar to CD4$^+$ Th17 cells (data not shown). MOG-specific CD4$^+$ Th17 cells were generated by incubation of irradiated (4,000 rad) wild-type C57BL6 mouse splenocytes with CD4$^+$ T cells purified from spleens of MOG_{35-55} immunization-induced EAE mice with clinical score ≥2.5 at 1:1 ratio in presence of MOG_{35-55} peptide (20 μg/ml), IL-6 (10 ng/ml), IL-23 (10 ng/ml), and TGF-β (5 ng/ml). MOG-specific CD4$^+$ MOG-TCR-Th17 cells were generated by co-stimulating naïve CD4$^+$ T cells (2×10^5 cells/ml) from MOG-TCR transgenic mice with irradiated (4,000 rads) DC_{MOG} (1×10^5 cells/ml) in the presence of IL-6 (10 ng/ml), IL-23 (10 ng/ml), TGF-β (5 ng/ml), and anti-IFN-γ antibody (20 μg/ml) for 3 days. Three days subsequent to incubation, MOG-specific CD4$^+$ Th17 and CD4$^+$ MOG-TCR-Th17 cells were purified by positive selection using CD4-microbeads.

Phenotypic Characterization of OVA- and MOG-specific CD4$^+$ Th17 Cells

The above CD4$^+$ Th17 cells were stained with a panel of Abs and analyzed for expression of various cell-surface

molecules by flow cytometry. To measure intracellular expression of cytokines, $CD4^+$ Th17 cells were processed using a commercial kit (Cytofix/CytoPerm Plus with GolgiPlug; BD-Biosciences, San Diego, CA) and stained with PE-conjugated anti-IFN-γ, IL-17, perforin, T-bet, and RORγt Abs. Culture supernatants of these OVA-specific and MOG-specific $CD4^+$ Th17 cells re-stimulated with irradiated (4,000 rad) OVAII peptide-pulsed and MOG peptide-pulsed LB27 cells, respectively, were analyzed for cytokine expression using ELISA kits (Endogen, Cambridge, MA).

$CD8^+$ T Cell Proliferation Assays

To assess the functional effect of OVA-specific $CD4^+$ Th17, we performed in vitro $CD8^+$ T cell proliferation assay. Irradiated (4,000 rad) DC_{OVA}, $CD4^+$ Th17, $CD4^+$ Th17 with anti-Il-2 antibody, and ($K^{b-/-}$)Th17 cells (0.4×10^5 cells/well) were co-cultured with a constant number of naive OT I $CD8^+$ T cells (1×10^5 cells/well). After culturing for 48 h, an overnight thymidine incorporation was determined by liquid scintillation counting. In in vivo proliferation assay, irradiated (400 rad) RIP-mOVA or perforin$^{-/-}$ mice (six mice per group) were i.v. adoptively transferred with DC_{OVA} (1×10^6 cells), $CD4^+$ Th17 cells (3×10^6 cells), or $CD4^+$ ($K^{b-/-}$)Th17 cells. Six days subsequent to adoptive transfer, mouse tail blood samples and pancreatic lymph node cell suspensions were stained with FITC-anti-CD8 Ab and PE-H-2K^b/OVAI tetramer, and analyzed by flow cytometry. In another set of experiments, tail blood samples of mice immunized with MOG_{35-55} peptide or injected with MOG-specific $CD4^+$ Th17 cells (3×10^6 cells) and $CD4^+$ MOG-TCR-Th17 cells (3×10^6 cells) were stained using FITC-anti-CD8 Ab and PE-H2-D^b/RSPFSRVVHL pentamer (Proimmune, Oxford, UK) and analyzed by flow cytometry 6 days after MOG_{35-55} peptide immunization or Th17 cell injection.

Cytotoxicity Assays

In in vitro cytotoxicity assay, Th17-activated OTI $CD8^+$ Tc1 cells were used as effector (E) cells, while ^{51}Cr-labeled EG7 and EL4 cells were used as target (T) cells in a chromium release assay. For testing the killing mechanisms, the effector cells were preincubated with CMA (1 μM) and emetin (5 μM) for 2 h before incubation with the target cells to prevent perforin and Fas/FasL interaction-mediated cytotoxicity. Specific killing was calculated as: 100× [(experimental cpm−spontaneous cpm)/(maximal cpm−spontaneous cpm) as previously described [34]. In in vivo cytotoxicity assay, C57BL/6 mouse splenocytes were incubated with either high (3.0 μM, $CFSE^{high}$) and low (0.6 μM, $CFSE^{low}$) concentrations of CFSE, and pulsed with OVAI or MOGI peptide and Mut1 peptide, respectively, and i.v. injected at 1:1 ratio into the OVA-specific or MOG-specific $CD4^+$ Th17 cell-transferred and MOG peptide-immunized mice. Sixteen hours after target cell delivery, the spleens were removed, and residual $CFSE^{high}$ and $CFSE^{low}$ target cells remaining in recipient spleens were sorted and analyzed by flow cytometry.

RT-PCR

Total RNA was extracted with Qiagen RNeasy purification kit (Qiagen, Mississauga, Ontario, Canada) as per manufacturer's protocol. Qiagen quantitative reverse transcription kit (Qiagen) was used to synthesize cDNA. Later, RORγt and GAPDH were analyzed by conventional PCR analysis. Following primer pairs were used for RT-PCR; RORγt: 5′ GCGGAGCAGACACACTTACA 3′, 5′ TTGGCAAACTC CACCACATA 3′ and GAPDH: 5′ CAGGTTGTCTCCTGC GACTT 3′, 5′ CTTGCTCAGTGTCCTTGCTG.3′. The protocol employed for amplification of mRNA comprised: 1 cycle of 94°C (5 min) and 25 cycles of 94°C (1 min), 52°C (1 min), and 72°C (1 min). All PCR reaction products were resolved using ethidium bromide stained 1% agarose gels.

EAE Induction

EAE was induced in wild-type C57BL/6 mice (15 mice/group) by s.c. injection over four sites in the flank with MOG_{35-55} peptide (200 μg/mouse) emulsified in CFA containing 0.6 mg mycobacterium tuberculosis (BD-Biosciences, San Diego, CA). To assess whether $CD4^+$ or $CD8^+$ T cells are the major pathogenic effector T cells in EAE, $Ia^{b-/-}$, and H-2$K^{b-/-}$ gene KO mice (ten mice/group) lacking $CD4^+$ and $CD8^+$ T cells were similarly s.c. immunized with MOG_{35-55} peptide. Two days after immunization, these MOG-sensitized mice were i.p. injected with 400 ng pertussis toxin (PT; Sigma) [31]. EAE was also induced by i.v. adoptive transfer of MOG-specific $CD4^+$ Th17 and $CD4^+$ MOG-TCR-Th17 cells (5×10^6 cells) into C57BL/6 or H-2$K^{b-/-}$ mice. Mice were examined daily for clinical signs. Mice were scored on scale of 0 to 5: 0, no clinical sign; 0.5, partially limp tail; 1, limp/flaccid tail; 2, moderate hind limb weakness; 2.5, one hind limb paralyzed; 3, both hind limbs paralyzed; 3.5, hind limbs paralyzed and weakness in forelimbs; 4, forelimbs paralyzed; and 5, moribund/death [31]. The analysis was performed on the raw data that included all clinical scores for each mouse at each time point in each group.

Histopathology

Pancreas was collected in 10% formalin from RIP-mOVA mice injected with $CD4^+$ Th17 and PBS. Pancreatic tissue

sections were stained with hematoxylin and eosin (H&E) and slides were assessed for inflammatory cell infiltration and tissue destruction in a blind fashion. EAE mice were extensively perfused with ice-cold PBS with 2 U/ml heparin (Sigma), and spinal cords were harvested and fixed in formalin. Sections (6 mm) were stained with Luxol fast blue (myelin stain) along with H&E counterstaining. Slides were assessed in a blind fashion for inflammation: 0, none; 1, a few inflammatory cells; 2, organization of perivascular infiltrates; and 3, increasing severity of perivascular cuffing with extension into adjacent tissue. For demyelination: 0, none; 1, rare foci; 2, a few areas of demyelination; and 3, large (confluent) areas of demyelination.

Statistical Analysis

Mouse survival and clinical score were analyzed using Log rank and Mann–Whitney *U* test [41, 42], respectively, and all other experiments were tested for statistical differences using unpaired, two tailed, Student's *t* test. Differences were considered significant if $p<0.05$.

Results

$CD4^+$ Th17 Cells Acquire pMHC I Complexes from DC_{OVA} in the Course of Activation

To activate naïve OT II $CD4^+$ T cells, we co-incubated them with irradiated DC_{OVA} in the presence of the IL-23/IL-6/TGF-β/anti-IFN-γ antibody cocktail. While naïve OT II $CD4^+$ T cells did not express CD25, CD40L, CD69 and Ia^b, the co-incubated $CD4^+$ lymphocytes acquired the above molecules (Fig. 1a), which clearly confirmed their activation status. The activated $CD4^+$ also expressed the cell-surface FasL, intranuclear RORγt [43], and intracellular perforin, IL-17 (Fig. 1a, b), but not IL-4, indicating that they represented the $CD4^+$ Th17 cells. To further confirm this, we performed RT-PCR analysis to show that these cells express transcription factor RORγt (Fig. 1c), but not T-bet (data not shown). ELISA assays also revealed the $CD4^+$ Th17 nature of the activated cell, since they proved to secrete the IL-2 (2.8 ng/ml), IL-6 (4.5 ng/ml), IL-17 (1.8 ng/ml), and TGF-β (0.2 ng/ml) cytokines. No $CD11c^+$ DC_{OVA} contamination could be observed in these $CD4^+$ Th17 cell populations (Fig. 1d). We previously showed that $CD4^+$ Th1 cells acquired DC's pMHC complexes in the course of DC activation [35]. In this study, we also showed that $CD4^+$ Th17 cells resulting from DC_{OVA} activation did display some DC's molecules such as pMHC I complexes (Fig. 1a), whereas $CD4^+$ ($K^{b-/-}$) Th17 cells obtained by co-incubation with pMHC I-deficient ($K^{b-/-}$)DC_{OVA} did not (Fig. 1e) but were activated similar to $CD4^+$ Th17 cells (data not shown), indicating that $CD4^+$ T cells acquire pMHC I complexes from DC_{OVA} upon co-culturing.

$CD4^+$ Th17 Cells Stimulate Effector $CD8^+$ CTL Responses In Vitro

Our further work showed that DC_{OVA}-activated $CD4^+$ Th17 cells with acquired pMHC I also stimulated in vitro OT I $CD8^+$ T cell proliferation in a dose-dependent fashion (Fig. 2a). Interestingly, $CD4^+$ ($K^{b-/-}$)Th17 cells without acquired pMHC I failed in stimulation of $CD8^+$ T cell proliferation. To assess whether $CD4^+$ Th17-activated $CD8^+$ T cells have any functional effect, we performed a chromium release assay, in which $CD4^+$ Th17-activated $CD8^+$ T cells and OVA-expressing EG7 tumor cells were used as effector and target cells, respectively. We found that $CD4^+$ Th17-activated $CD8^+$ T cells showed killing activity to OVA-expressing EG7 tumor cells, but not to the control EL4 tumor cells without OVA expression (Fig. 2b), indicating that their killing activities are specific for OVA. To assess the pathway responsible for the killing activity of $CD8^+$ T cells, we preincubated effector $CD8^+$ T cells with CMA or emetin to prevent perforin- and Fas/FasL interaction-mediated cytotoxicity. We found that CMA but not emetin treatment significantly abolished $CD8^+$ T cells' killing activity ($p<0.05$), indicating that the killing activity of $CD4^+$ Th17-stimulated CTLs was mediated by the perforin pathway.

$CD4^+$ Th17 Cells Stimulate Effector $CD8^+$ CTL Responses In Vivo in RIP-mOVA Mice

To assess the ability of $CD4^+$ Th17 cells to induce in vivo $CD8^+$ T cell proliferation, we performed an OVA-specific tetramer staining assay in transgenic RIP-mOVA mice adoptively transferred with $CD4^+$ Th17 cells [35]. As shown in Fig. 2c, $CD4^+$ Th17 cells stimulated in vivo proliferation of OVA-specific $CD8^+$ T cells accounting for 0.68% and 1.18% of total $CD8^+$ T cell population in peripheral blood and pancreatic lymph nodes, respectively. To investigate the role of acquired pMHC I, we repeated the above assay using ($K^{b-/-}$)DC_{OVA}-activated $CD4^+$ ($K^{b-/-}$)Th17 cells, lacking acquired pMHC I. We found that $CD4^+$ ($K^{b-/-}$)Th17 cells completely lost their in vivo stimulatory effect, indicating that the acquired pMHC I complexes play an important role in targeting $CD4^+$ Th17's stimulatory effect onto $CD8^+$ T cells. To assess the influence of $CD4^+$ Th17 cell-induced $CD8^+$ T cell differentiation into CTLs, we performed the in vivo cytotoxicity assay. This assay monitored eradication of an adoptively transferred target population of splenocytes in RIP-mOVA mice adoptively transferred with $CD4^+$

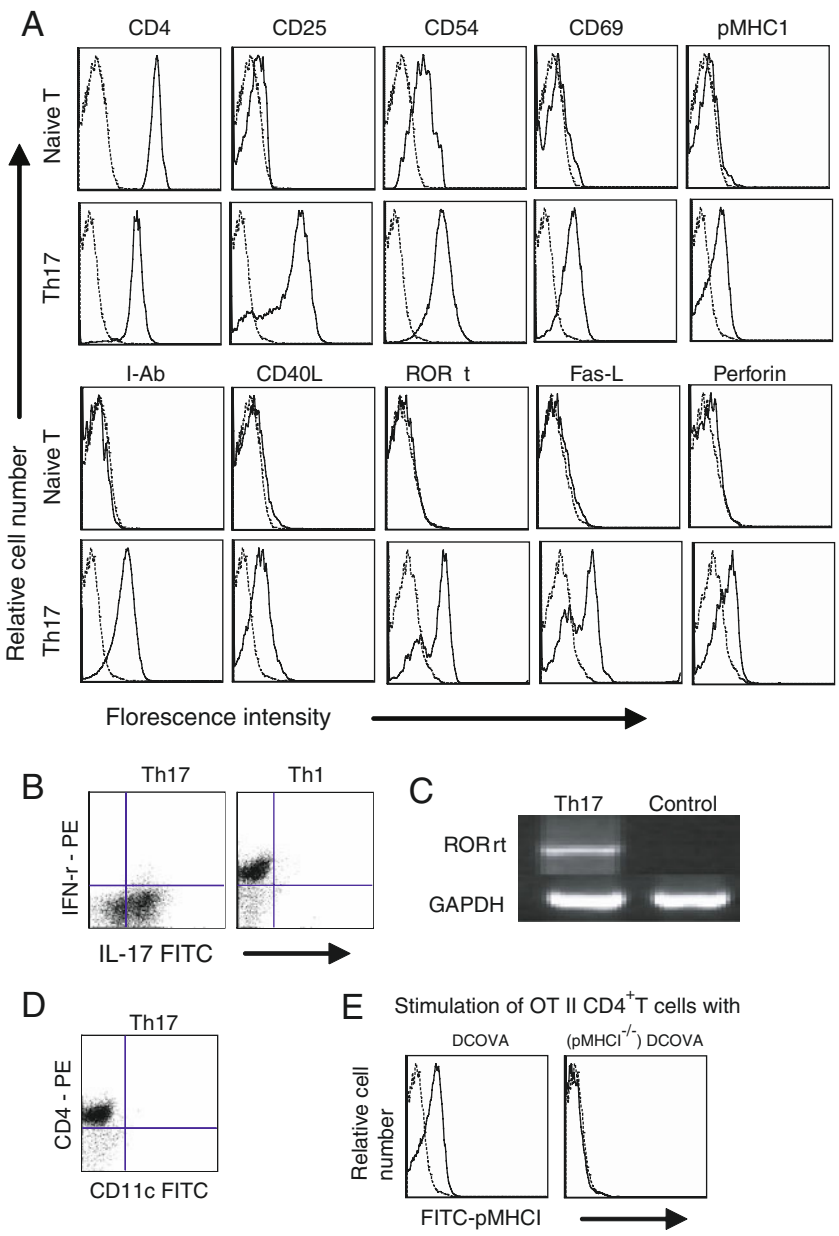

Fig. 1 Phenotypic characterization of OVA-specific $CD4^+$ Th17 cells. **a** Naïve $CD4^+$ T cells and DC_{OVA}-activated $CD4^+$ Th17 cells derived from OT II mice were stained with a panel of biotin-conjugated Abs (*solid lines*) followed by staining with FITC-conjugated avidin and analyzed by flow cytometry. Irrelevant isotype-matched biotin-conjugated Abs were used as controls (*light dotted lines*). **b** In vitro DC_{OVA}-activated $CD4^+$ Th1 and Th17 cells were double-stained with FITC-anti-IL-17 Ab and PE-anti-IFN-γ Ab, and analyzed by flow cytometry. **c** RNA extracted from DC_{OVA}-activated $CD4^+$ Th17 and Con A-stimulated $CD4^+$ T (control) cells were analyzed by RT-PCR for expression of Th17 cell specific transcription factor ROR-γt. **d** Purified active $CD4^+$ Th17 cells were stained with PE-anti-CD4 and FITC-anti-CD11c Abs and analyzed by flow cytometry. **e** $CD4^+$ Th17 and ($K^{b-/-}$)Th17 cells were stained with FITC-anti-pMHC I antibody (*solid lines*) and irrelevant isotype-matched antibody was used as control (*dotted lines*). One representative experiment of two experiments is shown

Th17 cells. Six days following the adaptive transfer of $CD4^+$ Th17, these mice were infused with syngeneic splenocytes pulsed with OVA I peptide and labeled with a high concentration of CFSE ($CFSE^{high}$) or pulsed with an irrelevant Mut1 peptide and labeled with low concentration of CFSE ($CFSE^{low}$) as OVA-specific or control target cells at a 1:1 ratio [35]. Sixteen hours later, the remaining CFSE-labeled target cells were enumerated and their numbers compared with the reference population. We found that there was substantial loss of the OVA-specific and $CFSE^{high}$-labeled target cells in Th17 cell-immunized (43.9%) mice (Fig. 2c), indicating that $CD4^+$ Th17 cells can stimulate $CD8^+$ T cell differentiation into effector CTLs with killing activity for OVA I-pulsed target cells. In addition, the $CD4^+$ ($K^{b-/-}$)Th17 cell-vaccinated mice did not display any killing activity for the OVA-specific and $CFSE^{high}$-labeled target cells in cytotoxicity assay. To assess the pathway responsible for the killing activity of $CD4^+$ Th17-stimulated $CD8^+$ T cells in vivo, we repeated the above experiments using $perforin^{-/-}$ mice in tetramer staining and in vivo cytotoxicity assays. We found that OVA-specific $CD8^+$ T cell responses in C57BL/6 and

perforin$^{-/-}$ mice with transfer of CD4$^+$ Th17 cells were similar (Fig. 2d). However, CD8$^+$ T cell-induced killing activity to OVA-specific CFSE^{high} target cells was lost in perforin$^{-/-}$ mice (Fig. 2d), indicating that the in vivo CD4$^+$ Th17-stimulated CD8$^+$ T cell-induced killing activity to OVA-specific target cells is also via perforin-dependent pathway.

CD4$^+$ Th17 Cell-Induced Diabetes in Transgenic RIP-mOVA Mice is Mediated by Th17-Stimulated CD8$^+$ CTLs

Interestingly, all (6/6) RIP-mOVA mice adoptively transferred with CD4$^+$ Th17 cells carrying acquired pMHC I developed diabetes, while none (0/6) of the mice adoptively transferred with CD4$^+$ (K$^{b-/-}$)Th17 cells without acquired pMHC I complexes developed diabetes (Fig. 2e). As expected, pancreatic islet tissues were destroyed and infiltrated with numerous lymphocytes in the diabetic mice (Fig. 2f). To assess the importance of CD8$^+$ T cells in pathogenesis of diabetes, we treated adoptively transferred mice with anti-CD8 Ab to deplete CD4$^+$ Th17-stimulated OVA-specific CD8$^+$ T cells. We found that this treatment completely prevented diabetes development in the experimental animals (0/6). The effect proved to be very specific, since similar treatment with control irrelevant antibody has not suppressed disease onset (Fig. 2e), indicating that CD4$^+$ Th17 cell-induced diabetes in transgenic RIP-mOVA mice is mainly mediated by Th17-stimulated CD8$^+$ CTLs.

EAE Induction by MOG_{35-55} Peptide Immunization is Mainly by CD4$^+$ T Cells

To induce EAE, we s.c. administered MOG_{35-55} peptide to C57BL/6 mice, following previously described procedures [30]. All treated animals were found to develop chronic-progressive EAE with apparent clinical scores subsequent to MOG_{35-55} immunization (Fig. 3a). To assess the MOG-specific CD8$^+$ T cell responses, we performed MOG-specific PE-pentamer staining using tail blood from mice 2 weeks subsequent to the immunization with MOG_{35-55} peptide. We found that MOG_{35-55} peptide immunization stimulated in vivo proliferation of MOG-specific CD8$^+$ T cells accounting for 0.56% of total CD8$^+$ T cell population (Fig. 3b), and these MOG-specific CD8$^+$ T cells are cytotoxic effector cells since there was a substantial loss of the MOG-specific and CFSE^{high}-labeled target cells (38%) in MOG_{35-55}-immunized mice (Fig. 3c). Consistent with the above clinical finding, the histological examination of the spinal column revealed dramatic pathological changes in the immunized mice, with multiple inflammatory foci and extensive demyelination in the white matter of the spinal cord (Fig. 3d). Mean inflammation and demyelination scores were 2.6 and 1.5, respectively (Fig. 3e), which were significantly higher than in control animals ($p<0.01$). To assess whether MOG-specific CD4$^+$ or CD8$^+$ T cells play a major role in EAE pathogenesis, we immunized H-2K$^{b-/-}$ and Ia$^{b-/-}$ KO mice with MOG peptide for EAE induction. As shown in Fig. 3a, Ia$^{b-/-}$ and H-2K$^{b-/-}$ mice, lacking CD4$^+$ and CD8$^+$ T cells, revealed practically no clinical score ($p<0.01$) and slightly lower clinical scores ($p<0.05$), respectively, compared to the control MOG_{35-55}-immunized C57BL/6 mice, indicating that CD4$^+$ T cells, but not CD8$^+$ T cells, are predominantly involved in EAE induction.

In Vitro-Generated CD4$^+$ MOG-TCR-Th17 Cells Play a Major Role in Pathogenesis of EAE

To assess whether CD4$^+$ Th17 cells have a pathogenic function in EAE, we generated MOG-specific CD4$^+$ MOG-TCR-Th17 cells in vitro by co-incubating DC_{MOG} with CD4$^+$ T cells derived from MOG-TCR transgenic mice in presence of the IL-23, IL-6, TGF-β cytokines, and anti-IFN-γ antibody. The resulting activated CD4$^+$ T cells were subjected to flow cytometry, ELISA, and RT-PCR analysis. These CD4$^+$ T cells proved to be, indeed, efficiently activated as they strongly expressed CD25 and CD69 on their membranes (Fig. 4a). They also produced intracellular IL-17, but not IL-4, and expressed intranuclear RORγt, but not T-bet, indicating that they represent the CD4$^+$ Th17 subset (Fig. 4a). The RT-PCR analysis also revealed RORγt expression, thus further confirming the CD4$^+$ Th17 nature of the obtained population (Fig. 4b). As expected, they secreted the IL-2 (1.8 ng/ml), IL-6 (3.3 ng/ml), IL-17 (1.5 ng/ml), and TGF-β (0.1 ng/ml), confirming that they belong to CD4$^+$ Th17 cells. To assess their potential stimulatory effect, we i.v. injected them into C57BL/6 mice and performed MOG-specific PE-pentamer staining assay. As shown in Fig. 4c, MOG-specific CD8$^+$ T cells were detected in CD4$^+$ MOG-TCR-Th17-immunized mice and accounted for 0.36% of the total CD8$^+$ T cell population, indicating that MOG-specific CD4$^+$ MOG-TCR-Th17 cells are capable of stimulating MOG-specific CD8$^+$ T cell responses. To assess MOG-specific CD8$^+$ T cell killing activity, we performed the in vivo cytotoxicity assay. A moderate (20%) loss of the MOG-specific, CFSE^{high}-labeled target cells was observed in CD4$^+$ MOG-TCR-Th17 cell-transferred mice (Fig. 4d), indicating that CD4$^+$ MOG-TCR-Th17 immunization was likely to stimulate CD8$^+$ T cell differentiation into effector CTLs with cytotoxic activity specific for MOG peptide-pulsed target cells. To examine their ability to induce EAE, we injected C57BL/6 mice with the in vitro-amplified CD4$^+$ MOG-TCR-Th17 cells. All mice injected with MOG-specific CD4$^+$ Th17 cells, but not with OVA-specific Th17 control,

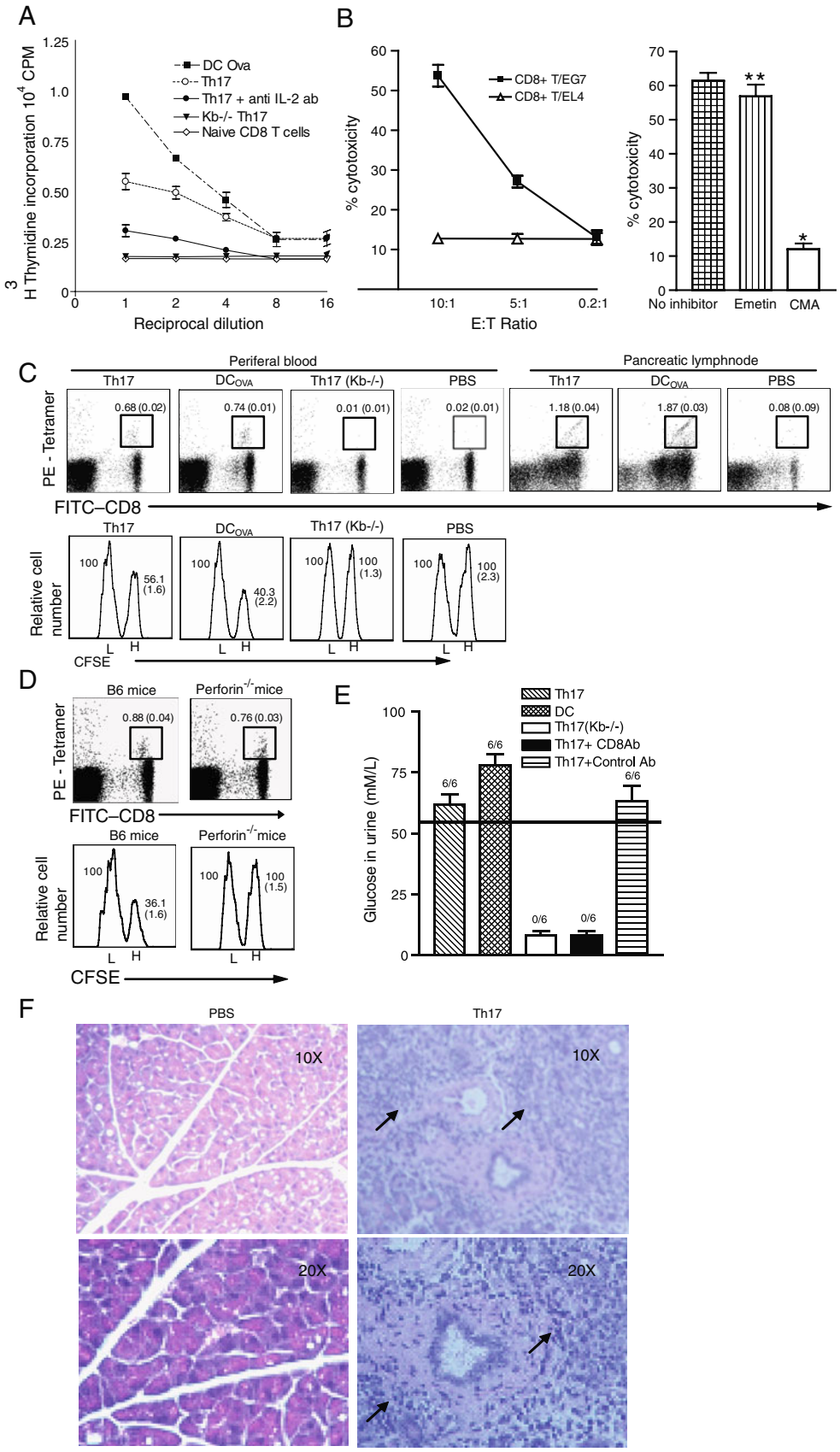
A
B
C
D
E
F
DC Ova
Th17
Th17 + anti IL-2 ab
Kb-/- Th17
Naive CD8 T cells
Reciprocal dilution
CD8+ T/EG7
CD8+ T/EL4
E:T Ratio
No inhibitor
Emetin
CMA
Periferal blood
Pancreatic lymphnode
PE - Tetramer
FITC–CD8
Relative cell number
CFSE
B6 mice
Perforin-/- mice
Th17(Kb-/-)
Th17+ CD8Ab
Th17+Control Ab
Glucose in urine (mM/L)
PBS
10X
20X

Fig. 2 $CD4^+$ Th17 cells induced CTL leads to diabetes in transgenic RIP-mOVA mice. **a** In vitro $CD8^+$ T cell proliferation assay. Irradiated DC_{OVA}, $CD4^+$ Th17, $CD4^+$ Th17 with anti-IL-2 Ab and $(K^{b-/-})$Th17 cells, and their twofold dilutions were co-cultured with naïve OTI $CD8^+$ T cells. After 2 days, the proliferative responses of $CD8^+$ T cells were determined by overnight ^{3}H-thymidine uptake assay. **b** In vitro cytotoxicity assay. Th17-activated OTI $CD8^+$ Tc1 cells were used as effector (E) cells and in another experiment, Th17-activated $CD8^+$ T cells with or without preincubation of concanamycin A (CMA, 1 μM) or emetin (5 μM) for 2 h were used as effector (E) cells, while ^{51}Cr-labeled EG7 and EL4 cells were used as target (T) cells in a chromium release assay. **c** In tetramer staining assay, the tail blood samples and pancreatic lymph node cells of transgenic RIP-mOVA mice adoptively transferred with $CD4^+$ Th17 cells, DC_{OVA}, $(K^{b-/-})CD4^+$ Th17 cells, and PBS (controls) were stained with PE-H-$2K^b$/OVAI (PE-tetramer) and FITC-CD8 Ab (FITC-CD8), and then analyzed by flow cytometry. The *values in each panel* represent the percentage of tetramer-positive $CD8^+$ T cells versus the total $CD8^+$ T cell population. The *value in parenthesis* represents the standard deviation. In in vivo cytotoxicity assay, 16 h after target cell delivery, the residual OVAI-pulsed $CFSE^{high}$ and Mut1-pulsed $CFSE^{low}$ target cells remaining in the spleens of the above cohorts of mice were sorted and analyzed by flow cytometry. The *value in parenthesis* represents the standard deviation; (n=6, average±SD), *p<0.05 versus cohorts of mice adoptively transferred with DC_{OVA} (Student's t test). **d** In tetramer staining assay, the tail blood samples of wild-type C57BL/6 and $perforin^{-/-}$ mice adoptively transferred with $CD4^+$ Th17 cells were stained with PE-H-$2K^b$/OVAI (PE-tetramer) and FITC-CD8 Ab (FITC-CD8), and then analyzed by flow cytometry. In in vivo cytotoxicity assay, 16 h after target cell delivery, the residual OVAI-pulsed $CFSE^{high}$ and Mut1-pulsed $CFSE^{low}$ target cells remaining in the spleens of the above cohorts of mice were sorted and analyzed by flow cytometry. The *value in parenthesis* represents the standard deviation; (n=6, average±SD), *p<0.05 versus cohorts of $perforin^{-/-}$ mice (Student's t test). **e** Urine test for diabetes. Glucose levels in urine samples from transgenic RIP-mOVA mice adoptively transferred with irradiated $CD4^+$ Th17 cells, DC_{OVA}, $(K^{b-/-})CD4^+$ Th17 cells, and PBS (controls). The cutoff line of urine glucose concentration for diabetes is shown. **f** Hematoxylin and eosin-stained sections from Th17- and PBS-injected mice at higher magnification showing extensive cellular infiltration in Th17-injected mice compared to control. Magnifications, ×10 and ×20. One representative experiment of two in the above different experiments is shown

developed chronic-progressive EAE, indicating that $CD4^+$ Th17-induced EAE was MOG specific (Fig. 4e). In addition, no significant difference in EAE initiation between C57BL/6 mice and H-$2K^{b-/-}$ mice lacking $CD8^+$ T cells could be observed (Fig. 4e), suggesting that $CD4^+$ Th17 cells, but not Th17-stimulated $CD8^+$ T cells, played a major role in EAE pathogenesis. Consistent with this clinical finding, histological examination of CNS tissues revealed pathological changes in C57BL/6 mice adoptively transferred with MOG-specific $CD4^+$ MOG-TCR-Th17 cells (Fig. 4f). In these adoptively transferred mice, multiple inflammatory foci and demyelination were observed in the white matter of the spinal cord, and mean inflammation and demyelination scores were 1.6 and 0.6, respectively, which was significantly higher than the control mice (p<0.01; Fig. 4g).

$CD4^+$ Th17 Cells Derived from EAE Mice also Play a Major Role in Pathogenesis of EAE

It has been demonstrated that in vivo-generated $CD8^+$ T cells derived from MOG_{35-55} peptide-immunized mice can induce EAE after they are amplified in vitro by MOG_{35-55} peptide stimulation and then i.v. transferred into C57BL/6 mice [31]. To assess whether the in vivo-generated $CD4^+$ Th17 cells derived from MOG_{35-55} peptide-immunized mice could also induce EAE, we amplified the MOG-specific $CD4^+$ Th17 cells by culturing $CD4^+$ T cells obtained from MOG_{35-55}-immunized mice with MOG_{35-55} peptide-pulsed splenocytes in the presence of IL-6, IL-23, and TGF-β. The amplified $CD4^+$ T cells were purified using CD4-microbeads and phenotypically analyzed by flow cytometry, ELISA, and RT-PCR approaches. The purified $CD4^+$ T cells proved to express CD25 and CD69 and produced IL-17 and RORγt, but not IL-4 or T-bet, confirming that they are active $CD4^+$ Th17 cells (Fig. 5a, b). In agreement, they also secreted IL-2 (1.2 ng/ml), IL-6 (3.0 ng/ml), IL-17 (1.3 ng/ml), and TGF-β (0.1 ng/ml). To assess their potential stimulatory effect, we injected MOG-specific $CD4^+$ Th17 cells into C57BL/6 mice and followed it by the MOG-specific PE-pentamer staining assay. As shown in Fig. 5c, MOG-specific $CD8^+$ T cells accounting for 0.46% of the total $CD8^+$ T cell population were detected in the injected mice, indicating that in vivo-generated MOG-specific $CD4^+$ Th17 cells are also capable of stimulating MOG-specific $CD8^+$ T cell responses. To assess MOG-specific $CD8^+$ T cell killing activity, we performed the in vivo cytotoxicity assay. We observed a 22% reduction within the MOG-specific, $CFSE^{high}$-labeled target cells in the MOG-$CD4^+$ Th17 transferred mice (Fig. 5d), showing again that MOG-$CD4^+$ Th17 cells could stimulate $CD8^+$ T cell differentiation into effector CTLs specifically targeting MOG peptide-pulsed target cells. To determine whether they are capable of inducing EAE, we injected C57BL/6 mice with the in vitro-amplified MOG-$CD4^+$ Th17 cells originally obtained from MOG_{35-55}-immunized mice. As shown in Fig. 5e, all C57BL/6 mice developed chronic-progressive EAE with apparent clinical scores occurring subsequent to adoptive transfer of MOG-specific $CD4^+$ Th17 cells, but not OVA-specific Th17 control. In addition, there is no significant difference in EAE between C57BL/6 mice and $CD8^+$ T cell-deficient H-$2K^{b-/-}$ mice, indicating that $CD4^+$ Th17 cells, rather than Th17-stimulated $CD8^+$ T cells, play a central role in EAE pathogenesis. Consistent with this clinical finding, histological examination of CNS tissues revealed pathological changes in C57BL/6 mice immunized with MOG-specific $CD4^+$ Th17 cells (Fig. 5f). In particular, multiple inflammatory foci and demyelination were observed in the white matter of the spinal cord of the immunized animals, with mean inflammation and demyelination scores of

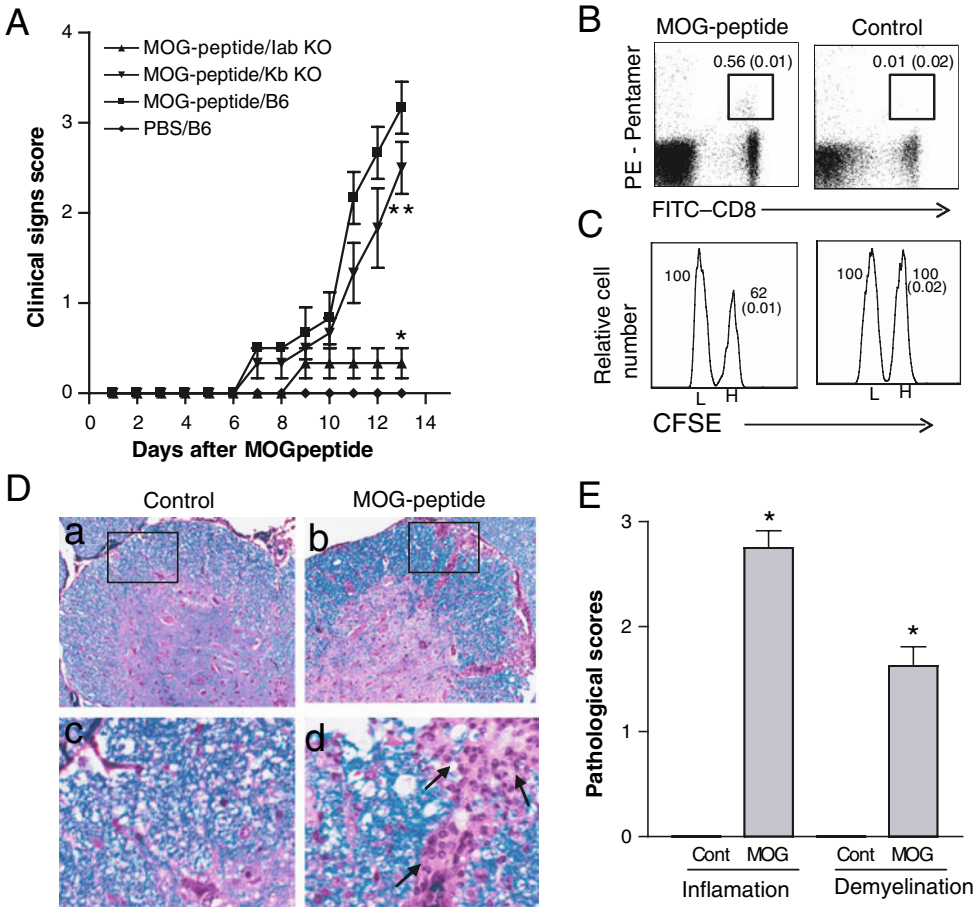

Fig. 3 MOG peptide immunization stimulate MOG-specific CTL responses and induce EAE. **a** Wild-type C57BL/6 and $CD4^+$ T cell- or $CD8^+$ T cell-deficient Ia^b and $H\text{-}2K^b$ mice were immunized with $MOG_{35\text{-}55}$ + CFA. C57BL/6 mice immunized with CFA only were used as control. Clinical EAE was scored according to 0–5 scale. The difference between C57BL/6 and $CD4^+$ T cell-depleted mice (*two asterisks*) or $CD8^+$ T cell-depleted C57BL/6 mice (*single*) is very significant ($p<0.01$) or significant ($p<0.05$; Mann–Whitney *U* test). **b** The tail blood samples of mice immunized with MOG peptide or OVAI peptide (control) were stained with PE-H-$2D^b$/MOGI pentamer (PE-pentamer) and FITC-anti-CD8 Ab (FITC-CD8), and then analyzed by flow cytometry. The *value in each panel* represents the percentage of pentamer-positive $CD8^+$ T cells versus the total $CD8^+$ T cell population. **c** In in vivo cytotoxicity assay, 16 h after target cell delivery, the residual MOGI-pulsed $CFSE^{high}$ and Mut1-pulsed $CFSE^{low}$ target cells remaining in the spleens of the above immunized mice were sorted and analyzed by flow cytometry. The *value in parenthesis* represents the standard deviation. **d** Photographs of sections of spinal cords derived from mice with EAE; tissue sections were stained with Luxol fast blue along with H&E counterstaining. Control mice (*a* and *c*) and MOG-immunized mice (*b* and *d*). Magnifications, ×5 (*a* and *b*) and ×20 (*c* and *d*). Inflammatory infiltration and demyelination are shown with *arrows*. **e** Mean scores of inflammation and demyelination±SD. *$p<0.01$ versus cohorts of the control groups (Student's *t* test). One representative experiment of three in the above experiments is shown

1.1 and 0.6, respectively, which was significantly higher than the scores in control mice ($p<0.01$; Fig. 5g).

Discussion

T1D is an organ-specific autoimmune disease characterized by predominantly T cell-mediated destruction of insulin-producing β-cells of the islets of Langerhans, culminating in the lifelong insulin dependence [44]. Before 1990, vast range of evidence favored a sole role of $CD4^+$ T cells in T1D: (a) $CD4^+$ T cells could be detected in abundance in islet cell infiltrates, (b) transfer of $CD4^+$ T cells from NOD mice caused diabetes in disease free young mice [45], and (c) the genetic region to which the defective genes mapped was the MHC II that interacts specifically with $CD4^+$ T cells [46, 47]. The development of T1D has usually been ascribed to a $CD4^+$ Th1 response with disease transfer in animal models being mediated by Th1 clones and lines [48, 49]. In addition, a potential involvement of Th17 cells in the course of T1D has recently been demonstrated in the mouse model [17]. However, over the time, new evidence has mounted implicating $CD8^+$ T cells in T1D initiation and progression. The primacy of $CD8^+$ T cells in autoimmunity,

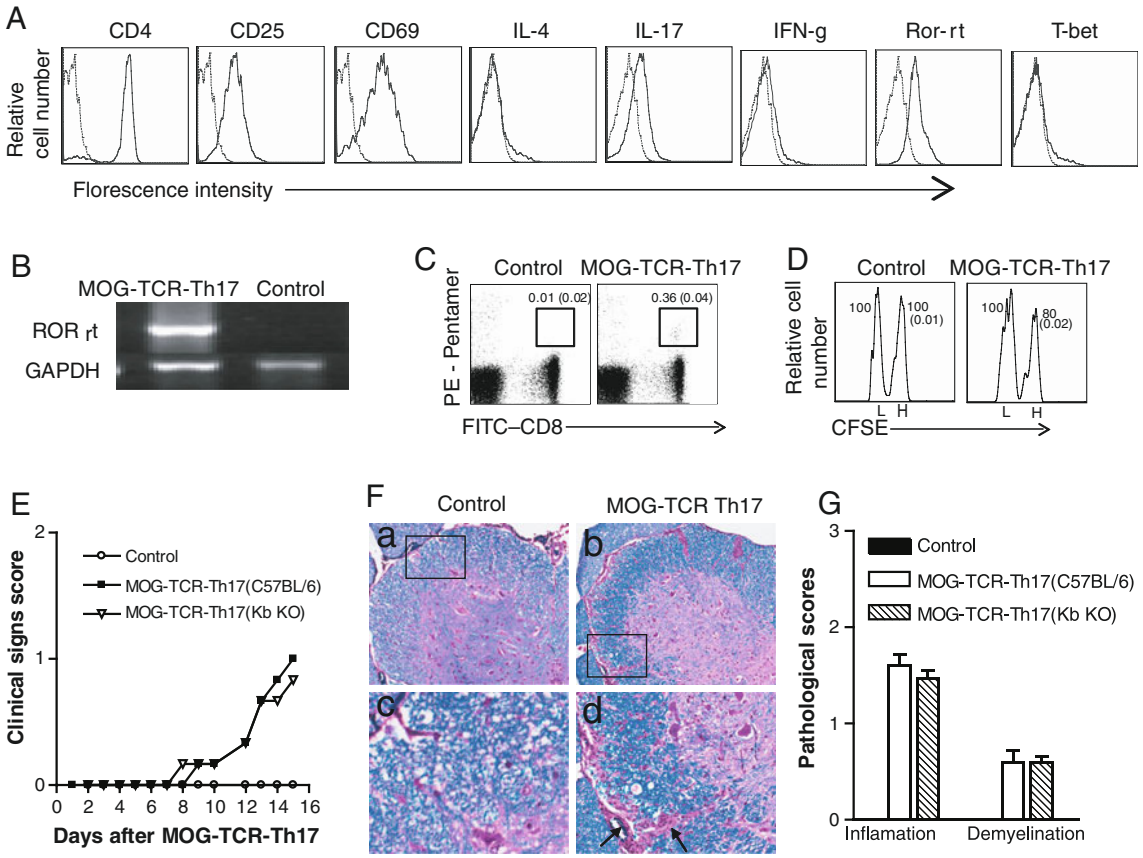

Fig. 4 In vitro-activated MOG-specific $CD4^+$ MOG-TCR-Th17 cells stimulate MOG-specific $CD8^+$ CTL responses and induce EAE. **a** Phenotypic analysis of MOG-specific $CD4^+$ MOG-TCR-Th17 cells. MOG-specific $CD4^+$ MOG-TCR-Th17 cells derived from transgenic MOG-TCR mice were stained with a panel of biotin-conjugated Abs (*solid lines*) followed by staining with FITC-conjugated avidin and analyzed by flow cytometry. Irrelevant isotype-matched biotin-conjugated Abs were used as controls (*light dotted lines*). **b** RNA extracted from MOG-specific $CD4^+$ MOG-TCR-Th17 and Con A-stimulated $CD4^+$ T (*control*) cells were analyzed by RT-PCR for assessment of expression of RORγt. **c** Pentamer staining assay. The tail blood samples of mice adaptively transferred with $CD4^+$ MOG-TCR-Th17 cells or Con A-stimulated $CD4^+$ T (control) cells were stained with PE-H-$2D^b$/MOGI pentamer (PE-pentamer) and FITC-anti-CD8 Ab (FITC-CD8), and then analyzed by flow cytometry. The *value in each panel* represents the percentage of pentamer-positive $CD8^+$ T cells versus the total $CD8^+$ T cell population. The *value in parenthesis* represents the standard deviation. **d** In vivo cytotoxicity assay. Sixteen hours after target cell delivery, the residual MOGI-pulsed $CFSE^{high}$ and Mut1-pulsed $CFSE^{low}$ target cells remaining in the spleens of the above cohorts of mice were sorted and analyzed by flow cytometry. The *value in parenthesis* represents the standard deviation. **e** Wild-type C57BL/6 mice were adoptively transferred with MOG-specific MOG-TCR-Th17 cells or OVA-specific Th17 cells (control). The clinical EAE was scored according to 0–5 scale. **f** Photographs of sections of spinal cords derived from mice with EAE; tissue sections were stained with Luxol fast blue along with H&E counterstaining. Control mice (*a* and *c*) and MOG-immunized mice (*b* and *d*). Magnifications, ×5 (*a* and *b*) and ×20 (*c* and *d*). Inflammatory infiltration and demyelination are shown with *arrows*. **g** Mean scores of inflammation and demyelination±SD. *$p<0.01$ versus cohorts of the control groups (Student's *t* test). One representative experiment of three in the above experiments is shown

including diabetes, came into focus with a study of human monozygotic twins and NOD mice [50, 51], that expressed low density of certain types of class I protein on the surface of APCs. Remarkably, similar studies in NOD mice also confirmed that the APCs of this spontaneously autoimmune animal model also had defects in MHC class I presentation [52]. It has been demonstrated that defects in loading of self antigens into class I polypeptides are associated with T1D pathogenesis [53, 54], indicating that defects in class I assembly and loading could lead to T1D, as a result of a negative selection defect. It has also been shown that $CD8^+$ T cells killed beta-cells expressing self-peptides in class I groove in murine models [55, 56], suggesting that $CD8^+$ T cells exert a strong role in the etiology of T1D.

To assess (a) the potential relationship between the pathogenic $CD4^+$ Th17 and $CD8^+$ T cells in T1D and (b) to determine the extent of their effect on pathogenesis of T1D, we generated RORγt- and IL-17-expressing OVA-specific $CD4^+$ Th17 cells by co-culturing OVA-specific TCR transgenic OTII $CD4^+$ T cells with OVA-pulsed DC_{OVA} in presence of IL-6, IL-23, TGF-β, and anti-IFN-γ antibody. We found that (a) OVA-specific Th17 cells stimulated OVA-specific CTL responses via IL-2 and acquired pMHC I signaling when transferred into RIP-

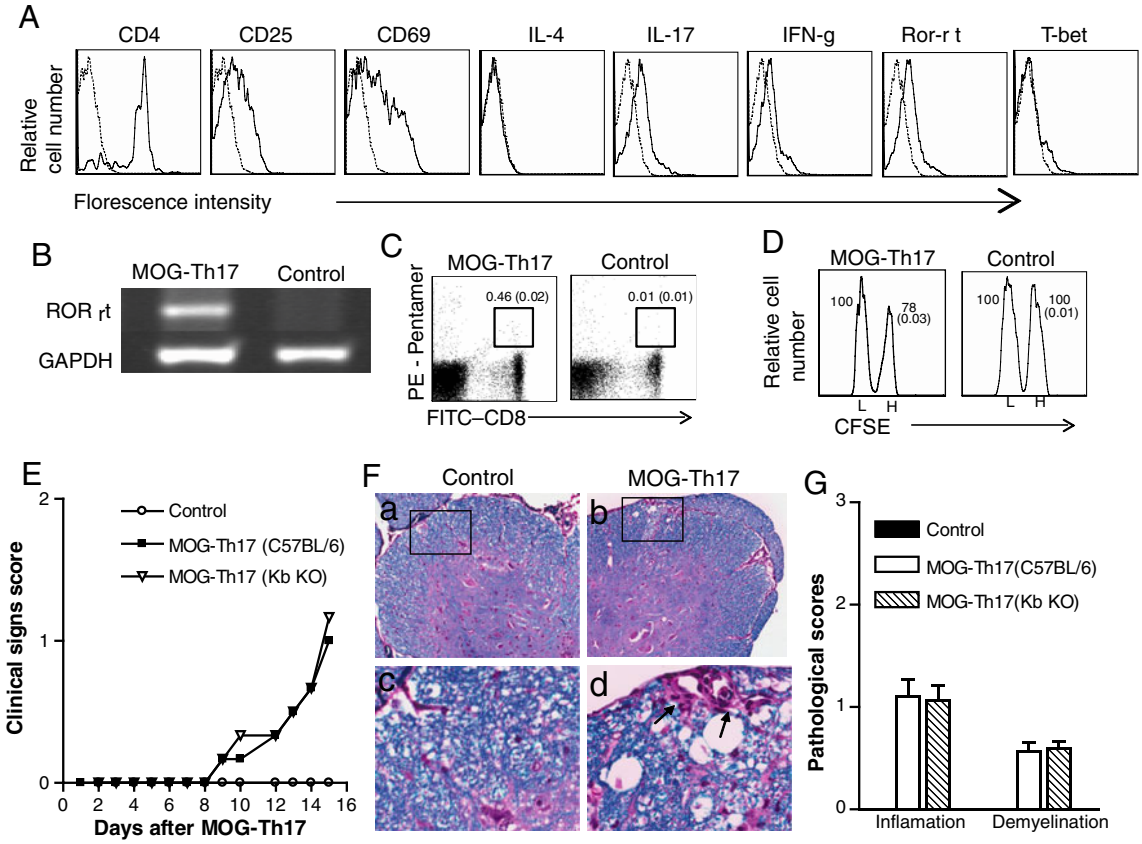

Fig. 5 In vivo-generated MOG-specific $CD4^+$ Th17 cells stimulate MOG-specific $CD8^+$ CTL responses and induce EAE. **a** Phenotypic analysis of in vivo-generated MOG-specific $CD4^+$ Th17 cells. MOG-specific $CD4^+$ Th17 cells derived from MOG peptide-immunized mice with EAE and expanded in vitro by co-culturing with MOG peptide-pulsed splenocytes were stained with a panel of biotin-conjugated Abs (*solid lines*) followed by staining with FITC-conjugated avidin and analyzed by flow cytometry. Irrelevant isotype-matched biotin-conjugated Abs were used as controls (*light dotted lines*). **b** RNA extracted from MOG-specific $CD4^+$ Th17 and Con A-stimulated $CD4^+$ T (control) cells were analyzed by RT-PCR for assessment of expression of RORγt. **c** Pentamer staining assay. The tail blood samples of mice adoptively transferred with $CD4^+$ Th17 cells or Con A-stimulated $CD4^+$ T (control) cells were stained with PE-H-$2D^b$/MOGI pentamer (PE-pentamer) and FITC-anti-CD8 Ab (FITC-CD8), and then analyzed by flow cytometry. The *value in each panel* represents the percentage of pentamer-positive $CD8^+$ T cells versus the total $CD8^+$ T cell population. The *value in parenthesis* represents the standard deviation. **d** In vivo cytotoxicity assay. Sixteen hours after target cell delivery, the residual MOGI-pulsed $CFSE^{high}$ and Mut1-pulsed $CFSE^{low}$ target cells remaining in the spleens of the above cohorts of mice were sorted and analyzed by flow cytometry. The *value in parenthesis* represents the standard deviation. **e** Wild-type C57BL/6 mice were adoptively transferred with MOG-specific Th17 cells or OVA-specific Th17 cells (control). The clinical EAE was scored according to 0–5 scale. **f** Photographs of sections of spinal cords derived from mice with EAE; tissue sections were stained with Luxol fast blue along with H&E counterstaining. Control mice (*a* and *c*) and MOG-immunized mice (*b* and *d*). Magnifications, ×5 (*a* and *b*) and ×20 (*c* and *d*). Inflammatory infiltration and demyelination are shown with *arrows*. **g** Mean scores of inflammation and demyelination±SD. *$p<0.01$ versus cohorts of the control groups (Student's *t* test). One representative experiment of three in the above experiments is shown

mOVA mice, and (b) Th17-stimulated $CD8^+$ T cells were capable of killing OVA-expressing target cells via perforin pathway. To assess the pathogenesis of OVA-specific $CD4^+$ Th17 cells or $CD8^+$ CTLs in T1D, we transferred these Th17 cells into RIP-mOVA mice or RIP-mOVA mice with anti-CD8 Ab treatment to deplete Th17-stimulated $CD8^+$ T cells. We showed that Th17-stimulated $CD8^+$ CTLs, but not Th17 cells themselves were required for T1D induction in RIP-mOVA mice (Fig. 6). Our findings are consistent with some previous reports showing that (a) transfer of islet-specific Th17 cells failed in diabetes induction, though it caused an extensive insulitis [57], and (b) treatment with neutralizing IL-17-specific Abs did not prevent T1D in NOD/SCID mice, which were derived from transfer of highly purified Th17 cells from BDC2.5 transgenic mice [49]. Furthermore, autoreactive $CD8^+$ T cells have been shown to play an important role in the pathogenesis of T1D [47, 58]. $CD8^+$ CTLs kill target cells through two distinct cytolytic pathways, the perforin-dependent granule exocytosis and the Fas/FasL interaction pathways [59]. The perforin in the presence of calcium has the ability to insert into lipid bilayer membrane, polymerize, and form structural and functional pores that can lead to cell lysis, whereas the binding of FasL on CTLs to Fas initiates the death pathway of apoptosis in the Fas-

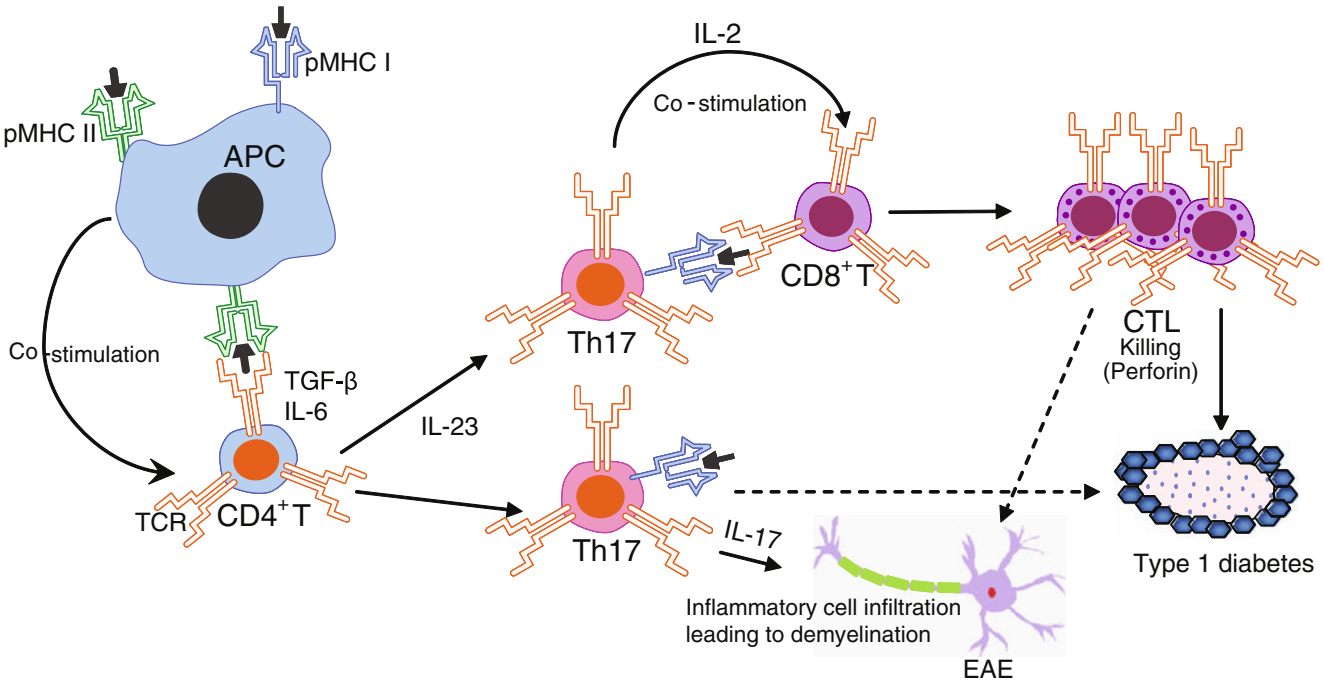

Fig. 6 Distinct role of $CD4^+$ Th17- and Th17-stimulated $CD8^+$ CTL in pathogenesis of T1D and EAE. Both $CD4^+$ Th17 cells and Th17-stimulated $CD8^+$ CTLs are involved in pathogenesis of T1D and EAE. However, T1D is directly mediated by Th17-stimulated $CD8^+$ CTLs to destroy OVA-expressing pancreatic islets of RIP-mOVA mice via perforin-mediated cytotoxicity. On the contrary, $CD4^+$ Th17 cells play a major role in pathogenesis of EAE by Th17 cytokine-mediated tissue inflammation leading to demyelination in the central nervous system

bearing target cells. In this study, we demonstrated that $CD4^+$ Th17-stimulated $CD8^+$ T cells were able to kill OVA-expressing target cells both in vitro and in vivo via perforin-dependent pathway [60], indicating that $CD4^+$ Th17 induces diabetes in RIP-mOVA mice, may be through OVA-expressing pancreatic beta-cells killing by $CD4^+$ Th17-stimulated $CD8^+$ CTLs via perforin-dependent pathway.

In addition to T1D, we used EAE in our work as a model of human multiple sclerosis induced by autoreactive $CD4^+$ Th cells that mediate tissue inflammation and demyelination in the central nervous system. EAE can be induced through adjuvant and pertussis toxin-based immunization of C57BL/6 mice with a peptide, representing a fragment of an external myelin component, the encephalitogenic MOG peptide. Following the immunization, myelin sheaths of oligodendrocytes are attacked [61]. Although the predominant evidence has shown the critical role of $CD4^+$ Th17 cells in EAE pathogenesis [22–26], a potential involvement of $CD8^+$ T cells in EAE has also been recognized [28]. Whereas, the work of Abdul-Majid et al. has previously demonstrated that both $CD4^+$ and $CD8^+$ T cells were involved in EAE pathogenesis in MOG-immunized DBA/1 mice [62].

To assess whether $CD4^+$ or $CD8^+$ T cells were involved in pathogenesis of EAE, we immunized wild-type C57BL/6 mice or H-$2K^{b-/-}$ and $Ia^{b-/-}$ mice lacking $CD8^+$ and $CD4^+$ T cells with MOG35-55 peptide. Our experiments showed that MOG immunization-induced EAE only in C57BL/6 and H-$2K^{b-/-}$ mice, but not in $CD4^+$ T cell-deficient $Ia^{b-/-}$ mice, indicating that $CD4^+$ T cells are likely to play a critical role in MOG immunization-induced EAE in C57BL/6 mice. The apparent discrepancy between our findings and the previous report [62] may potentially result from genetic differences between different strains (DBA/1 and C57BL/6) of mice used in these two studies. DBA/1 mice are very sensitive to MOG immunization leading to EAE induction even in the absence of PT treatment, whereas C57BL/6 mice only develop MOG immunization-induced EAE, when mice are boosted with pertussis toxin, which greatly enhances $CD4^+$ T cell responses [62].

To further dissect (a) the potential relationship between the pathogenic $CD4^+$ Th17 and $CD8^+$ T cells in EAE and (b) to establish the extent of their influence on EAE pathogenesis, we generated two types of RORγt- and IL-17-expressing MOG-specific $CD4^+$ Th17 cells by cultivation of (a) naïve $CD4^+$ T cells derived from MOG-specific TCR transgenic mice and (b) primed MOG-specific $CD4^+$ T cells derived from EAE mice. We found that both MOG-specific $CD4^+$ Th17 cells were capable of stimulating MOG-specific $CD8^+$ CTL responses when transferred into C57BL/6 mice. To assess EAE induction by these MOG-specific $CD4^+$ Th17 cells or MOG-specific Th17-stimulated

$CD8^+$ T cells, we transferred them into C57BL/6 mice or into H-$2K^{b-/-}$ mice with deficiency in the production of Th17-stimulated $CD8^+$ T cells. We have showed that the adoptively transferred $CD4^+$ Th17 cells, but not in vivo $CD4^+$ Th17-stimulated $CD8^+$ CTLs, are responsible for EAE initiation in C57BL/6 mice, indicating that $CD4^+$ Th17 cells play a crucial role in pathogenesis of EAE (Fig. 6). The failure of in vivo $CD4^+$ Th17-stimulated $CD8^+$ CTLs to trigger EAE may be due to their efficiency being lower than the efficiency of in vitro expanded MOG-specific $CD8^+$ T cells that were successful in EAE induction, when adoptively transferred into experimental mice [30–32]. Nevertheless, our observations indicate that $CD4^+$ Th17 cells, but not in vivo Th17-stimulated $CD8^+$ CTLs are likely to induce EAE under physiological conditions. Our data are also consistent with some recent reports showing that (a) IL-17A significantly contributes to the induction of EAE in immunized mice [63] and (b) adoptive transfer of MOG-specific Th17 cells induce EAE in C57BL/6 mice leading to the induction of EAE in wild-type C57BL/6 mice [22]. Increasing evidence suggests that Th17 cells mediate inflammatory responses through selective migration, accumulative retention at specific sites and secretion of inflammatory cytokines, such as IL-17 [64] inducing tissue inflammation, eventually leading to demyelination in the central nervous system [65–67].

EAE has long been considered the prototypic IFN-γ-secreting Th1-mediated autoimmune disease [68, 69]. Until some findings suggested a primary role for IL-17-secreting Th17 cells in this model [70, 71]. It has been shown that Th1 cells facilitate the entry of Th17 cells to the central nervous system during EAE [72]. Th1 and Th17 cells are shown to have different regulatory roles in inflammation of the brain and spinal cord [26] and EAE with different pathological phenotypes [73]. IFN-β was effective in reducing EAE symptoms induced by Th1 cells, but exacerbated disease induced by Th17 cells [74]. Therefore, this paradigm shift has sparked a rapid and remarkable change in emphasis in the search for disease-modifying drugs away from the Th1 pathway toward the Th17 pathway [75].

Conclusions

Taken together, our study shows that $CD4^+$ Th17 cells acquired pMHC I in the process of activation by DCs and became capable of stimulating OVA or MOG-specific $CD8^+$ CTL responses, when transferred into the mice. Our data also elucidate a distinct role of $CD4^+$ Th17 and Th17-stimulated $CD8^+$ T cells in autoimmune diseases, that T1D being directly mediated by Th17-stimulated $CD8^+$ cells, whereas EAE is likely to be triggered by $CD4^+$ Th17 cells. Therefore, this work may have great impact on the overall understanding of $CD4^+$ Th17 cells in the pathogenesis of autoimmune diseases.

Acknowledgment This study was supported by Canadian Institute of Health Research (MOP 405674). Manjunatha Ankathatti Munegowda was supported by Dean's Scholarship of University of Saskatchewan.

References

1. Dong C. TH17 cells in development: an updated view of their molecular identity and genetic programming. Nat Rev Immunol. 2008;8:337–48.
2. Weaver CT, Harrington LE, Mangan PR, Gavrieli M, Murphy KM. Th17: an effector CD4 T cell lineage with regulatory T cell ties. Immunity. 2006;24:677–88.
3. Bettelli E, Korn T, Kuchroo VK. Th17: the third member of the effector T cell trilogy. Curr Opin Immunol. 2007;19:652–7.
4. Steinman L. A brief history of T(H)17, the first major revision in the T(H)1/T(H)2 hypothesis of T cell-mediated tissue damage. Nat Med. 2007;13:139–45.
5. Ivanov II, McKenzie BS, Zhou L, Tadokoro CE, Lepelley A, Lafaille JJ, et al. The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. Cell. 2006;126:1121–33.
6. Fukushima T, Zapata JM, Singha NC, Thomas M, Kress CL, Krajewska M, et al. Critical function for SIP, a ubiquitin E3 ligase component of the beta-catenin degradation pathway, for thymocyte development and G1 checkpoint. Immunity. 2006;24:29–39.
7. Veldhoen M, Hocking RJ, Atkins CJ, Locksley RM, Stockinger B. TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. Immunity. 2006;24:179–89.
8. Mangan PR, Harrington LE, O'Quinn DB, Helms WS, Bullard DC, Elson CO, et al. Transforming growth factor-beta induces development of the T(H)17 lineage. Nature. 2006;441:231–4.
9. Korn T, Bettelli E, Gao W, Awasthi A, Jager A, Strom TB, et al. IL-21 initiates an alternative pathway to induce proinflammatory T(H)17 cells. Nature. 2007;448:484–7.
10. Zhou L, Ivanov II, Spolski R, Min R, Shenderov K, Egawa T, et al. IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. Nat Immunol. 2007;8:967–74.
11. Peck A, Mellins ED. Plasticity of T-cell phenotype and function: the T helper type 17 example. Immunology. 2010;129:147–53.
12. Taplin CE, Barker JM. Autoantibodies in type 1 diabetes. Autoimmunity. 2008;41:11–8.
13. Di Lorenzo TP, Peakman M, Roep BO. Translational mini-review series on type 1 diabetes: systematic analysis of T cell epitopes in autoimmune diabetes. Clin Exp Immunol. 2007;148:1–16.
14. Kay TW, Parker JL, Stephens LA, Thomas HE, Allison J. RIP-beta 2-microglobulin transgene expression restores insulitis, but not diabetes, in beta 2-microglobulin null nonobese diabetic mice. J Immunol. 1996;157:3688–93.
15. Serreze DV, Gallichan WS, Snider DP, Croitoru K, Rosenthal KL, Leiter EH, et al. MHC class I-mediated antigen presentation and induction of CD8+ cytotoxic T-cell responses in autoimmune diabetes-prone NOD mice. Diabetes. 1996;45:902–8.
16. Wang B, Gonzalez A, Benoist C, Mathis D. The role of CD8+ T cells in the initiation of insulin-dependent diabetes mellitus. Eur J Immunol. 1996;26:1762–9.

17. Vukkadapu SS, Belli JM, Ishii K, Jegga AG, Hutton JJ, Aronow BJ, et al. Dynamic interaction between T cell-mediated beta-cell damage and beta-cell repair in the run up to autoimmune diabetes of the NOD mouse. Physiol Genomics. 2005;21:201–11.
18. Jain R, Tartar DM, Gregg RK, Divekar RD, Bell JJ, Lee HH, et al. Innocuous IFNgamma induced by adjuvant-free antigen restores normoglycemia in NOD mice through inhibition of IL-17 production. J Exp Med. 2008;205:207–18.
19. Sawcer S, Jones HB, Feakes R, Gray J, Smaldon N, Chataway J, et al. A genome screen in multiple sclerosis reveals susceptibility loci on chromosome 6p21 and 17q22. Nat Genet. 1996;13:464–8.
20. Ebers GC, Kukay K, Bulman DE, Sadovnick AD, Rice G, Anderson C, et al. A full genome search in multiple sclerosis. Nat Genet. 1996;13:472–6.
21. Haines JL, Ter-Minassian M, Bazyk A, Gusella JF, Kim DJ, Terwedow H, et al. A complete genomic screen for multiple sclerosis underscores a role for the major histocompatability complex. The Multiple Sclerosis Genetics Group. Nat Genet. 1996;13:469–71.
22. Yang Y, Weiner J, Liu Y, Smith AJ, Huss DJ, Winger R, et al. T-bet is essential for encephalitogenicity of both Th1 and Th17 cells. J Exp Med. 2009;206:1549–64.
23. Sutton C, Brereton C, Keogh B, Mills KH, Lavelle EC. A crucial role for interleukin (IL)-1 in the induction of IL-17-producing T cells that mediate autoimmune encephalomyelitis. J Exp Med. 2006;203:1685–91.
24. Komiyama Y, Nakae S, Matsuki T, Nambu A, Ishigame H, Kakuta S, et al. IL-17 plays an important role in the development of experimental autoimmune encephalomyelitis. J Immunol. 2006;177:566–73.
25. Bettelli E, Oukka M, Kuchroo VK. T(H)-17 cells in the circle of immunity and autoimmunity. Nat Immunol. 2007;8:345–50.
26. Stromnes IM, Cerretti LM, Liggitt D, Harris RA, Goverman JM. Differential regulation of central nervous system autoimmunity by T(H)1 and T(H)17 cells. Nat Med. 2008;14:337–42.
27. Tzartos JS, Friese MA, Craner MJ, Palace J, Newcombe J, Esiri MM, et al. Interleukin-17 production in central nervous system-infiltrating T cells and glial cells is associated with active disease in multiple sclerosis. Am J Pathol. 2008;172:146–55.
28. Ford ML, Evavold BD. Specificity, magnitude, and kinetics of MOG-specific CD8+ T cell responses during experimental autoimmune encephalomyelitis. Eur J Immunol. 2005;35:76–85.
29. Friese MA, Fugger L. Autoreactive CD8+ T cells in multiple sclerosis: a new target for therapy? Brain. 2005;128:1747–63.
30. Sun D, Zhang Y, Wei B, Peiper SC, Shao H, Kaplan HJ. Encephalitogenic activity of truncated myelin oligodendrocyte glycoprotein (MOG) peptides and their recognition by CD8+ MOG-specific T cells on oligomeric MHC class I molecules. Int Immunol. 2003;15:261–8.
31. Sun D, Whitaker JN, Huang Z, Liu D, Coleclough C, Wekerle H, et al. Myelin antigen-specific CD8+ T cells are encephalitogenic and produce severe disease in C57BL/6 mice. J Immunol. 2001;166:7579–87.
32. Huseby ES, Liggitt D, Brabb T, Schnabel B, Ohlen C, Goverman J. A pathogenic role for myelin-specific CD8(+) T cells in a model for multiple sclerosis. J Exp Med. 2001;194:669–76.
33. Ahmed KA, Munegowda MA, Xie Y, Xiang J. Intercellular trogocytosis plays an important role in modulation of immune responses. Cell Mol Immunol. 2008;5:261–9.
34. Xiang J, Huang H, Liu Y. A new dynamic model of CD8+ T effector cell responses via CD4+ T helper-antigen-presenting cells. J Immunol. 2005;174:7497–505.
35. Umeshappa CS, Huang H, Xie Y, Wei Y, Mulligan SJ, Deng Y, et al. CD4+ Th-APC with acquired peptide/MHC class I and II complexes stimulate type 1 helper CD4+ and central memory CD8+ T cell responses. J Immunol. 2009;182:193–206.
36. Ye Z, Ahmed KA, Hao S, Zhang X, Xie Y, Munegowda MA, et al. Active CD4+ helper T cells directly stimulate CD8+ cytotoxic T lymphocyte responses in wild-type and MHC II gene knockout C57BL/6 mice and transgenic RIP-mOVA mice expressing islet beta-cell ovalbumin antigen leading to diabetes. Autoimmunity. 2008;41:501–11.
37. Ahmed KA, Xie Y, Zhang X, Xiang J. Acquired pMHC I complexes greatly enhance CD4(+) Th cell's stimulatory effect on CD8(+) T cell-mediated diabetes in transgenic RIP-mOVA mice. Cell Mol Immunol. 2008;5:407–15.
38. Kurts C, Heath WR, Kosaka H, Miller JF, Carbone FR. The peripheral deletion of autoreactive CD8+ T cells induced by cross-presentation of self-antigens involves signaling through CD95 (Fas, Apo-1). J Exp Med. 1998;188:415–20.
39. Chen Z, Dehm S, Bonham K, Kamencic H, Juurlink B, Zhang X, et al. DNA array and biological characterization of the impact of the maturation status of mouse dendritic cells on their phenotype and antitumor vaccination efficacy. Cell Immunol. 2001;214:60–71.
40. Xia D, Hao S, Xiang J. CD8+ cytotoxic T-APC stimulate central memory CD8+ T cell responses via acquired peptide-MHC class I complexes and CD80 costimulation, and IL-2 secretion. J Immunol. 2006;177:2976–84.
41. Xiang J, Moyana T. Cytotoxic CD4+ T cells associated with the expression of major histocompatibility complex class II antigen of mouse myeloma cells secreting interferon-gamma are cytolytic in vitro and tumoricidal in vivo. Cancer Gene Ther. 1998;5:313–20.
42. Sas S, Chan T, Sami A, El-Gayed A, Xiang J. Vaccination of fiber-modified adenovirus-transfected dendritic cells to express HER-2/neu stimulates efficient HER-2/neu-specific humoral and CTL responses and reduces breast carcinogenesis in transgenic mice. Cancer Gene Ther. 2008;15:655–66.
43. Stockinger B, Veldhoen M. Differentiation and function of Th17 T cells. Curr Opin Immunol. 2007;19:281–6.
44. Atkinson MA, Eisenbarth GS. Type 1 diabetes: new perspectives on disease pathogenesis and treatment. Lancet. 2001;358:221–9.
45. Haskins K, Portas M, Bradley B, Wegmann D, Lafferty K. T-lymphocyte clone specific for pancreatic islet antigen. Diabetes. 1988;37:1444–8.
46. Jones EY, Fugger L, Strominger JL, Siebold C. MHC class II proteins and disease: a structural perspective. Nat Rev Immunol. 2006;6:271–82.
47. Faustman DL, Davis M. The primacy of CD8 T lymphocytes in type 1 diabetes and implications for therapies. J Mol Med. 2009;87:1173–8.
48. Katz JD, Benoist C, Mathis D. T helper cell subsets in insulin-dependent diabetes. Science. 1995;268:1185–8.
49. Bending D, De La Peña H, Veldhoen M, Phillips JM, Catherine U, Stockinger B, et al. Highly purified Th17 cells from BDC2.5NOD mice convert into Th1-like cells in NOD/SCID recipient mice. J Clin Invest. 2009;119.
50. Faustman D, Li XP, Lin HY, Fu YE, Eisenbarth G, Avruch J, et al. Linkage of faulty major histocompatibility complex class I to autoimmune diabetes. Science. 1991;254:1756–61.
51. Yan G, Fu Y, Faustman DL. Reduced expression of Tap1 and Lmp2 antigen-processing genes in the nonobese diabetic (NOD) mouse due to a mutation in their shared bidirectional promoter. J Immunol. 1997;159:3068–80.
52. Li F, Guo J, Fu Y, Yan G, Faustman D. Abnormal class I assembly and peptide presentation in the nonobese diabetic mouse. Proc Natl Acad Sci USA. 1994;91:11128–32.
53. Yan G, Shi L, Faustman D. Novel splicing of the human MHC-encoded peptide transporter confers unique properties. J Immunol. 1999;162:852–9.
54. Qu HQ, Lu Y, Marchand L, Bacot F, Frechette R, Tessier MC, et al. Genetic control of alternative splicing in the TAP2 gene: possible implication in the genetics of type 1 diabetes. Diabetes. 2007;56:270–5.

55. Christianson SW, Shultz LD, Leiter EH. Adoptive transfer of diabetes into immunodeficient NOD-scid/scid mice. Relative contributions of CD4+ and CD8+ T-cells from diabetic versus prediabetic NOD.NON-Thy-1a donors. Diabetes. 1993;42:44–55.
56. Wong FS, Karttunen J, Dumont C, Wen L, Visintin I, Pilip IM, et al. Identification of an MHC class I-restricted autoantigen in type 1 diabetes by screening an organ-specific cDNA library. Nat Med. 1999;5:1026–31.
57. Martin-Orozco N, Chung Y, Chang SH, Wang YH, Dong C. Th17 cells promote pancreatic inflammation but only induce diabetes efficiently in lymphopenic hosts after conversion into Th1 cells. Eur J Immunol. 2009;39:216–24.
58. Pinkse GG, Tysma OH, Bergen CA, Kester MG, Ossendorp F, van Veelen PA, et al. Autoreactive CD8 T cells associated with beta cell destruction in type 1 diabetes. Proc Natl Acad Sci USA. 2005;102:18425–30.
59. Russell JH, Ley TJ. Lymphocyte-mediated cytotoxicity. Annu Rev Immunol. 2002;20:323–70.
60. Bolitho P, Voskoboinik I, Trapani JA, Smyth MJ. Apoptosis induced by the lymphocyte effector molecule perforin. Curr Opin Immunol. 2007;19:339–47.
61. Merkler D, Oertle T, Buss A, Pinschewer DD, Schnell L, Bareyre FM, et al. Rapid induction of autoantibodies against Nogo-A and MOG in the absence of an encephalitogenic T cell response: implication for immunotherapeutic approaches in neurological diseases. FASEB J. 2003;17:2275–7.
62. Abdul-Majid KB, Wefer J, Stadelmann C, Stefferl A, Lassmann H, Olsson T, et al. Comparing the pathogenesis of experimental autoimmune encephalomyelitis in CD4−/− and CD8−/− DBA/1 mice defines qualitative roles of different T cell subsets. J Neuroimmunol. 2003;141:10–9.
63. Ishigame H, Kakuta S, Nagai T, Kadoki M, Nambu A, Komiyama Y, et al. Differential roles of interleukin-17A and -17 F in host defense against mucoepithelial bacterial infection and allergic responses. Immunity. 2009;30:108–19.
64. Kolls JK, Linden A. Interleukin-17 family members and inflammation. Immunity. 2004;21:467–76.
65. Park H, Li Z, Yang XO, Chang SH, Nurieva R, Wang YH, et al. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. Nat Immunol. 2005;6:1133–41.
66. Furuzawa-Carballeda J, Vargas-Rojas MI, Cabral AR. Autoimmune inflammation from the Th17 perspective. Autoimmun Rev. 2007;6:169–75.
67. Guo B, Chang EY, Cheng G. The type I IFN induction pathway constrains Th17-mediated autoimmune inflammation in mice. J Clin Invest. 2008;118:1680–90.
68. Ando DG, Clayton J, Kono D, Urban JL, Sercarz EE. Encephalitogenic T cells in the B10.PL model of experimental allergic encephalomyelitis (EAE) are of the Th-1 lymphokine subtype. Cell Immunol. 1989;124:132–43.
69. Wensky AK, Furtado GC, Marcondes MC, Chen S, Manfra D, Lira SA, et al. IFN-gamma determines distinct clinical outcomes in autoimmune encephalomyelitis. J Immunol. 2005;174:1416–23.
70. Cua DJ, Sherlock J, Chen Y, Murphy CA, Joyce B, Seymour B, et al. Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. Nature. 2003;421:744–8.
71. Langrish CL, Chen Y, Blumenschein WM, Mattson J, Basham B, Sedgwick JD, et al. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. J Exp Med. 2005;201:233–40.
72. O'Connor RA, Prendergast CT, Sabatos CA, Lau CW, Leech MD, Wraith DC, et al. Cutting edge: Th1 cells facilitate the entry of Th17 cells to the central nervous system during experimental autoimmune encephalomyelitis. J Immunol. 2008;181:3750–4.
73. Jager A, Dardalhon V, Sobel RA, Bettelli E, Kuchroo VK. Th1, Th17, and Th9 effector cells induce experimental autoimmune encephalomyelitis with different pathological phenotypes. J Immunol. 2009;183:7169–77.
74. Axtell RC, de Jong BA, Boniface K, van der Voort LF, Bhat R, De Sarno P, et al. T helper type 1 and 17 cells determine efficacy of interferon-beta in multiple sclerosis and experimental encephalomyelitis. Nat Med. 2010;16:406–12.
75. Kikly K, Liu L, Na S, Sedgwick JD. The IL-23/Th(17) axis: therapeutic targets for autoimmune inflammation. Curr Opin Immunol. 2006;18:670–5.

CHAPTER 4

4. $CD4^+$ Th2 cells function alike effector Tr1 and Th1 cells through the deletion of a single cytokine IL-6 and IL-10 gene.

Brief introduction to chapter 4

To demonstrate that a single signature cytokine gene deletion might lead to functional conversion of naive $CD4^+$ T helper cells cultured under Th2 differentiation conditions, into different subsets, we generated OVA-specific wild-type (WT) Th2, and Th2(IL-5 KO), or Th2(IL-5 KO), or Th2(IL-6 KO), or Th2(IL-10 KO) cells, and assessed their capacity in modulating DC_{OVA}-induced $CD8^+$ cytotoxic T lymphocyte (CTL) responses, and antitumor immunity in WT C57BL/6 mice. We conclusively demonstrate that GATA-3-expressing Th2 cells enhance DC_{OVA}-induced CTL responses via IL-6 secretion. We also show that IL-6 and IL-10 gene deficient Th2(IL-6 KO) and Th2(IL-10 KO) cells, but not IL-4 and IL-5 gene deficient Th2(IL-4 KO) and Th2(IL-5 KO) cells, behave like functional Tr1 and Th1 cells by inhibiting and enhancing DC_{OVA}-induced OVA-specific $CD8^+$ CTL responses and antitumor immunity, respectively. We have further demonstrated that inhibition and enhancement of DC_{OVA}-induced OVA-specific CTL responses by Th2(IL-6 KO) and Th2(IL-10 KO) cells are mediated by their immune suppressive IL-10 and pro-inflammatory IL-6 secretions, respectively. Taken together, our experiments suggest that deletion of a single cytokine gene IL-6 and IL-10 converts' naive $CD4^+$ T helper cells cultured under Th2 differentiation condition into functional $CD4^+$ Tr1 and Th1 cells. Our data thus not only provide new evidence for another type of $CD4^+$ T cell plasticity, but also have a potential to impact the development of a new direction in immunotherapy of allergic diseases.

Manuscript is in press for publication in Molecular Immunology, 2012.

$CD4^+$ Th2 cells function alike effector Tr1 and Th1 cells through the deletion of a single cytokine IL-6 and IL-10 gene

Manjunatha Ankathatti Munegowda, Shuling Xu, Andrew Freywald* and Jim Xiang

Cancer Research Unit, Research Division, Saskatchewan Cancer Agency, Departments of Oncology and *Pathology, University of Saskatchewan, Saskatoon, Saskatchewan, Canada.

Key Words: Th2, Th1, Tr1, IL-10, IL-6, CTL

Correspondence should be addressed to Dr. Jim Xiang, Saskatoon Cancer Center, 20 Campus Drive, Saskatoon, Saskatchewan S7N 4H4, Canada, Tel: 306 6552917, Fax: 306 6552635, email: jim.xiang@saskcancer.ca

4.2 ABSTRACT

Depending on polarizing cytokine signals during activation by antigen, naïve $CD4^+$ T cells can be stimulated and differentiated into distinct functional $CD4^+$ T cell subsets such as Th1, Th2 and Tr1 cells. Among them, Th2 cells are pathogenic in allergic diseases such as asthma, which are characterized by transcription factor GATA3 expression and IL-4, IL-5, IL-6, and IL-10 cytokine secretion. The overlapping expression of some signature cytokines by Th2 and other subsets of $CD4^+$ T cells may not only indicate the plasticity of $CD4^+$ T cells, but could also suggest the possibility of the deletion of a single signature cytokine gene leading to the functional differentiation of naïve $CD4^+$ T cells into effector Th1 or Tr1 cells under Th2 differentiation conditions. In this work, we stimulated naïve $CD4^+$ T cells derived from OT II mice or OT II mice that were deficient in individual cytokines (IL-4, IL-5, IL-6 and IL-10) with OVA-pulsed dendritic cells (DC_{OVA}) in the presence of IL-4 and anti-IFN-γ, to generate OVA-specific wild-type (WT) Th2, and Th2(IL-4 KO), or Th2(IL-5 KO), or Th2(IL-6 KO), or Th2(IL-10 KO) cells, and to assess their capacity in modulating DC_{OVA}-induced $CD8^+$ cytotoxic T lymphocyte (CTL) responses, and antitumor immunity in WT C57BL/6 mice. We conclusively demonstrate that GATA-3-expressing Th2 cells enhance DC_{OVA}-induced CTL responses via IL-6 secretion. We also show that IL-6 and IL-10 gene deficient Th2(IL-6 KO) and Th2(IL-10 KO) cells, but not IL-4 and IL-5 gene deficient Th2(IL-4 KO) and Th2(IL-5 KO) cells, behave like functional Tr1 and Th1 cells by inhibiting and enhancing DC_{OVA}-induced OVA-specific $CD8^+$ CTL responses and antitumor immunity, respectively. We further elucidate that inhibition and enhancement of DC_{OVA}-induced OVA-specific CTL responses by Th2(IL-6 KO) and Th2(IL-10 KO) cells are mediated by their immune suppressive IL-10 and pro-inflammatory IL-6 secretion, respectively. Taken together, our study suggests that deletion of a single cytokine gene IL-6 and IL-10 makes $CD4^+$ Th2 cells become effector $CD4^+$ Tr1- and Th1-like cells, respectively. Our data thus not only provide new evidence for another type of $CD4^+$ T cell plasticity, but also have a potential to impact the development of a new direction in immunotherapy of allergic diseases.

4.3 **INTRODUCTION**

In response to infections, various types of immune cells are involved in both innate and adoptive immune responses, and are activated to control and eliminate the invading pathogens. Among them, $CD4^+$ T cells play an important role in the defensive immunity. These lymphocytes activate macrophages to develop enhanced microbicidal activity and recruit neutrophils, eosinophils and basophils to sites of inflammation via their secreted cytokines or chemokines thus assisting the innate immunity. In addition, they also help B cells to produce antibodies and license dendritic cells (DCs) to modulate different types of $CD8^+$ cytotoxic T lymphocyte (CTL) immune responses in adoptive immunity (1).

Depending on polarizing cytokine signals during activation by antigen (Ag), naïve $CD4^+$ T cells can initiate various differentiation programs that lead to the development of distinct functional $CD4^+$ T cell subsets. Key transcriptional factors act as lineage-specifying regulators coordinating expression of specific cytokine genes (2). For example, the transcriptional factor T-box-containing protein expressed in T cells (T-bet) controls type 1 T help (Th1) cell differentiation program associated with the production of Th1 signature cytokine IFN-γ required for efficient immune responses against intracellular pathogens (3) and tumors (4). In contrast, GATA-binding protein 3 (GATA-3) is a regulator of the development of type 2 T help (Th2) cells that express IL-4, IL-5, IL-6 and IL-10. These signature cytokines are critical for the immunity against helminthes and other extracellular pathogens (5) or for antibodies production (6). Unfortunately, Th2 cells are also pathogenic in allergic diseases such as asthma (7). Another subset of $CD4^+$ T cells is the $Foxp3^+$ IL-10-and IFN-γ-secreting $CD4^+$ type 1 regulatory T (Tr1) cells that are generated from naive T cells in the periphery after encounter with Ag presented by DCs in the status distinct from those for promoting the differentiation of Th1 and Th2 cells (8). TGF-β plays a major role in Tr1 differentiation (9, 10) and development (11) by activation of Smad3 to promote Foxp3 expression (12). These $CD4^+$ Tr cells, which are specific for pathogen-derived Ags, suppressed immune responses via their signature cytokine IL-10 secretion (13).

We previously established an ovabumin (OVA)-specific animal model using OVA-specific T cell receptor (TCR) transgenic OT II $CD4^+$ T cell subsets to study the functional

effect and molecular mechanisms associated with different subsets of $CD4^+$ T cells (13-15). In this study, we investigate whether manipulation of a single signature cytokine can differentiate naïve $CD4^+$ T cells into functional Th1 and Tr1 cells under Th2 differentiation conditions. To assess the role of specific cytokines, we have generated OVA-specific $CD4^+$ Th2 cells by *in vitro* stimulation of naïve $CD4^+$ T cells derived from OT II mice or OT II mice with deficiency in individual cytokine (IL-4, IL-6, IL-5 or IL-10) with OVA-pulsed DCs (DC_{OVA}) in the presence of IL-4 and anti-IFN-γ antibody, and then compared their capacities in stimulating or inhibiting OVA-specific $CD8^+$ T cell responses, and antitumor immunity induced by DC_{OVA} immunization in wild-type C57BL/6 mice.

4.4 **MATERIALS AND METHODS**

Tumor cells, reagents and animals.

The OVA-transfected BL6-10 (BL6-10_{OVA}) cell lines were generated in our laboratory (16). The biotin-labeled antibodies (Abs) specific for CD4 (GK1.5), CD11c (HL3), CD25 (7D4), CD40 (K19), CD69 (H1.2F3) and CD80 (16-10A) were obtained from BD Pharmingen, Mississauga, ON, Canada. The FITC conjugated avidin was obtained from Jackson Immuno Research Laboratory Inc. (West Grove, PN). The anti-H-$2K^b$/OVAI (pMHC I) Ab was obtained from Dr. Germain, National Institute of Health, Bethesda, MD (17). Recombinant cytokines IL-2, IL-4, GM-CSF and TGF-β were purchased from R&D systems Inc. (Minneapolis, MN). IL-6 (MP5-20F3) neutralizing antibody was purchased from eBiosciences (San Diego, CA) and IL-10 (JES5-2A5) neutralizing antibody was purchased from BD Pharmingen, Mississauga, ON, Canada. The PE-H-$2K^b$/OVAI tetramer and FITC-anti-CD8 Ab (PK135) were obtained from Beckman Coulter, Mississauga, Ontario, Canada. The OVAI (SIINFEKL) peptide specific for H-$2K^b$ and Mut1 (FEQNTAQP) specific for H-$2K^b$ of irrelevant 3LL lung carcinoma (16) were synthesized by Multiple Peptide Systems (San Diego, CA). The C57BL/6 (B6, CD45.2^+), OVA-specific TCR-transgenic OT II mice, and IL-4, IL-5, IL-6 and IL-10 gene knockout (KO) mice on a C57BL/6 background were obtained from the Jackson Laboratory (Bar Harbor, MA). Homozygous OT II/IL-$4^{-/-}$, OT II/IL-$5^{-/-}$, OT II/IL-$6^{-/-}$, and OT II/IL-$10^{-/-}$ mice were generated by backcrossing the designated gene KO mice onto the OT II background for three generations; homozygosity was confirmed by polymerase-chain reaction (PCR) according to Jackson laboratory's protocols.

Preparation of dendritic cells.

Mature bone marrow-derived DCs, expressing high levels of CD11c, CD40, CD80 and pMHC class I, were generated as described previously (16). Briefly, bone marrow cells were collected from femora and tibiae of wild type C57BL/6 mice and RBCs are depleted with 0.84% ammonium chloride. Obtained cells were plated in DC culture medium (DMEM plus 10% FCS, 20ng/ml

GM-CSF and 20ng/ml IL-4). On day 3 non adherent granulocytes, T cells and B cells were gently removed and replaced with medium. Two days later, loosely adherent DC's were dislodged and replated and grown till 6 days. On 6^{th} day non adherent DCs were harvested and pulsed overnight at 37°C with 0.1 mg/ml OVA (Sigma, St. Louis, MO) and termed DC_{OVA}. Inhibitory DC_{OVA} was derived expressing high levels of IL-10 as described earlier (18). Briefly, bone marrow cells depleted with RBCs were cultured in presence of 20ng/ml GM-CSF in 10% FCS containing DMEM for 10 days, carefully changing medium every 48 hrs. After 10 days they were cultured for 3 days in presence of 7.5ng/ml GM-CSF, 50ng/ml IL-10 in 10% FCS containing DMEM and loosely bound DCs are harvested on last day and pulsed overnight with 0.1 mg/ml OVA.

Preparation of OT II $CD4^+$ Th2 cells.

Naïve OVA-specific $CD4^+$ T cells were isolated from OT II mouse spleens. T cells were enriched by passage through nylon wool columns and then $CD4^+$ cells were purified by negative selection using anti-mouse CD8 paramagnetic beads (DYNAL Inc, Lake Success, NY) to yield populations that were >95% $CD4^+/V\alpha2V\beta5^+$ T cells. To generate *in vitro* DC_{OVA}-activated $CD4^+$ Th2 cells, $CD4^+$ T cells ($2X10^5$ cells/ml) from OT II mice or designated gene-deleted OT II mice were stimulated for three days with irradiated (4,000 rad) bone marrow derived DC_{OVA} ($1X10^5$ cells/ml) in presence of IL-4 (20 ng/ml) and anti-IFN-γ Ab (10 μg/ml) (R&D Systems, Minneapolis, MN). *In vitro* DC_{OVA}-activated $CD4^+$ T cells derived from OT II with respective IL-4, IL-5, IL-6 and IL-10 gene KO mice were referred to as Th2(IL-4 KO), Th2(IL-5 KO), Th2(IL-6 KO) and Th2(IL-10 KO), respectively.

Preparation of OT II $CD4^+$ Th1 cells.

Naïve OVA-specific $CD4^+$ T cells isolated as explained earlier. To generate *in vitro* DC_{OVA}-activated $CD4^+$ Th1 cells, $CD4^+$ T cells ($2X10^5$ cells/ml) from OT II mice were stimulated for three days with irradiated (4,000 rad) bone marrow derived DC_{OVA} ($1X10^5$ cells/ml) in presence of IL-2 (20U/ml), IL-12 (5ng/ml) and anti-IL-4 Ab (10μg/ml) (R&D Systems, Minneapolis, MN).

Preparation of OT II $CD4^+$ Tr1 cells.

Naïve OVA-specific $CD4^+$ T cells isolated as explained earlier. To generate *in vitro* DC_{OVA}-activated $CD4^+$ Tr1 cells, $CD4^+$ T cells ($2X10^5$ cells/ml) from OT II mice were stimulated for five days with irradiated (4,000 rad) bone marrow derived inhibitory IL-10 secreting DC_{OVA} ($1X10^5$ cells/ml) in presence of IL-2 (20U/ml), IL-10 (20ng/ml) and TGF-β (20ng/ml) (R&D Systems, Minneapolis, MN).

Phenotypic characterization of $CD4^+$ Th1, Th2 and Tr1 cells.

For the phenotypic analyses, the above Th1, Th2 and Tr1 cells were stained with a panel of biotin-conjugated Abs. After washing with PBS, these cells were further stained with R-phycoerythrin (PE)-conjugated avidin and analyzed by flow cytometry. Their culture supernatants were analyzed for cytokine expression using ELISA kits (Endogen, Cambridge, MA), as previously described (16).

RT-PCR.

Total RNA was extracted from Th1, Th2 and Tr1 cells with Qiagen RNeasy purification kit (Qiagen, Mississauga, Ontario, Canada) as per manufacturer's protocols. Qiagen quantitative reverse transcription kit (Qiagen) was used to synthesize cDNA, which was then analysed by semi-quantitative PCR in triplicates.

In vivo $CD8^+$ T cell proliferation assay.

Wild type C57BL/6 (n=6) mice were i.v. immunized with irradiated (4,000 rad) DC_{OVA} ($0.5X10^6$ cells) or DC_{OVA} ($0.5X10^6$ cells) plus Th1 or Tr1 or Th2 or Th2(KO) ($3X10^6$ cells), respectively. In another experiment wild type C57BL/6 mice were i.v. immunized with irradiated (4,000 rad) DC_{OVA} ($0.5X10^6$ cells) plus Th2(IL-6 KO) or Th2(IL-10 KO) ($3X10^6$ cells) along with anti-IL-10

or anti-IL-6 neutralizing antibodies on 0 and 3rd day after immunization, respectively. Six days subsequent to immunization, the mouse blood samples were harvested and stained with PE-H-2K^b/OVAI tetramer and FITC-anti-CD8 Ab (PK135) (Beckman Coulter). The erythrocytes were then lysed using lysis/fixed buffer (Beckman Coulter) and samples were analyzed by flow cytometry according to the company's protocol.

In vivo CD8$^+$ T cell cytotoxicity assay.

In *in vivo* cytotoxicity assay, six days after immunization, the cohort of above immunized mice were i.v. co-injected with 1:1 ratio of splenocytes labeled with high (3.0 µM, CFSE^{high}) and low (0.6 µM, CFSE^{low}) concentrations of CFSE and pulsed with OVAI and Mut1, respectively (16). Sixteen hours after target cell delivery, the spleens were removed and residual CFSE^{high} and CFSE^{low} target cells remaining in the recipients' spleens were sorted and analyzed by flow cytometry.

Animal studies.

Wild-type C57BL/6 mice (n=8) were i.v. immunized with irradiated (4,000 Rad) DC_{OVA} ($0.5X10^6$ cells per mouse) alone or in combination with Th1, Tr1, Th2, Th2(IL-6 KO) and Th2(IL-10 KO) ($3X10^6$ cells per mouse), respectively. Eight days later, the mice were i.v. challenged with BL6-10_{OVA} ($0.3X10^6$ cells per mouse) tumor cells. The mice were sacrificed 3 weeks after tumor cell challenge and lung metastatic tumor colonies were counted.

Statistical analysis.

All experiments were tested for statistical significance using unpaired, two tailed, *student's t test*. Differences were considered significant if $p<0.05$.

4.5 RESULTS

4.5.1 Phenotypic characterization of OVA-specific CD4$^+$ Th1, Th2 and Tr1 cells

To generate different subsets of ovalbumin (OVA)-specific Th1, Th2 and Tr1 cells, naive OT II mouse CD4$^+$ T cells were cultured with OVA-pulsed dendritic cells (DC_{OVA}) expressing CD11c, Ia^b, CD40, CD80 and pMHC I (**Fig 4.1A**) in the presence of subset specific differentiation medium. To phenotypically characterize them, these DC_{OVA}-activated CD4$^+$ T cells were analyzed by flow cytometry, RT-PCR and ELISA for assessment of expression of cell surface markers, intracellular transcription factors and cytokine secretion, respectively. We demonstrated that these T cells were positive for CD4, CD25, and CD69 expression (**Fig 4.1B**), indicating that they are active CD4$^+$ T cells. CD4$^+$ Th1 cells expressed transcription factor T-bet (**Fig 4.1C**) and secreted IL-2 (2.5 ng/ml), IL-6 (0.9 ng/ml), IFN-γ (3.4 ng/ml) but no IL-4, IL-5 and IL-10 (**Fig 4.1D**), whereas CD4$^+$ Th2 cells expressed transcription factor GATA-3 and secreted IL-2 (1.4 ng/ml), IL-4 (1.3 ng/ml), IL-5 (1.5 ng/ml), IL-6 (1 ng/ml), IL-10 (0.4 ng/ml) and undetectable levels of IFN-γ, which are consistent with typical phenotypes of CD4$^+$ Th1 and Th2 cells, respectively. In contrast, CD4$^+$ Tr1 cells expressed transcription factor Foxp3 and secreted IL-5 (0.5 ng/ml), IL-10 (2.5 ng/ml), IFN-γ (1.9 ng/ml), as we previously described (13).

4.5.2 Th1 and Th2 cells augment whereas Tr1 cells inhibit DC_{OVA}-induced CTL responses

To assess the functional effect of different CD4$^+$ T subsets on DC_{OVA}-induced CTL responses, DC_{OVA} were injected intravenously into wild type C57BL/6 mice. DC_{OVA}-immunized mice showed OVA-specific CD8$^+$ T cell proliferation accounting for 0.79 % of the total CD8$^+$ T cell population in the peripheral blood (**Fig 4.2A**). When Th2 cells were co-injected with DC_{OVA}, OVA-specific CD8$^+$ T cells were increased to 0.97 % ($p<0.05$), indicating that *in vitro* generated Th2 cells enhance DC_{OVA}-induced CTL responses (**Fig 4.2A**). To assess the functional effect of activated CTLs, we adoptively transferred OVAI peptide-pulsed wild-type (WT) C57BL/6 mouse splenocytes strongly labeled with CFSE ($CFSE^{high}$) and equal proportion of control peptide Mut1 pulsed wild type splenocytes weakly labeled with CFSE ($CFSE^{low}$) to wild type C57BL/6 mice

that were immunized 6 days earlier with DC_{OVA} alone or co-injected with DC_{OVA} and Th1 or Th2 or Tr1 cells. Sixteen hrs after their transfer, we found that 50.3% of $CFSE^{high}$ labeled cells were killed and none of the $CFSE^{low}$ labeled cells were killed in DC_{OVA}-immunized mice, whereas in Th2 and DC_{OVA} co-injected mice 68.7% of $CFSE^{high}$ cells were killed (**Fig 4.2B**). In concurrence with $CD8^+$ T cell proliferative responses (**Fig 4.2A**), there was significantly increased cytotoxicity associated with Th2 co-injection with DC_{OVA} ($p<0.05$), indicating that Th2-enhanced CTLs are functional effectors with killing activity to OVA-specific target cells.

4.5.3 IL-6 gene deficient $CD4^+$ Th2 cells behave like functional Tr1 cells by inhibiting DC_{OVA}-induced CTL responses in an IL-10 dependent manner

IL-6 gene deficient $CD4^+$ Th2 [Th2(IL-6 KO)] cells were obtained by culturing DC_{OVA} with naïve $CD4^+$ T cells derived from OTII/IL-$6^{-/-}$ mice in the presence of IL-4 and anti-IFN-γ Ab. These Th2(IL-6 KO) cells had similar phenotypes, including expression of cell surface markers and transcription factor, as WT Th2 cells (data not shown). In addition, while being IL-6 deficient, they secreted IL-2 (0.5 ng/ml), IL-4 (0.6 ng/ml), IL-5 (1.1 ng/ml), IL-10 (2.5 ng/ml), IFN-γ (1.4 ng/ml) (**Fig 4.3A**). Interestingly, we found that DC_{OVA}-induced OVA-specific CTL responses dramatically declined from the original 0.79% (DCova) to only 0.18% [DCoav+Th2(IL6KO)] (**Fig 4.3B**). In contrast, control, IL-4 gene deficient $CD4^+$ Th2 [Th2(IL-4 KO)] cells and IL-5 gene deficient $CD4^+$ Th2 [Th2(IL-5 KO)] cells did not modulate DC_{OVA}-stimulated OVA-specific CTL responses. To elucidate the molecular mechanism underlying immune suppression by Th2(IL-6 KO) cells, we i.v. injected mice with neutralizing anti-IL-10 Ab simultaneously with Th2(IL-6 KO) cells to block IL-10 signal. We found anti-IL-10 Ab to completely inhibit the immune suppressive effect of Th2(IL-6 KO) cells on DC_{OVA}-induced OVA-specific CTL responses (**Fig 4.3C**), indicating that Th2(IL-6 KO) cells behaving in analogy to Tr1 cells suppress immune responses by secretion of IL-10 cytokine, which is consistent with our previously published observation (13).

4.5.4 IL-10 gene deficient $CD4^+$Th2 cells behave like functional Th1 cells by enhancing DC_{OVA}-induced CTL responses through IL-6 cytokine

IL-10 gene deficient $CD4^+$ Th2 [Th2(IL-10 KO)] cells were obtained by culturing DC_{OVA} with naïve $CD4^+$ T cells derived from OTII/IL-$10^{-/-}$ mice in the presence of IL-4 and anti-IFN-γ Ab. Based on the expression of cell surface markers and of the GATA-3 transcription factor, these Th2(IL-10 KO) cells had a phenotype resembling WT Th2 cells (data not shown). In addition, despite IL-10 deficiency, they secreted IL-2 (0.5 ng/ml), IL-4 (1.2 ng/ml), IL-5 (2.5 ng/ml), IL-6 (1.5 ng/ml), IFN-γ (2.4 ng/ml) (**Fig 4.3A**). Interestingly, we found that DC_{OVA}-stimulated OVA-specific CTL responses significantly increased from the original 0.97%, triggered by DC_{OVA}, to 1.28% ($P<0.01$) in the presence of DC_{OVA} and Th2(IL-10 KO) co-stimulation (**Fig 4.3B**), which is similar to enhancement of CTL responses by Th1 cells **(Fig 4.2A)**. To elucidate the molecular mechanism underlying the immune enhancement by Th2(IL-10 KO) cells, we co-injected the mice with neutralizing anti-IL-6 Ab and Th2(IL-10 KO) cells to block IL-6 signal. In this experiment, anti-IL-6 Ab completely blocked the enhancement of DC_{OVA}-induced CTL responses initiated by Th2(IL-10 KO) cells (**Fig 4.3C**), indicating that Th2(IL-10 KO) behave similar to Th1 population and augment immune responses by secreting IL-6.

4.5.5 IL-6 gene deficient $CD4^+$ Th2 cells inhibit and IL-10 deficient cells enhance DC_{OVA}-triggered OVA-specific antitumor immunity

To confirm the above observations that IL-6 gene deficient $CD4^+$ Th2 cells suppress, while IL-10 deficient cells promote DC_{OVA}-induced OVA-specific CTL responses, we conducted animal experiments and monitored antitumor immunity. Wild-type C57BL/6 mice were immunized with DC_{OVA} and DC_{OVA} accompanied by Th1, Th2, Tr1 or Th2(KO) cells. All immunized mice were challenged with BL6-10_{OVA} tumor cells eight days following the immunization. DC_{OVA} immunized mice demonstrated complete protection (8/8) from lung tumor metastasis (**Table 4.2**). DC_{OVA}-immunized mice with the co-injection of Th1, Th2 or Th2(IL-10 KO) cells were equally protected (8/8), however mice co-injected with Tr1 or Th2(IL-6 KO) cells all (8/8) died of lung tumor metastasis, confirming that Th2(IL-10 KO) cells act like functional Th1 and Th2(IL-6 KO) behave similar to Tr1 cells in stimulating, and suppressing DC_{OVA}-induced antitumor immunity.

4.6 **DISCUSSION**

It is canonically accepted that Th1 and Th2 responses are counter-regulated by one another. Cross-regulation of Th1- and Th2-specific cytokines has been documented, and Th1 cytokines IFN-γ and IL-12 have been shown to dampen IL-4-mediated granuloma formation in schistasomiasis (19-21). Originally, Khoruts el al showed that Th2 cells were inefficient in suppression of Th1 cell-mediated experimental autoimmune encephalomyelitis (EAE) (22). Fernando et al demonstrated that Th2 cells neither enhanced nor suppressed antitumor CTL responses (23). Later, however, another group showed Th2 cells to play role in suppressing Th1 responses during helminth infection (24). In addition, Th2 cells also inhibited alloantigen-specific delayed-type hypersensitivity (25) and marrow graft rejection (26). In this study, we generated OVA-specific $CD4^+$ T-bet-expressing Th1, GATA-3-expressing Th2 and Foxp3-expressing Tr1 cells by culturing OVA-pulsed DC_{OVA} with OTII $CD4^+$ T cells under different culture conditions and assessed their modulatory effect on DC_{OVA}-induced OVA-specific $CD8^+$ CTL responses. We demonstrate that OVA-specific $CD4^+$ Th1 and Tr1 cells enhance and suppress DC_{OVA}-induced CTL responses, respectively, consistent with our previous reports (13). Interestingly, we clearly show that OVA-specific $CD4^+$ Th2 cells also enhance DC_{OVA}-induced CTL responses via inflammatory IL-6 secretion possibly due to the stimulatory effect on T cell proliferation (27) and counteractive effect on $CD4^+CD25^+$ Tr cell-mediated inhibition (28) mediated by the IL-6 cytokine.

The overlapping expression of some signature cytokines in different subsets of $CD4^+$ T cells not only indicate the plasticity of $CD4^+$ T cells (29), but also suggest the possibility of deletion of a single signature cytokine gene leading to functional differentiation of naïve $CD4^+$ T cells into Th1 or Tr1 cells under Th2 cell differentiation conditions. In this study, we show that IL-6 and IL-10 gene deficient $CD4^+$ Th2(IL-6 KO) and Th2(IL-10 KO) cells behave like functional Tr1 and Th1 cells by inhibiting and enhancing DC_{OVA}-induced OVA-specific $CD8^+$ CTL responses and antitumor immunity, respectively. We demonstrate that the inhibition of DC_{OVA}-induced OVA-specific CTL responses by $CD4^+$ Th2(IL-6 KO) cells is mediated by suppressive IL-10 secretion, consistent with previous reports (13, 30, 31), since the neutralizing anti-IL-10 Ab treatment completely blocked its inhibitory effect. We also demonstrate that the

enhancement of DC_{OVA}-induced OVA-specific CTL responses by $CD4^+$ Th2(IL-10 KO) cells is mediated by inflammatory IL-6 secretion since neutralizing anti-IL-6 Ab treatment completely blocked the enhancive effect. Our data indicate that potential immune stimulatory and suppressive effect of Th2 cells are balanced by IL-6 and IL-10 cytokines, and shift in this balance is likely lead to potential generation of either immunogenic Th1-like or immune-suppressive Tr1-like cells. Therefore, our study provides the first evidence for another type of $CD4^+$ T cell plasticity with converting one type of functional T subset into another by depletion of one single key signature cytokine gene. Since Th2 cells are pathogenic in allergic diseases such as asthma (7), converting Th2 cell lineage commitment with different mechanisms becomes a key target for allergen-specific immunotherapy (32, 33). Therefore, our study may provide another direction for immunotherapeutic approach to allergic diseases by converting pathogenic Th2 cells into immune suppressive Tr1 cells via blocking IL-6 by neutralizing anti-IL-6 Ab treatment.

Conclusions

Taken together, our study suggests that deletion of a single cytokine gene IL-6 and IL-10 makes $CD4^+$ Th2 cells become effector $CD4^+$ Tr1- and Th1-like cells, respectively. Our data therefore, not only provide new evidence for another type of $CD4^+$ T cell plasticity, but also may have positive impact on the development of a new immunotherapy approach to the treatment of allergic diseases.

4.7 **Acknowledgements:**

This study was supported by Canadian Institute of Health Research (MOP 405674). Manjunatha Ankathatti Munegowda was supported by Dean's Scholarship of University of Saskatchewan. We thank Mark Boyd for helping in flowcytometrtic analysis.

4.8 References:

1. Skapenko, A., J. R. Kalden, P. E. Lipsky, and H. Schulze-Koops. 2005. The IL-4 receptor alpha-chain-binding cytokines, IL-4 and IL-13, induce forkhead box P3-expressing CD25+CD4+ regulatory T cells from CD25-CD4+ precursors. *J Immunol* 175:6107-6116.
2. Wilson, C. B., E. Rowell, and M. Sekimata. 2009. Epigenetic control of T-helper-cell differentiation. *Nat Rev Immunol* 9:91-105.
3. Szabo, S. J., S. T. Kim, G. L. Costa, X. Zhang, C. G. Fathman, and L. H. Glimcher. 2000. A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell* 100:655-669.
4. Goedegebuure, P. S., and T. J. Eberlein. 1995. The role of CD4+ tumor-infiltrating lymphocytes in human solid tumors. *Immunol Res* 14:119-131.
5. Zheng, W., and R. A. Flavell. 1997. The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. *Cell* 89:587-596.
6. Carter, L. L., and R. W. Dutton. 1996. Type 1 and type 2: a fundamental dichotomy for all T-cell subsets. *Curr Opin Immunol* 8:336-342.
7. Gordon, J. R., F. Li, A. Nayyar, J. Xiang, and X. Zhang. 2005. CD8 alpha+, but not CD8 alpha-, dendritic cells tolerize Th2 responses via contact-dependent and -independent mechanisms, and reverse airway hyperresponsiveness, Th2, and eosinophil responses in a mouse model of asthma. *J Immunol* 175:1516-1522.
8. Roncarolo, M. G., S. Gregori, M. Battaglia, R. Bacchetta, K. Fleischhauer, and M. K. Levings. 2006. Interleukin-10-secreting type 1 regulatory T cells in rodents and humans. *Immunol Rev* 212:28-50.
9. Chen, W., W. Jin, N. Hardegen, K. J. Lei, L. Li, N. Marinos, G. McGrady, and S. M. Wahl. 2003. Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J Exp Med* 198:1875-1886.
10. Bettelli, E., Y. Carrier, W. Gao, T. Korn, T. B. Strom, M. Oukka, H. L. Weiner, and V. K. Kuchroo. 2006. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* 441:235-238.
11. Liu, Y., P. Zhang, J. Li, A. B. Kulkarni, S. Perruche, and W. Chen. 2008. A critical function for TGF-beta signaling in the development of natural CD4+CD25+Foxp3+

regulatory T cells. *Nat Immunol* 9:632-640.

12. Tone, Y., K. Furuuchi, Y. Kojima, M. L. Tykocinski, M. I. Greene, and M. Tone. 2008. Smad3 and NFAT cooperate to induce Foxp3 expression through its enhancer. *Nat Immunol* 9:194-202.
13. Hao, S., J. Yuan, S. Xu, M. A. Munegowda, Y. Deng, J. Gordon, Z. Xing, and J. Xiang. 2008. Antigen specificity acquisition of adoptive CD4+ regulatory T cells via acquired peptide-MHC class I complexes. *J Immunol* 181:2428-2437.
14. Umeshappa, C. S., H. Huang, Y. Xie, Y. Wei, S. J. Mulligan, Y. Deng, and J. Xiang. 2009. CD4+ Th-APC with acquired peptide/MHC class I and II complexes stimulate type 1 helper CD4+ and central memory CD8+ T cell responses. *J Immunol* 182:193-206.
15. Ankathatti Munegowda, M., Y. Deng, S. J. Mulligan, and J. Xiang. Th17 and Th17-stimulated CD8(+) T cells play a distinct role in Th17-induced preventive and therapeutic antitumor immunity. *Cancer Immunol Immunother* 60:1473-1484.
16. Xiang, J., H. Huang, and Y. Liu. 2005. A new dynamic model of CD8+ T effector cell responses via CD4+ T helper-antigen-presenting cells. *J Immunol* 174:7497-7505.
17. Porgador, A., J. W. Yewdell, Y. Deng, J. R. Bennink, and R. N. Germain. 1997. Localization, quantitation, and in situ detection of specific peptide-MHC class I complexes using a monoclonal antibody. *Immunity* 6:715-726.
18. Huang, H., W. Dawicki, X. Zhang, J. Town, and J. R. Gordon. Tolerogenic dendritic cells induce CD4+CD25hiFoxp3+ regulatory T cell differentiation from CD4+CD25-/loFoxp3- effector T cells. *J Immunol* 185:5003-5010.
19. Lukacs, N. W., and D. L. Boros. 1993. Lymphokine regulation of granuloma formation in murine schistosomiasis mansoni. *Clin Immunol Immunopathol* 68:57-63.
20. Oswald, I. P., P. Caspar, D. Jankovic, T. A. Wynn, E. J. Pearce, and A. Sher. 1994. IL-12 inhibits Th2 cytokine responses induced by eggs of Schistosoma mansoni. *J Immunol* 153:1707-1713.
21. Boros, D. L., and J. R. Whitfield. 1999. Enhanced Th1 and dampened Th2 responses synergize to inhibit acute granulomatous and fibrotic responses in murine schistosomiasis mansoni. *Infect Immun* 67:1187-1193.
22. Khoruts, A., S. D. Miller, and M. K. Jenkins. 1995. Neuroantigen-specific Th2 cells are

inefficient suppressors of experimental autoimmune encephalomyelitis induced by effector Th1 cells. *J Immunol* 155:5011-5017.

23. Fernando, G. J., T. J. Stewart, R. W. Tindle, and I. H. Frazer. 1998. Th2-type CD4+ cells neither enhance nor suppress antitumor CTL activity in a mouse tumor model. *J Immunol* 161:2421-2427.
24. McKee, A. S., and E. J. Pearce. 2004. CD25+CD4+ cells contribute to Th2 polarization during helminth infection by suppressing Th1 response development. *J Immunol* 173:1224-1231.
25. Waaga, A. M., M. Gasser, J. E. Kist-van Holthe, N. Najafian, A. Muller, J. P. Vella, K. L. Womer, A. Chandraker, S. J. Khoury, and M. H. Sayegh. 2001. Regulatory functions of self-restricted MHC class II allopeptide-specific Th2 clones in vivo. *J Clin Invest* 107:909-916.
26. Erdmann, A. A., U. Jung, J. E. Foley, Y. Toda, and D. H. Fowler. 2004. Co-stimulated/Tc2 cells abrogate murine marrow graft rejection. *Biol Blood Marrow Transplant* 10:604-613.
27. Takeda, K., T. Kaisho, N. Yoshida, J. Takeda, T. Kishimoto, and S. Akira. 1998. Stat3 activation is responsible for IL-6-dependent T cell proliferation through preventing apoptosis: generation and characterization of T cell-specific Stat3-deficient mice. *J Immunol* 161:4652-4660.
28. Pasare, C., and R. Medzhitov. 2003. Toll pathway-dependent blockade of CD4+CD25+ T cell-mediated suppression by dendritic cells. *Science* 299:1033-1036.
29. Zhu, J., and W. E. Paul. Heterogeneity and plasticity of T helper cells. *Cell Res* 20:4-12.
30. Fiorentino, D. F., A. Zlotnik, P. Vieira, T. R. Mosmann, M. Howard, K. W. Moore, and A. O'Garra. 1991. IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells. *J Immunol* 146:3444-3451.
31. Steinbrink, K., M. Wolfl, H. Jonuleit, J. Knop, and A. H. Enk. 1997. Induction of tolerance by IL-10-treated dendritic cells. *J Immunol* 159:4772-4780.
32. Hawrylowicz, C. M., and A. O'Garra. 2005. Potential role of interleukin-10-secreting regulatory T cells in allergy and asthma. *Nat Rev Immunol* 5:271-283.
33. Holgate, S. T., and R. Polosa. 2008. Treatment strategies for allergy and asthma. *Nat Rev Immunol* 8:218-230.

Table 4.1. Polarizing culture conditions of different subsets of T-helper cells.

T-helper subset	polarizing culture conditions condition
Th1	IL-2 (20 U/ml), IL-12 (5 ng/ml) and anti-IL-4 Ab (10 μg/ml)
Th2	IL-4 (20 ng/ml) and anti-IFN-γ Ab (10 μg/ml)
Tr1	IL-2 (20 U/ml), IL-10 (20 ng/ml) and TGF-β (20 ng/ml)

Table 4.2. Inhibitory or stimulatory effects of Th2 cells on DC_{OVA}-induced immunity against lung tumor metastases

Immunization	Tumor bearing mice (%)	Median number of colonies
DC_{OVA}	0/8 (0)	0
Th2 + DC_{OVA}	0/8 (0)	0
Th1 + DC_{OVA}	0/8 (0)	0
Tr1 + DC_{OVA}	8/8 (100)	>100
Th2(IL-6 KO) + DC_{OVA}	8/8 (100)	>100
Th2(IL-10 KO) + DC_{OVA}	0/8 (0)	0
PBS	8/8 (100)	>100

C57BL/6 mice (8 per group) were i.v. immunized with DC_{OVA} and injected with Th2 or Th1 or Tr1or Th2 with cytokine gene deficiency. Eight days after injection, mice were challenged with BL6-10_{OVA} cells. The mice were sacrificed 3 weeks after tumor cell challenge and lung metastatic tumor colonies were counted. One representative experiment of two is shown.

Figure 4.1.

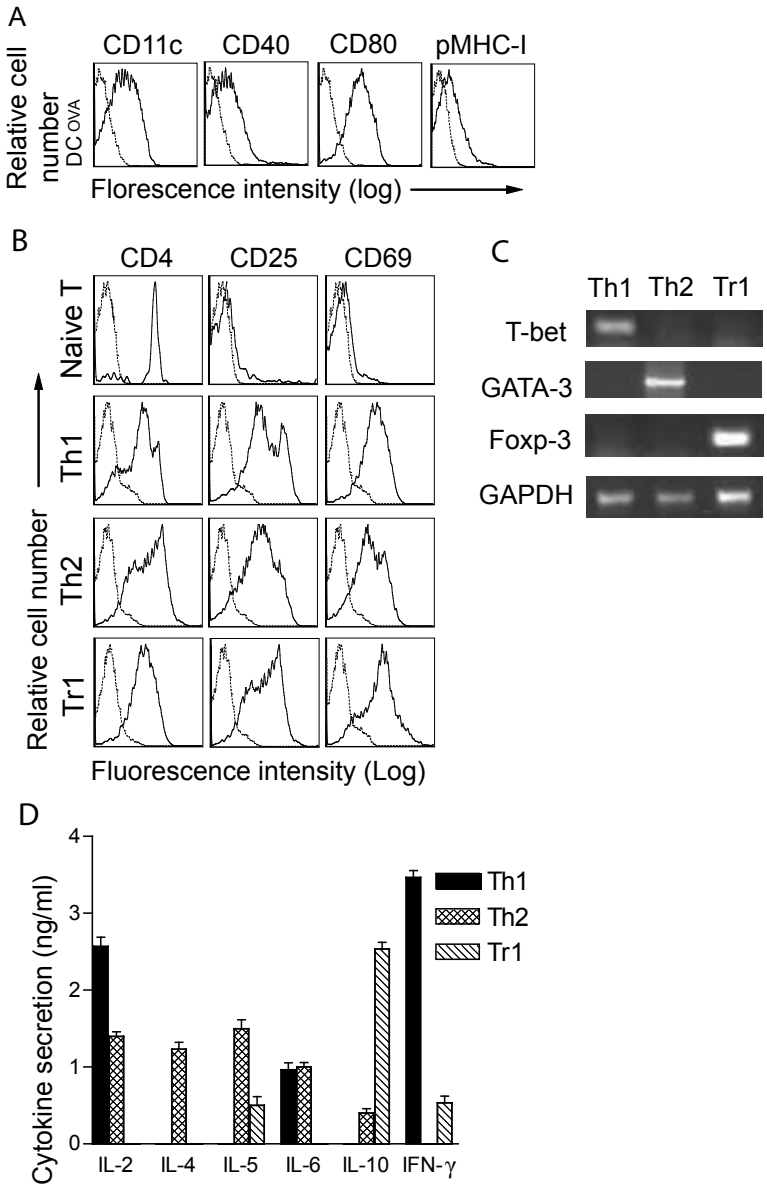
A
CD11c
CD40
CD80
pMHC-I
Relative cell number
DC OVA
Florescence intensity (log)
B
CD4
CD25
CD69
Naive T
Th1
Th2
Tr1
Relative cell number
Fluorescence intensity (Log)
C
Th1
Th2
Tr1
T-bet
GATA-3
Foxp-3
GAPDH
D
Cytokine secretion (ng/ml)
0
1
2
3
4
Th1
Th2
Tr1
IL-2
IL-4
IL-5
IL-6
IL-10
IFN-γ

Figure 4.1. Phenotypic characterization of Th1, Th2, Tr1 and DC_{OVA}. (A) Flow cytometric analysis of DC_{OVA}. *In vitro* generated DC_{OVA} were stained with panel of Abs for analysis of cell surface expression of CD11c, CD40, CD80 and pMHC-I (thick solid lines). Isotype-matched irrelevant Abs were used as controls (dotted lines). **(B)** Flow cytometric analysis. *In vitro* DC_{OVA}-activated Th1 and Th2, and *in vitro* inhibitory DC_{OVA} activated-Tr1 population were stained with a panel of Abs for analysis of cell surface expression of CD4, CD25 and CD69 (thick solid lines). Isotype-matched irrelevant Abs were used as controls (dotted lines). **(C)** RNA extracted from Th1, Th2 and Tr1 cells were analyzed by RT-PCR to assess the expression of T-bet, GATA-3 and Foxp-3. **(D)** The supernatants of Th1, Th2 and Tr1 cells were assayed for IL-2, IL-4, IL-5, IL-6, IL-10 and IFN-γ secretion by ELISA. One representative experiment of three is displayed.

Figure 4.2

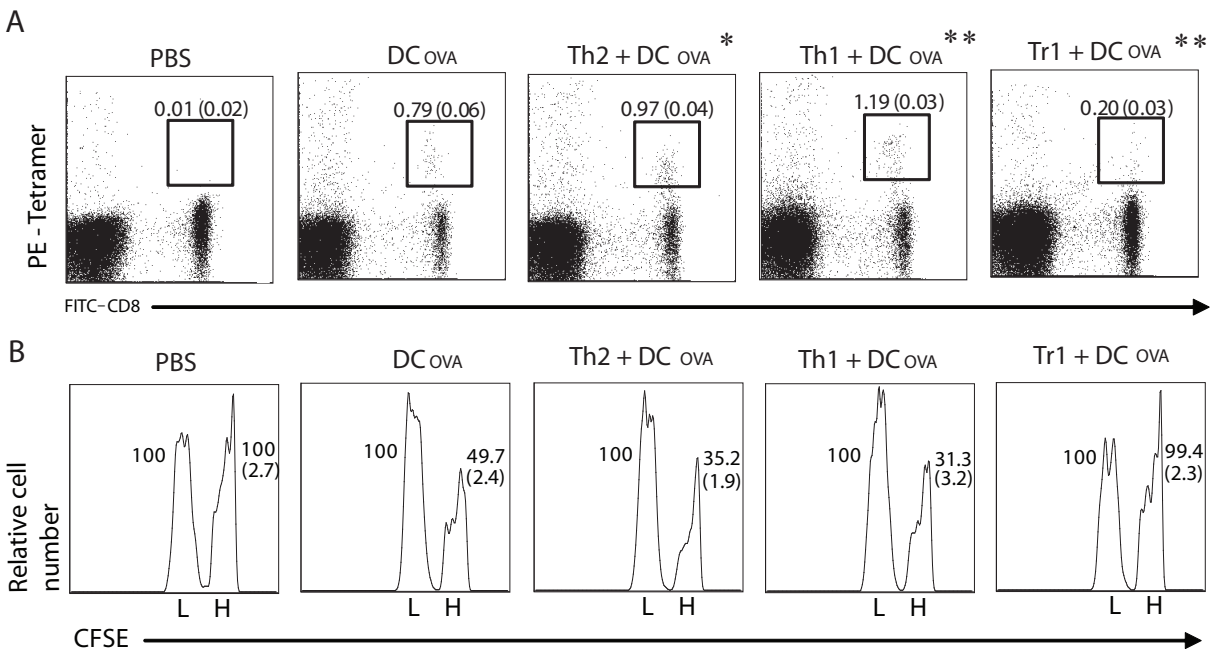
A
PBS
DC OVA
Th2 + DC OVA *
Th1 + DC OVA **
Tr1 + DC OVA **
0.01 (0.02)
0.79 (0.06)
0.97 (0.04)
1.19 (0.03)
0.20 (0.03)
PE - Tetramer
FITC-CD8
B
PBS
DC OVA
Th2 + DC OVA
Th1 + DC OVA
Tr1 + DC OVA
100
100 (2.7)
100
49.7 (2.4)
100
35.2 (1.9)
100
31.3 (3.2)
100
99.4 (2.3)
L H
Relative cell number
CFSE

Figure 4.2. Stimulatory or inhibitory effects of Th1, Th2 and Tr1 cells on DC_{OVA} induced $CD8^+$ T cell response. (A) Tetramer staining assay. Wild type C57BL/6 mice (6 mice per group) were immunized with PBS, DC_{OVA}, DC_{OVA} along with Th1/Th2/Tr1 cells. Six days after immunization tail blood samples of immunized mice were stained with PE-H-$2K^b$/OVAI tetramer and FITC-anti-CD8 Ab, and analyzed by flow cytometry. The value in each panel represents the percentage of tetramer-positive $CD8^+$ T cells versus the total $CD8^+$ T cells with standard deviation in parenthesis. The differences between DC_{OVA} and Th2+DC_{OVA} mice (*) or Th1+DC_{OVA} and Tr1+DC_{OVA} mice (**) are significant ($p<0.05$) or very significant ($p<0.01$) (students t test). **(B)** *In vivo* cytotoxicity assay. The residual $CFSE^{high}$ (H) and $CFSE^{low}$ (L) target cells remaining in the recipient spleens were analyzed by flow cytometry. The value in each panel represents the percentage of $CFSE^{high}$ vs $CFSE^{low}$ target cells remaining in the spleen with standard deviation in parenthesis. (n=6, average±SD). One representative experiment of two is shown.

Figure 4.3.

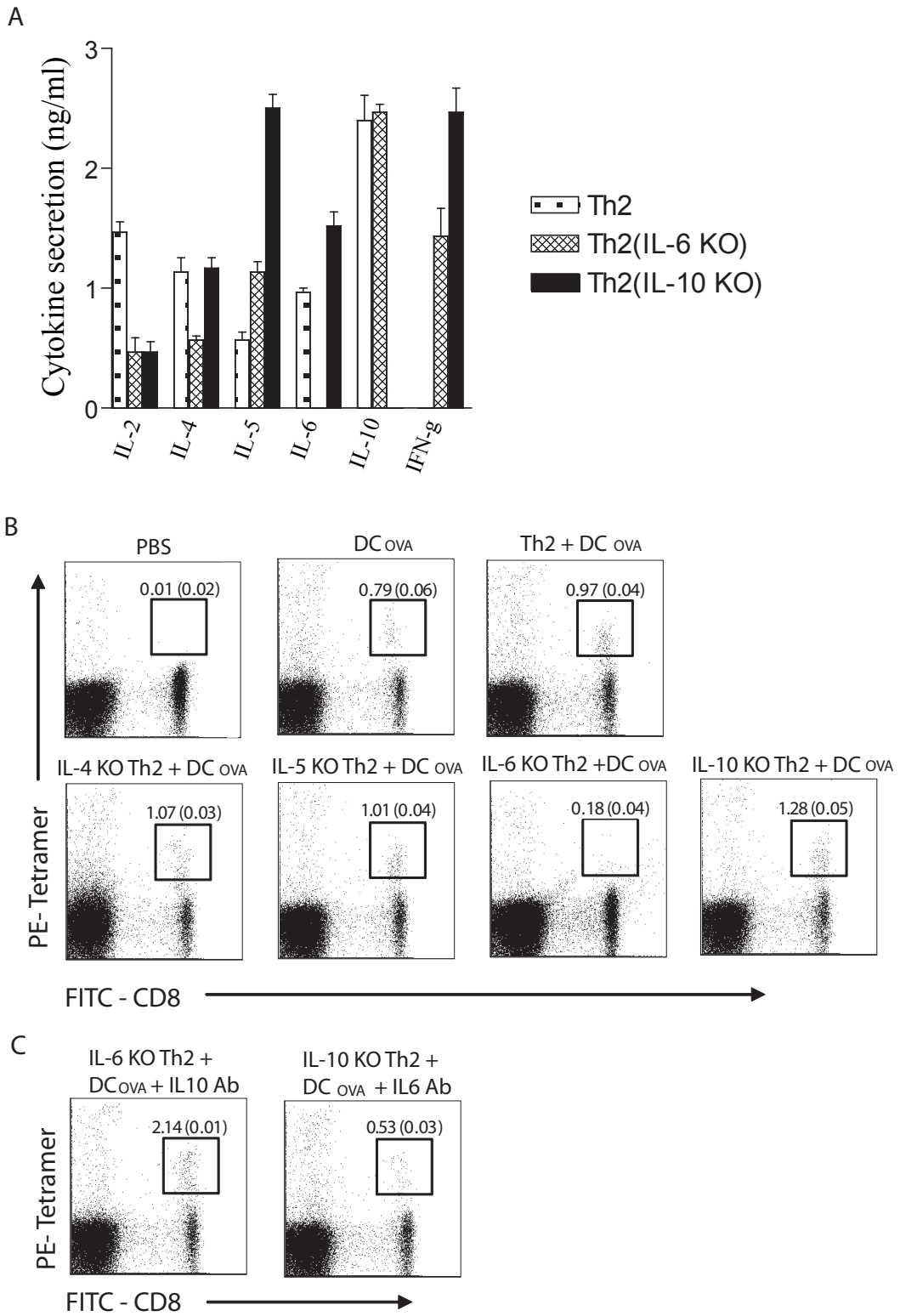
A
Cytokine secretion (ng/ml)
3
2
1
0
IL-2
IL-4
IL-5
IL-6
IL-10
IFN-g
Th2
Th2(IL-6 KO)
Th2(IL-10 KO)
B
PBS
DC OVA
Th2 + DC OVA
0.01 (0.02)
0.79 (0.06)
0.97 (0.04)
IL-4 KO Th2 + DC OVA
IL-5 KO Th2 + DC OVA
IL-6 KO Th2 +DC OVA
IL-10 KO Th2 + DC OVA
1.07 (0.03)
1.01 (0.04)
0.18 (0.04)
1.28 (0.05)
PE- Tetramer
FITC - CD8
C
IL-6 KO Th2 +
DC OVA + IL10 Ab
IL-10 KO Th2 +
DC OVA + IL6 Ab
2.14 (0.01)
0.53 (0.03)
PE- Tetramer
FITC - CD8

Figure 4.3. Stimulatory or inhibitory impacts of cytokine gene deficient Th2 cells on DC_{OVA} induced $CD8^+$ T cell responses. (A) The supernatants of Th2, Th2(IL-6 KO) and Th2(IL-10 KO) cells were analyzed cytokines IL-2, IL-4, IL-5, IL-6, IL-10 and IFN-γ secretion by ELISA. **(B & C)** Tetramer staining assay. In experiment **B,** wild type C57BL/6 mice (6 mice per group) were immunized with DC_{OVA} and different knockout Th2. In experiment **C,** C57BL/6 mice (6 mice per group) were immunized with DC_{OVA} and different knockout Th2, and simultaneously these mice were injected twice (day 0 and day 3) with depicted neutralizing antibodies. Six days after immunization tail blood samples of immunized mice were stained with PE-H-$2K^b$/OVAI tetramer and FITC-anti-CD8 Ab, and analyzed by flow cytometry. The value in each panel represents the percentage of tetramer-positive $CD8^+$ T cells versus the total $CD8^+$ T cells with standard deviation in parenthesis (n=6, average±SD).

CHAPTER 5

DISCUSSION, CONCLUSIONS, AND FUTURE DIRECTIONS

5.1 General discussion

Specific discussions associated with each objective pursued in this thesis work are presented under each chapter (chapters 2 to 4). The purpose of this discussion is to comprehensively understand the thesis as whole. Though each chapter deals with different aspects, they all come under the aegis of $CD4^+$ T cell biology. $CD4^+$ T helper cells are the conductors of immune responses, controlling/helping other cells in eliciting favourable immune-response and in keeping memories of antigen encounters. A naive $CD4^+$ T cell develops into different types of $CD4^+$ T helper cell subsets under different microenvironments. Recent evidence has also shown that some of the terminally differentiated $CD4^+$ T cells can re-differentiate into a different subtype because of the plasticity associated with these cells. An infection or an aberrant growth of tumor cells or autoimmunity occurs when there is an imbalance in immune responses. $CD4^+$ T cells being the conductors controlling different arms of immune-response, most of the time an imbalance in immune responses in the above said conditions occurs from deregulated $CD4^+$ T cell response. Because of the importance associated with $CD4^+$ T cells, it is very important to understand the patho-physiology and biology associated with $CD4^+$ T cells. In our study, we have deciphered the role of $CD4^+$ Th17 cells in tumor immunity (chapter 2), and in autoimmune T1D and EAE (chapter 3). We have also understood the biology associated with $CD4^+$ Th2 cells (chapter 4).

5.2 $CD4^+$ Th17 cells in tumor immunity

During the course of this thesis work, there has been a flow of enormous information related to Th17-cells. When we started our study, there was no literature indicating antitumor responses of Th17 cells, though there were various studies showing the presence of these cells in different tumors. There were controversial results regarding the role of Th17 cells and Th17-cytokine IL-17, since transgenic expression of IL-17 induced tumor regression through enhanced antitumor immunity in immune-competent mice (1, 2) or promoted tumor growth through

increased inflammatory angiogenesis in immune deficient mice (3). When we understand the studies relating to IL-17, we have to treat IL-17 and Th17 cells as different factors because, apart from Th17 cells, various other cells like smooth muscle, NK, γδ T cells also secrete IL-17. There were studies showing the role of Th17 cells in autoimmunity and Th17 cells as a pro-inflammatory subset. Understanding this pro-inflammatory nature of Th17 cells, we speculated that they might be beneficial cells in tumors. During the course of our study, there were other studies suggesting the antitumor response of Th17 cells (4, 5). In Dr. Jim Xiang's laboratory, it was demonstrated that $CD4^+$ Th1 cells acquire DC's molecules through trogocytosis during their interaction; in our study, we speculated that $CD4^+$ Th17 cells which are functionally and phenotypically different from $CD4^+$ Th1 cells will acquire DC's molecules (6-8). Moreover, for the first time we have demonstrated that in vitro DC_{OVA} activated Th17 cells expressing Ror-γt and secreting IL-17, IL-2 also acquired pMHC I and stimulated antigen-specific $CD8^+$ CTL response and long term memory via IL-2 and pMHC I, but not via IL-17. We have also demonstrated that Th17-induced preventive antitumor immunity is mediated through Th17-stimulated $CD8^+$ CTLs. Our study elucidates that the molecular mechanism of Th17 cells has a stimulatory effect on $CD8^+$ CTL response; apart from that, we have also shown that it is the Th17-stimulated $CD8^+$ CTLs, but not Th17 cells themselves, which have direct in vitro killing activity on tumor cells. In the therapeutic model, we found that it is the Th17-activated $CD8^+$ T cells that play a major role in the eradication of metastatic lung tumors, where Th17-cytokine IL-17, but not host IFN-γ, was associated with the Th17- induced therapeutic effect. We have also demonstrated that Th17 cells aid in the recruitment of various inflammatory cells (DCs, $CD4^+$ and $CD8^+$ T cells) to the tumor site through CCL2/20 chemoattraction. Although Th17 cell cytokine IL-17 helps in the chemoattraction of various inflammatory cells to a tumor site, it was the $CD8^+$ T cells which played the major role in tumor therapy through their perforin mediated killing. Overall, our study demonstrated a distinct role played by Th17 cell and Th17-stimulated $CD8^+$ T cells in preventive and therapeutic antitumor immunity. To date, adaptive T-cell immunotherapy for tumors by infusing antigen-specific $CD8^+$ T cells had seen some success; however, the major problem was the targeting of these cells to a tumor site. With our findings, we speculate that injecting Th17 cells may help in targeting those cells and also aid in tumor therapy through other mechanisms elucidated in our findings. To further perceive our speculation, Dr. Jim Xiang's lab is working to find out the possibility of enhancing antitumor

immunity in adoptive $CD8^+$ T cell therapy by co-immunizing with Th17 cells. With our findings we could also further evaluate the use of Th17 based therapies for tumor therapy by modifying the tumor immune environment to favour Th17 differentiation at tumor sites. Various studies have shown the involvement of TGF-β in the differentiation of Th17 cells as well as Treg cells. At tumor sites, tumors maintain immune suppressive conditions through various suppressor cells and immune suppressive cytokines. One of such immune suppressive cytokines is TGF-β. We speculate that we can use the TGF-β present at the tumor site to elicit Th17 responses. By local expression of IL-6 at the tumor site, we might increase Th17 differentiation through the local environment containing both IL-6 and TGF-β, which may help in tumor destruction through various mechanisms deciphered in our study.

5.3 $CD4^+$ Th17 cells in autoimmune T1D and EAE

During the course of this study, there has been accumulated data suggesting the role of Th17 cells in autoimmune T1D and EAE. However, there was a lacuna in understanding the mechanism of the pathogenic role played by Th17 cells. In our study, we have addressed this lacuna by deciphering the potential involvement of Th17 cells and Th17-stimulated $CD8^+$ T cells in the pathogenesis of T1D and EAE and we also addressed the potential relationship between Th17 and $CD8^+$ T cells.

Type 1 diabetes (T1D) is an organ-specific autoimmune disease characterized by predominantly T cell-mediated destruction of insulin producing β cells of the islets of Langerhans, culminating in lifelong insulin dependence (9). Before 1990, a vast range of evidence favoured the sole role of $CD4^+$ T cells in T1D (10). The development of T1D has usually been ascribed to a $CD4^+$ Th1 response with disease transfer in animal models being mediated by Th1 clones and lines (11, 12). In addition, a potential involvement of Th17 cells in the course of T1D was also demonstrated in the mouse model (13). However, over time, new evidence has mounted to implicate $CD8^+$ T cells in T1D initiation and progression, suggesting that $CD8^+$ T cells exert a strong role in the aetiology of T1D (14-20). Nonetheless, the potential relationship between the pathogenic $CD4^+$ Th17 and $CD8^+$ T cells and their relative effect on pathogenesis of T1D remained elusive. In our study, we found that OVA-specific Th17 cells

stimulated OVA-specific CTL responses via IL-2 and acquired pMHC I signalling when transferred into RIP-mOVA mice, and Th17-stimulated $CD8^+$ T cells were capable of killing OVA-expressing target cells via the perforin pathway. We also showed that Th17-stimulated $CD8^+$ CTLs, but not Th17 cells themselves, were required for T1D induction in RIP-mOVA mice. Our findings are consistent with some previous reports showing that transfer of islet-specific Th17 cells failed in diabetes induction, though it caused an extensive insulitis (21); treatment with neutralizing IL-17-specific Abs did not prevent T1D in NOD/SCID mice, which were derived from transfer of highly purified Th17 cells from BDC2.5 transgenic mice (12). Supporting our findings, autoreactive $CD8^+$ T cells have been shown to play an important role in the pathogenesis of T1D (22, 23). We have also demonstrated that $CD4^+$ Th17-stimulated $CD8^+$ T cells were able to kill OVA-expressing target cells both in vitro and in vivo via the perforin-dependent pathway (24), indicating that $CD4^+$ Th17-induces diabetes in RIP-mOVA mice, perhaps through OVA-expressing pancreatic beta-cells killing by $CD4^+$ Th17-stimulated $CD8^+$ CTLs via perforin-dependent pathway.

EAE is a model of human multiple sclerosis induced by autoreactive $CD4^+$ Th cells that mediate tissue inflammation and demyelination in the central nervous system. Although the predominant evidence had shown the critical role of $CD4^+$ Th17 cells in EAE pathogenesis (25-29), a potential involvement of $CD8^+$ T cells in EAE had also been recognized (30). Whereas other studies had demonstrated that both $CD4^+$ and $CD8^+$ T cells are involved in EAE pathogenesis (31), we have assessed whether $CD4^+$ or $CD8^+$ T cells are involved in the pathogenesis of EAE. Our experiments reveal that MOG immunization induced EAE only in C57BL/6 and H-2Kb-/- mice, but not in $CD4^+$ T-deficient $Iab^{-/-}$ mice, indicating that $CD4^+$ T cells are likely to play a critical role in MOG immunization-induced EAE in C57BL/6 mice. Further, we have addressed the potential relationship between the pathogenic $CD4^+$ Th17 and $CD8^+$ T cells in EAE and the extent of their influence on EAE pathogenesis. By using two types of MOG-specific $CD4^+$ Th17 cells, we have showed that the adoptively transferred $CD4^+$ Th17 cells, but not in vivo $CD4^+$ Th17-stimulated $CD8^+$ CTLs, were responsible for the EAE initiation in C57BL/6 mice, indicating that $CD4^+$ Th17 cells play a crucial role in the pathogenesis of EAE. Our data is consistent with some reports showing that IL-17A significantly contributes to the induction of EAE in immunized mice (32), and the adoptive transfer of MOG-specific $CD4^+$

Th17 cells induce EAE in C57BL/6 mice leading to the induction of EAE in wild-type C57BL/6 mice (25).

Taken together, our data elucidated a distinct role of $CD4^+$ Th17 cells and Th17-stimulated $CD8^+$ T cells in autoimmune diseases: T1D is directly mediated by Th17-stimulated $CD8^+$ cells, whereas EAE is likely triggered by $CD4^+$ Th17 cells. Therefore, this work may have great impact on the overall understanding of $CD4^+$ Th17 cells in the pathogenesis of autoimmune diseases. Understanding the pathogenesis associated with different autoimmune diseases will help in designing disease-modifying drugs to treat autoimmunity. We have shown that T1D is majorly mediated through $CD8^+$ T cells, further studies could be designed to specifically target $CD8^+$ T cells. IN case of EAE we may have to specifically target Th17 cells to treat MS patients.

5.3 Regulation of $CD4^+$ Th2 cells

Various studies had shown that Th1 and Th2 responses are counter regulated by one another (33-35). The majority of the studies dealt with the counter balance of Th1/Th2 responses or inhibitory responses of cytokines in counter regulation. In our study, we looked at the direct evidence for comparative inhibitory or stimulatory responses of Th1, Th2, and Tr1 cells on DC_{OVA}-induced Th1 kind of $CD8^+$ T cell-proliferative response. Earlier studies had shown that Th2 cells were not affecting vaccine induced antitumor CTL activity (36, 37). We found that, in concurrence with earlier studies, Th2 cells were not inhibitory on DC_{OVA}-induced $CD8^+$ T cell response. The notion of T-helper cell plasticity (12, 21, 38) suggests that immune response is far more adaptable than previously thought and is therefore able to respond more appropriately to environmental stimuli. Historically, T-helper cells were deemed terminally differentiated cell lineages committed to their paths. Most in vitro differentiation models suggest that such commitment of T-helper cells provides simplified experimental models that allow us to understand how they are regulated. Emerging evidence suggested that under certain conditions, seemingly committed T cells possess plasticity and may convert into other types of effector cells. However, how $CD4^+$ T helper cells achieve such plasticity was not fully understood, but it was very well understood that under different microenvironments even a well differentiated T helper cell was ready to re-differentiate into a different T-helper subset. We speculated that it would be

fruitful to harvest this phenomenon to treat immune mediated disease conditions by designing effective immune balancing strategies. In our study, we showed that IL-6 and IL-10 gene-deficient $CD4^+$ Th2(IL-6 KO) and Th2(IL-10 KO) cells behave like functional Tr1 and Th1 cells by inhibiting and enhancing DC_{OVA}-induced OVA-specific $CD8^+$ CTL responses and antitumor immunity, respectively. We demonstrated that the inhibition of DC_{OVA}-induced OVA-specific CTL responses by $CD4^+$ Th2(IL-6 KO) cells was mediated by suppressive IL-10 secretion, consistent with previous reports (39-41), since the neutralizing anti-IL-10 Ab treatment completely blocked its inhibitory effect. We also demonstrated that the enhancement of DC_{OVA}-induced OVA-specific CTL responses by $CD4^+$ Th2(IL-10 KO) cells was mediated by inflammatory IL-6 secretion since the neutralizing anti-IL-6 Ab treatment completely blocked its enhancive effect. Our data indicated that the potential immune stimulatory and suppressive effects of Th2 cells are balanced by its IL-6 and IL-10 cytokine, and breakage of this balance can lead to either immunogenic Th1 or suppressive Tr1-like cells. Our study provided the first evidence for another type of $CD4^+$ T cell plasticity through converting one type of functional T subset into another one by depletion of one single key signature cytokine gene. Since the persistence of Th2 cytokines is associated with allergy and asthma (42), converting Th2 cell lineage commitment with different mechanisms is a key target of allergen-specific immunotherapy (43, 44). Therefore, our study may provide another direction for an immunotherapeutic approach to allergic diseases by converting pathogenic Th2 cells into immune-suppressive Tr1 cells via blocking IL-6 by a neutralizing anti-IL-6 Ab treatment.

Taken together, our study shows that the deletion of a single cytokine gene IL-6 and IL-10 converts effector $CD4^+$ Th2 cells into functional $CD4^+$ Tr1 and Th1 cells, respectively. Our data therefore not only provides new evidence for another type of $CD4^+$ T cell plasticity, but also may have a significant positive impact on the development of a new immunotherapy approach to allergy treatment. We speculate that by adaptively transferring single gene deficient naïve $CD4^+$ T cells, they may behave differently when they get the site of allergy. This phenomenon can be utilized to get favorable response for our benefit.

5.5 References

1. Benchetrit, F., A. Ciree, V. Vives, G. Warnier, A. Gey, C. Sautes-Fridman, F. Fossiez, N. Haicheur, W. H. Fridman, and E. Tartour. 2002. Interleukin-17 inhibits tumor cell growth by means of a T-cell-dependent mechanism. *Blood* 99:2114-2121.
2. Hirahara, N., Y. Nio, S. Sasaki, Y. Minari, M. Takamura, C. Iguchi, M. Dong, K. Yamasawa, and K. Tamura. 2001. Inoculation of human interleukin-17 gene-transfected Meth-A fibrosarcoma cells induces T cell-dependent tumor-specific immunity in mice. *Oncology* 61:79-89.
3. Numasaki, M., M. Watanabe, T. Suzuki, H. Takahashi, A. Nakamura, F. McAllister, T. Hishinuma, J. Goto, M. T. Lotze, J. K. Kolls, and H. Sasaki. 2005. IL-17 enhances the net angiogenic activity and in vivo growth of human non-small cell lung cancer in SCID mice through promoting CXCR-2-dependent angiogenesis. *J Immunol* 175:6177-6189.
4. Muranski, P., A. Boni, P. A. Antony, L. Cassard, K. R. Irvine, A. Kaiser, C. M. Paulos, D. C. Palmer, C. E. Touloukian, K. Ptak, L. Gattinoni, C. Wrzesinski, C. S. Hinrichs, K. W. Kerstann, L. Feigenbaum, C. C. Chan, and N. P. Restifo. 2008. Tumor-specific Th17-polarized cells eradicate large established melanoma. *Blood* 112:362-373.
5. Martin-Orozco, N., P. Muranski, Y. Chung, X. O. Yang, T. Yamazaki, S. Lu, P. Hwu, N. P. Restifo, W. W. Overwijk, and C. Dong. 2009. T helper 17 cells promote cytotoxic T cell activation in tumor immunity. *Immunity* 31:787-798.
6. Ahmed, K. A., M. A. Munegowda, Y. Xie, and J. Xiang. 2008. Intercellular trogocytosis plays an important role in modulation of immune responses. *Cell Mol Immunol* 5:261-269.
7. Xiang, J., H. Huang, and Y. Liu. 2005. A new dynamic model of CD8+ T effector cell responses via CD4+ T helper-antigen-presenting cells. *J Immunol* 174:7497-7505.
8. Umeshappa, C. S., H. Huang, Y. Xie, Y. Wei, S. J. Mulligan, Y. Deng, and J. Xiang. 2009. CD4+ Th-APC with acquired peptide/MHC class I and II complexes stimulate type 1 helper CD4+ and central memory CD8+ T cell responses. *J Immunol* 182:193-206.
9. Atkinson, M. A., and G. S. Eisenbarth. 2001. Type 1 diabetes: new perspectives on disease pathogenesis and treatment. *Lancet* 358:221-229.
10. Haskins, K., M. Portas, B. Bradley, D. Wegmann, and K. Lafferty. 1988. T-lymphocyte clone specific for pancreatic islet antigen. *Diabetes* 37:1444-1448.

11. Katz, J. D., C. Benoist, and D. Mathis. 1995. T helper cell subsets in insulin-dependent diabetes. *Science* 268:1185-1188.
12. Bending, D., H. De la Pena, M. Veldhoen, J. M. Phillips, C. Uyttenhove, B. Stockinger, and A. Cooke. 2009. Highly purified Th17 cells from BDC2.5NOD mice convert into Th1-like cells in NOD/SCID recipient mice. *J Clin Invest* 119:565-572.
13. Vukkadapu, S. S., J. M. Belli, K. Ishii, A. G. Jegga, J. J. Hutton, B. J. Aronow, and J. D. Katz. 2005. Dynamic interaction between T cell-mediated beta-cell damage and beta-cell repair in the run up to autoimmune diabetes of the NOD mouse. *Physiol Genomics* 21:201-211.
14. Faustman, D., X. P. Li, H. Y. Lin, Y. E. Fu, G. Eisenbarth, J. Avruch, and J. Guo. 1991. Linkage of faulty major histocompatibility complex class I to autoimmune diabetes. *Science* 254:1756-1761.
15. Yan, G., Y. Fu, and D. L. Faustman. 1997. Reduced expression of Tap1 and Lmp2 antigen-processing genes in the nonobese diabetic (NOD) mouse due to a mutation in their shared bidirectional promoter. *J Immunol* 159:3068-3080.
16. Li, F., J. Guo, Y. Fu, G. Yan, and D. Faustman. 1994. Abnormal class I assembly and peptide presentation in the nonobese diabetic mouse. *Proc Natl Acad Sci U S A* 91:11128-11132.
17. Yan, G., L. Shi, and D. Faustman. 1999. Novel splicing of the human MHC-encoded peptide transporter confers unique properties. *J Immunol* 162:852-859.
18. Qu, H. Q., Y. Lu, L. Marchand, F. Bacot, R. Frechette, M. C. Tessier, A. Montpetit, and C. Polychronakos. 2007. Genetic control of alternative splicing in the TAP2 gene: possible implication in the genetics of type 1 diabetes. *Diabetes* 56:270-275.
19. Christianson, S. W., L. D. Shultz, and E. H. Leiter. 1993. Adoptive transfer of diabetes into immunodeficient NOD-scid/scid mice. Relative contributions of CD4+ and CD8+ T-cells from diabetic versus prediabetic NOD.NON-Thy-1a donors. *Diabetes* 42:44-55.
20. Wong, F. S., J. Karttunen, C. Dumont, L. Wen, I. Visintin, I. M. Pilip, N. Shastri, E. G. Pamer, and C. A. Janeway, Jr. 1999. Identification of an MHC class I-restricted autoantigen in type 1 diabetes by screening an organ-specific cDNA library. *Nat Med* 5:1026-1031.

21. Martin-Orozco, N., Y. Chung, S. H. Chang, Y. H. Wang, and C. Dong. 2009. Th17 cells promote pancreatic inflammation but only induce diabetes efficiently in lymphopenic hosts after conversion into Th1 cells. *Eur J Immunol* 39:216-224.
22. Faustman, D. L., and M. Davis. 2009. The primacy of CD8 T lymphocytes in type 1 diabetes and implications for therapies. *J Mol Med (Berl)* 87:1173-1178.
23. Pinkse, G. G., O. H. Tysma, C. A. Bergen, M. G. Kester, F. Ossendorp, P. A. van Veelen, B. Keymeulen, D. Pipeleers, J. W. Drijfhout, and B. O. Roep. 2005. Autoreactive CD8 T cells associated with beta cell destruction in type 1 diabetes. *Proc Natl Acad Sci U S A* 102:18425-18430.
24. Bolitho, P., I. Voskoboinik, J. A. Trapani, and M. J. Smyth. 2007. Apoptosis induced by the lymphocyte effector molecule perforin. *Curr Opin Immunol* 19:339-347.
25. Yang, Y., J. Weiner, Y. Liu, A. J. Smith, D. J. Huss, R. Winger, H. Peng, P. D. Cravens, M. K. Racke, and A. E. Lovett-Racke. 2009. T-bet is essential for encephalitogenicity of both Th1 and Th17 cells. *J Exp Med* 206:1549-1564.
26. Sutton, C., C. Brereton, B. Keogh, K. H. Mills, and E. C. Lavelle. 2006. A crucial role for interleukin (IL)-1 in the induction of IL-17-producing T cells that mediate autoimmune encephalomyelitis. *J Exp Med* 203:1685-1691.
27. Komiyama, Y., S. Nakae, T. Matsuki, A. Nambu, H. Ishigame, S. Kakuta, K. Sudo, and Y. Iwakura. 2006. IL-17 plays an important role in the development of experimental autoimmune encephalomyelitis. *J Immunol* 177:566-573.
28. Bettelli, E., M. Oukka, and V. K. Kuchroo. 2007. T(H)-17 cells in the circle of immunity and autoimmunity. *Nat Immunol* 8:345-350.
29. Stromnes, I. M., L. M. Cerretti, D. Liggitt, R. A. Harris, and J. M. Goverman. 2008. Differential regulation of central nervous system autoimmunity by T(H)1 and T(H)17 cells. *Nat Med* 14:337-342.
30. Ford, M. L., and B. D. Evavold. 2005. Specificity, magnitude, and kinetics of MOG-specific CD8+ T cell responses during experimental autoimmune encephalomyelitis. *Eur J Immunol* 35:76-85.
31. Abdul-Majid, K. B., J. Wefer, C. Stadelmann, A. Stefferl, H. Lassmann, T. Olsson, and R. A. Harris. 2003. Comparing the pathogenesis of experimental autoimmune

encephalomyelitis in CD4-/- and CD8-/- DBA/1 mice defines qualitative roles of different T cell subsets. *J Neuroimmunol* 141:10-19.

32. Ishigame, H., S. Kakuta, T. Nagai, M. Kadoki, A. Nambu, Y. Komiyama, N. Fujikado, Y. Tanahashi, A. Akitsu, H. Kotaki, K. Sudo, S. Nakae, C. Sasakawa, and Y. Iwakura. 2009. Differential roles of interleukin-17A and -17F in host defense against mucoepithelial bacterial infection and allergic responses. *Immunity* 30:108-119.
33. Lukacs, N. W., and D. L. Boros. 1993. Lymphokine regulation of granuloma formation in murine schistosomiasis mansoni. *Clin Immunol Immunopathol* 68:57-63.
34. Oswald, I. P., P. Caspar, D. Jankovic, T. A. Wynn, E. J. Pearce, and A. Sher. 1994. IL-12 inhibits Th2 cytokine responses induced by eggs of Schistosoma mansoni. *J Immunol* 153:1707-1713.
35. Boros, D. L., and J. R. Whitfield. 1999. Enhanced Th1 and dampened Th2 responses synergize to inhibit acute granulomatous and fibrotic responses in murine schistosomiasis mansoni. *Infect Immun* 67:1187-1193.
36. Fernando, G. J., T. J. Stewart, R. W. Tindle, and I. H. Frazer. 1998. Th2-type CD4+ cells neither enhance nor suppress antitumor CTL activity in a mouse tumor model. *J Immunol* 161:2421-2427.
37. Doan, T., K. A. Herd, P. F. Lambert, G. J. Fernando, M. D. Street, and R. W. Tindle. 2000. Peripheral tolerance to human papillomavirus E7 oncoprotein occurs by cross-tolerization, is largely Th-2-independent, and is broken by dendritic cell immunization. *Cancer Res* 60:2810-2815.
38. Yang, X. O., R. Nurieva, G. J. Martinez, H. S. Kang, Y. Chung, B. P. Pappu, B. Shah, S. H. Chang, K. S. Schluns, S. S. Watowich, X. H. Feng, A. M. Jetten, and C. Dong. 2008. Molecular antagonism and plasticity of regulatory and inflammatory T cell programs. *Immunity* 29:44-56.
39. Hao, S., J. Yuan, S. Xu, M. A. Munegowda, Y. Deng, J. Gordon, Z. Xing, and J. Xiang. 2008. Antigen specificity acquisition of adoptive CD4+ regulatory T cells via acquired peptide-MHC class I complexes. *J Immunol* 181:2428-2437.
40. Fiorentino, D. F., A. Zlotnik, P. Vieira, T. R. Mosmann, M. Howard, K. W. Moore, and A. O'Garra. 1991. IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells. *J Immunol* 146:3444-3451.

41. Steinbrink, K., M. Wolfl, H. Jonuleit, J. Knop, and A. H. Enk. 1997. Induction of tolerance by IL-10-treated dendritic cells. *J Immunol* 159:4772-4780.
42. Gordon, J. R., F. Li, A. Nayyar, J. Xiang, and X. Zhang. 2005. CD8 alpha+, but not CD8 alpha-, dendritic cells tolerize Th2 responses via contact-dependent and -independent mechanisms, and reverse airway hyperresponsiveness, Th2, and eosinophil responses in a mouse model of asthma. *J Immunol* 175:1516-1522.
43. Hawrylowicz, C. M., and A. O'Garra. 2005. Potential role of interleukin-10-secreting regulatory T cells in allergy and asthma. *Nat Rev Immunol* 5:271-283.
44. Holgate, S. T., and R. Polosa. 2008. Treatment strategies for allergy and asthma. *Nat Rev Immunol* 8:218-230.