Advances in Experimental Medicine and Biology 841

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T Helper Cell Differentiation and Their Function

Springer

Advances in Experimental Medicine and Biology

Volume 841

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Springer

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ISSN 0065-2598 ISSN 2214-8019 (electronic)
ISBN 978-94-017-9486-2 ISBN 978-94-017-9487-9 (eBook)
DOI 10.1007/978-94-017-9487-9

Library of Congress Control Number: 2014951155

Springer Dordrecht Heidelberg New York London

Printed on acid-free paper

Springer is part of Springer Science+Business Media (www.springer.com)

Preface

This book focuses on the differentiation and regulation of various subsets of CD4+ T cells. It also covers CD4+ T cell subset plasticity and CD4+ T cell function in physiological and diseased states. Where the classic T cell model describes that every subset or subpopulation of T cells develops by producing its own unique cytokines and master regulators, more recent research has shown that the truth may be a bit more complicated. With new technologies such as ChIP-seq, RNA-seq, and data-based analysis, potential transcript factors and binding cites have been identified in different T cell subsets and differentiation stages. These results suggest that T cell differentiation is not a linear process from start to finish that can be switched "on" and "off," but is the result of complex molecular signaling cascades, subtly controlled by a variety of master regulators. Additionally, epigenetic regulation, the modification of DNA or histones, might help to decide T cell fate by facilitating or blocking the binding of transcript factors. While correct and moderate stimulation will lead to precise T cell responses, incorrect signals cause dysfunctions like autoimmune diseases and allergies. As CD4+ T cells play the key role in our adaptive immune system, examining chemokine receptor interference by small molecules or antibodies represents a new approach to disease treatment. This book provides researchers, graduate students, and clinic practitioners with a cutting-edge and comprehensive summary of research on CD4+ T cells and their potential applications in the clinic. I sincerely thank all the chapter authors for their great contribution to the book. I also thank Qiaoshi Lian, Haiyan Zhou, and Wenjing Xuan for their coordination and help.

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Chapter 1
Overview of Orchestration of CD4+ T Cell Subsets in Immune Responses

Bing Sun and Yuan Zhang

Abstract Adaptive immunity plays an important role in the host defense of pathogens, among which CD4+ T helper cell takes a major part. The regulation of Th cell differentiation, the function they exerts in immune response, autoimmune diseases, and allergic conditions, has long attracted much attention. Naive CD4+ T cells differentiate into distinct subsets after receiving TCR and costimulation signaling for activation and cytokine signaling to direct their differentiation. In this chapter, we will have a broad overview of all Th cell subsets, including Th1, Th2, Th17, Treg, Tfh, as well as Th9 and Th22.

Keywords CD4 T helper cell · Th cell subsets · Th1 · Th2 · Th17

1.1 Introduction

CD4+ T cells play critical roles in mediating adaptive immunity to a variety of pathogens. They are also involved in autoimmunity, asthma, and allergic responses as well as in tumor immunity. Twenty seven years ago, Robert Coffman and Timothy Mossman established "Th1-Th2" theory from their animal study, invented a classification method based on the cytokine profiles secreted by distinct CD4+ T cell subsets. This hypothesis was not substantially challenged until 2006, when Th17 and Tfh cells were recognized as new subsets of CD4+ T cells. In addition, Treg cells, Th9 cells, and Th22 cells also were found as new CD4+ lineages. These subsets comprise a wide and delicate network of CD4+ T cell family.

Naive T cells can differentiate into different subsets driving by the cytokines and signals in the environments where the T cells were activated. The stimuli from

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B. Sun (ed.), *T Helper Cell Differentiation and Their Function*,
Advances in Experimental Medicine and Biology 841,
DOI 10.1007/978-94-017-9487-9_1

dendritic cells will precisely lead to differentiation of CD4+ T cells, while incorrect signals will result in Th1/Th2 unbalance and consequently cause a dysfunction in immune system, inducing autoimmune diseases and allergy. Activation of CD4+ T cells takes place in secondary lymphoid organ, and chemokine receptor expression patterns on CD4+ T cells induce them to ingress, stay, and egress, which drive them to the right place, at the right time, and receive right signals. The main frame of how chemokines orchestrate has been established in recent years, and many studies showed some critical CCR molecules drive T cells toward where they were needed. This means same T cells subset with different chemokine expression will lead to divergent disease condition. Since CD4+ T cells plays a key role in adaptive immune system, chemokine receptors interference by small molecules or antibodies is new approach for disease treatment.

Classic Th cell model tells us every subset develops by producing its unique cytokines and master regulator. But recent researches show that things maybe a little complicated. With new technologies such as ChIP-seq, RNA-seq, and database analysis, potential transcript factors and binding sites were found in different subsets and stage of T cells. These results tell us T cell differentiation is not a clear surface from starting point to the end, but struggling from mass of signals and molecules regulation, under subtle controls by master regulator, instead of simply switch on and off. In epigenetic level, the modification of DNA or histone may help to deciding cell fate, by facilitating or blocking transcript factor binding.

Our book will also focus on the respects of CD4+ T cell research which has great progress in recent years, such as subsets plasticity and their function in physical and disease condition. Foxp3+ regulatory T cells is important in moderating immune responses, and we will discuss the recent findings in adjusting Foxp3+ and other key molecules in Treg.

Another star of CD4+ T cell subset is Th17 cells, which is very important in autoimmune disease, and the findings in Th17 in recent years show a dramatical development with the help of modern technologies.

Follicular help T cell (Tfh) is also a recently identified CD4 T cell subset. Tfh cells are specialized to provide help to B cell, necessary for a variety of processes including affinity maturation, class switching, antibody production, and memory B cell differentiation. Tfh cells depend on the expression of chemokine receptor CXCR5 to migration into B cell zones, and induction of the master regulator Bcl6 is essential for Tfh differentiation program.

Finally, two untypical subsets, Th22 and Th9, will be discussed. These 2 subsets share some common features as other subsets (producing IL-22 like Th17, and IL-9 like Th2), but they also have some unique functions under certain disease conditions. Understanding these rare subsets may lead us to have the full view of CD4+ T cells.

There are eight chapters in this book. In Chap. 1, it is an overview of orchestration of CD4+ T cell subsets in immune responses.

As we all know, the CD4+ T cell development in thymus is essential to maintain a health immune system. When matured T cells leave the thymus and move to second peripheral tissue organs, where they play a key regulatory role in adaptive

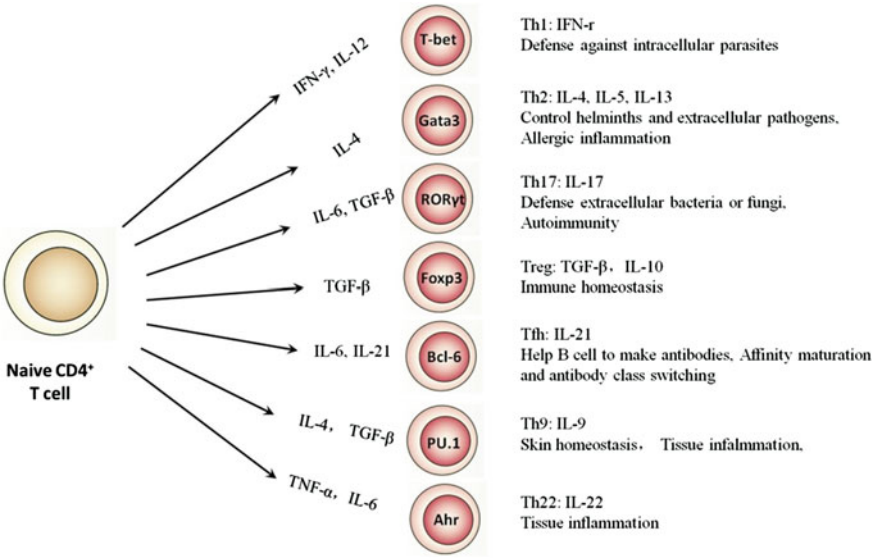

Fig. 1.1 The CD4+ T cell differentiation. Differentiation of naive CD4+ T cells into different T-helper-cell subsets is dependent on factors present in the local environment, most prominently cytokines. The specific stimulatory conditions influence transcription factor expression, which determines the differentiation program that the T cell will follow, and thus the cytokines that it will subsequently produce. The pattern of cytokine expression characterizes the individual T-helper-cell subsets, and dictates their function in host defenses

immunity and participate in many physiological or pathological processes, such as defense against pernicious and harmful invasions, allergic disease toward harmless alien antigens and other T cell mediated disease such as autoimmune disease and organ transplantation. The mechanism and regulation study on CD4+ T cell differentiation will provide us a new strategy to understand the disease pathogenesis, and create some therapeutic target accordingly (Fig. 1.1).

1.1.1 The Modification and Redefinition of CD4+ T Helper Cells Subset Models

Classic Th1/Th2 models of CD4+ T helper cells is hugely modified in recent years, and different scientists provide divergent classification standards and accept some of new Th subsets as new family members of Th1 and Th2, while others been taken as just a unstable status of CD4 helper T cells. With the new findings of these nonclassic CD4 T cell subsets, more and more mechanisms are uncovered in T cell development, and adaptive immune systems. We will overview these subsets in the followings.

1.1.1.1 Th1/Th2 Cell Overview

For several decades, it has been recognized that CD4 T cells specialize in response to antigens like microbial challenges [53]. The first subsets identified were Th1 and Th2 cells based on the selective production of their cytokines, IFN-γ and IL-4 [72], respectively. The details of the Th1/Th2 cytokines/chemokines production and regulation research will be discussed in Chap. 2.

The master regulator of T-bet in Th1 cells [74] plays critical role in driving Th1 cell differentiation. The T-bet works together with STAT1 triggered by IFNγ [2, 44] and STAT4 activated by IL-12 to promote Th1 cells fully development. The GATA3 is a major master regulator in Th2 cells differentiation [94]. The GATA3 results in a phosphorylation of STAT6 under IL-4 stimulation to control Th2 cell fully development [37, 68, 75].

Other key factors have been identified from Th1 or Th2 cells. These factors play an important function either in Th1/Th2 cells differentiation, proliferation, or migration. The detailed mechanism study for these key molecules has enlarged our knowledge to understand their regulatory role in Th cell network. One example of this is Runx (Runt-related transcript factor) family. There are 3 Runx family members, Runx1, Runx2, and Runx3 [20]. It is showed that the binding sites for Runt domain transcription factors are essential for CD4+ transcriptional silencer function, and that the different RUNX family members are required to fulfill unique functions at each stage for T cell activation.

Another important transcript factor is IRF4 (IFN regulatory factor family members 4). IRF4 is a transcription factor essential for the development of Th2 cells, Th17 cells, and Th9 cells [71]. It is reported that Ablation of a conditional Irf4 allele in T regulatory cells resulted in selective dysregulation of Th2 responses [95].

Among the key co-transcript factors, Dec2 (gene name: basic helix-loop-helix family, member e41; bhlhe41) is a transcriptional regulator that control a wide variety of biological processes, including cell proliferation and differentiation [6, 36, 54]. Dec2 is highly expressed in murine T cells, while Dec1 does not exhibit such a preference, and overexpression of Dec2 leads to higher CD25 (IL-2 receptor α), thus more Th2 differentiation, both in in vitro experiments and ex vivo results using Dec2 transgenic mice [46]. In the same year, it is also found that Dec2 is able to induce GATA3 expression, and Dec2 deficiency leads to impaired Th2 responses both in vitro and in vivo [90]. Considering that IL-2 is also required in Th2 development [15] just like IL-4 [72], and lack of IL-2/STAT5 signals leads to eliminated Th2 levels, even with normal IL-4 signals and STAT6 phosphorylation [98]. It is also found that IL-2 and STAT5 are necessary for maintain GATA3 expression [28] and regulates IL-4Rα expression, in the very beginning of Th2 cell development [43]. It is remain unsolved which mechanism is more significant of Dec2 in Th2 cells, as a co-factor of GATA3, or as a bridge of IL-4 and IL-2 signaling. With more and more finding in cytokines downstream signals crosstalk, it is for sure that more intermediary molecules like Dec2 will be found.

With the chip sequence and RNA sequence methods, the binding site and pattern, of transcript factors are widely and systematically identified, GATA3 is the

first master regulator in approaching this thoroughly analysis. Zhu found that GATA3 binding exhibited both common and cell-specific patterns among divergent T cell lineages, and it is surprisingly to find that many genes were either positively or negatively regulated by GATA3 in a cell type-specific manner, suggesting that GATA3-mediated gene regulation depends on existing or not of cofactors in different T cells [85]. As the same researcher found that intergenic long noncoding RNAs are much more significantly changed in different T-cell subsets [31], in the future, this difference of lnRNA environment and other intracellular milieu of divergent T cell subsets may successfully explain the subtle mechanisms in transcript factor function.

After differentiation, CD4 T cells need to migrate into focus of infection to exert its function. The research of T cell migration control obtains lots of harvests, and leads to discovering some drugs and medical methods with significant therapeutic effects. The history of CD4 T cell migration moves along with corresponding drug discoveries [82]. Critical determinants of the in vivo activities of chemokines in the immune system include their secretion by endothelial cells and together presenting with other extracellular matrix molecules, to recruit corresponding CD4 T helper cells and other immune cells. On the other side, there are cellular uptake via "silent" chemokine receptors (interceptors) leading either to their transcytosis or to degradation. Considering chemokines signals may contradict each other, and different chemokine receptors sometimes share the same chemokine ligand, the total image of T cell migration regulation is extremely complicated and hard to uncover, represent the complexity of T cell migration in tissue damage [3, 61]. But in recent years, the mosaic tiles of T-cells migration is on its way to finish. For Th1 cells, CCR5 (C–C chemokine receptor 5) will lead them into inflamed tissues [73].

Among all the chemokine signaling, there is an overwhelming one S1P (sphingosine-1-phosphate), S1P receptor is specifically expressed in divergent immune cells, in CD4+ T cells, S1Pr1 (sphingosine-1-phosphate receptor 1), and S1Pr4 (sphingosine-1-phosphate receptor 4) are primarily expressed [32, 80]. S1P receptors are all GPCR (G protein–coupled receptors). S1Pr1 and S1pr4 play the key role in driving naive CD4 T cell egress out of thymus and migrate into secondary lymph node after their maturation [69]. During the activating, S1Pr1 and S1pr4 quickly down regulated under TCR (T cell receptor), which keeps naive T cells stay in the lymph node, waiting the APC (antigen presenting cell) with the pathogen to activate them [26]. Then S1Pr1 and S1Pr4 re-expressed with their transcript factor KLF2 (kruppel-like factor 2) [86], driving effector T cells to lesion tissues. The mechanism of S1Pr1 and S1Pr4 stay uncovered until recently, Li and colleagues found that ECM1 (extracellular matrix protein 1), which is specifically expressed in Th2 cells, is responsible for KLF2 and S1Pr1 revival in Th2 cells, through interacting with CD122, IL-2 receptor β, and blocking late IL-2 signals [42]. These results showed that Th2 cells are under significant control of IL-2 signals, not only in the early differentiation, but also in the late activation.

1.1.1.2 Th17 Cell Overview

In 2005, a series of research established that Th17 cells were a true distinct lineage [29] whose in vitro generation was enhanced when IL-4 and IFN-γ were blocked and was independent of Th1-or Th2-related transcription factors (T-bet, Stat1, Stat4, and Stat6) [18]. Induction of the Th17 polarization program was initially attributed to the effect of IL-23 (a heterodimer of p40 shared with IL-12 and p19 subunit) signaling via its distinct IL-23R (IL-23 receptor) [97], triggering the Stat3 pathway; however, this process produced cultures containing only small fractions of IL-17A–secreting Th cells. Subsequently, Th17 cells were efficiently generated in the presence of transforming growth factor TGF-β1 (transforming growth factor, beta-1) and IL-6, which signaled via Smad family Proteins (Smads) and Stat3, respectively. But the finding of culture condition of Th17 cells is under a short but circuitous way. Firstly, TGF-β was consider necessary in Th17 development, implies this inhibitory cytokine also has an inflammatory function [9, 29, 47, 49, 78]. In a few years, TGF-β is found no longer necessary in human and mice Th17 cells development, and even reduce Th17 pathogenetic abilities [1, 51, 87, 97], then, in 2008, with the help of nonserum cell medium, the role of TGF-β is further confirmed [48, 84], and finally, pathogenic Th17 cells are generated under the absence of TGF-β, implies TGF-β are not critical in Th17 development [25].

1.1.1.3 Treg Cell Overview

Before Foxp3, a fork head transcription factor encoded by the X chromosome, is regarded as the master regulator of Treg, regulatory T cells (Treg cells) has been paid attention to for a long time. The initial finding that T cell not only functioned as a supporter but also a objector in immune responses is discovered in 1970 and the down-regulation is mediated by T cells that were different from helper T cells [24]. From then on, many studies focused on this T-cell population, called suppressor T cells. In the 1990s, Tr1 and Tr3 were discovered, which were IL-10-secreting suppressor T cells and TGF-b-secreting suppressor T cells, respectively [14, 27]. CD25 molecule (the IL-2 receptor a-chain, a cell surface molecule) was identified as the first candidate for Treg, because CD25+ T cells were confined in the $CD5^{high}$ and $CD45RB^{low}$ of CD4+ T cells and loss of CD25+ T cells can cause lots of autoimmune diseases [5, 62]. A key discovery in the Treg cell research is the function of Foxp3, which was identified in 2001 as the disease-causative gene in Scurfy mice. Foxp3 can endow the CD4+ CD25- T cells with in vivo and in vitro suppressive activity, and upregulation of CD25 and other Treg cell-associated molecules (such as CTLA-4 and GITR) [22, 30]. Burchill et al. [12] and Yao et al. [91] gave the evidence that stat5 can regulate Foxp3 by binding the Foxp3 gene directly. Foxp3 can activate or repress hundreds of genes by forming a transcription complex with other key transcription factors such as NFAT and AML1/Runx1 [50, 58, 88, 96]. There are 3 main suppressive mechanisms are mediated by Foxp3+ Treg cells, e.g., cell-contact dependent inhibition of the activation and proliferation

of T cells, killing or inactivation of APCs and/or T cells, and suppression via cytokines such as IL-10, IL-35, and TGF-b [67, 76, 81, 10].

Under certain inflammatory conditions, Foxp3+ Treg cells might loss its character to adopt a phenotype that is more characteristic of effector CD4+ T cells, so many scientists have paid more attention to this filed for its potential application of treatment [63]. Recently, a research study showed that a special function of tissue-resident Treg cells that can potentiate muscle repair, is different from the traditional idea of Treg cells [13].

With the latest find of IL-2 negatively regulation function in Tfh cells, Treg showed its positive roles in immune response and high-affinity antibody production, through consumption of IL-2 and cause high ration of Tfh and GC B cells afterward. These results suggest that in some conditions, Treg activate immune responses other than inhibition [40].

1.1.1.4 Tfh Cell Overview

Follicular helper CD4+ T (Tfh) cells were first found in human tossils [11, 39, 64], which organism are a secondary lymphoid organ similar to lymph node with constant exposure to antigens via the throat and upper respiratory tract. Several group have identified Bcl6 as a master regulator of Tfh differentiation [34, 55, 93]. These discovery made Tfh cells an individual CD4+ T helper cell subset. Tfh cells have a specific expression profile of Bcl6, CXCR5, PD1, SAP, IL21 and ICOS [16]. TFH cells play an unparalled role on class-switch and high affinity antibody secretion. With absence of Blimp-1 which act as an antagonist of Bcl6, that highly expressed in TH1, TH2, TH17 or other non-TFH subsets [34]. Ascl2 initiates TFH differentiation through up-regulate CXCR5 expression, while Bcl6 is not expressed in the early phase [45]. It has been shown that IL6/IL21 promotes TFH differentiation through activation of STAT3 signaling pathway [57, 83]. But IL-2-STAT5 signaling pathway suppress TFH differentiation through down-regulated expression of CXCR5, c-Maf, Batf, and IL21 [7, 33, 56]. The costimulatory molecule ICOS regulates the expression of CXCR5 and IL21 [8], implies to integrated models, which APCs and cognate B cells are needed for TFH differentiation [16].

1.1.1.5 Th9 and Th22 Cell Overview

Before the description of Th9 cells, it is reported that IL-2 signaling is crucial for IL-9 production by CD4+ T cells, IL-9 is promoted or enhanced by IL-4, transforming growth factor(TGF)-b) and IL-1, while IFN-γ represents a potent inhibitor of IL-9 expression [65, 66]. In 2008, Veldhoen et al. and Dardalhon et al. described the Th9 cells, which promoted the development of allergic and autoimmune diseases by preferentially producing interleukin (IL)-9 [17, 70, 79]. After that, many additional stimuli had been found to be contribute to Th9 cell development, such as IL-25 [4], Jagged2 [21], programmed cell death ligand (PD-L)2 [38], cyclooxygenase

(COX)-2 [41], 1,25-dihydroxyvitamin D3 [59], calcitonin gene-related peptide (CGRP) [52], tumor necrosis factor receptor superfamily member 4 (TNFRSF4 or Ox40) [89], and thymic stromal lymphopoietin (TSLP) [92].

A subset of human CD4+ T cells that specifically expresses IL22 has been identified in skin. These cells also highly express CCR4, CCR6, and CCR10, allowing them localize to the skin [19, 77]. Th22 cells have a specific profiles of TH1 and TH17-associated genes, such as IFN-γ,IL17A, T-bet and RORγt [19]. AHR plays an important role on IL22 expression [60]. IL6 and TNF-α promote priming of Th22 cells and addition of active vitamin D enhances IL22 expression. Langerhans cells, a specialized professional APCs found in the epidermis, are able to induce Th22 cell [23], as well as plasmacytoid dendritic cells [19]. These findings suggest that Th22 contributes not only to skin homeostasis, but also to the pathogenesis of skin disease. It has been reported that Th22 cells increased in psoriasis patients [35].

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Chapter 2
Th1/Th2 Cell Differentiation and Molecular Signals

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Abstract The distinctive differentiated states of the CD4+ T helper cells are determined by the set of transcription factors and the genes transcribed by the transcription factors. In vitro induction models, the major determinants of the cytokines present during the T-cell receptor (TCR)-mediated activation process. IL-12 and IFN-γ make Naive CD4+ T cells highly express T-bet and STAT4 and differentiate to TH1 cells, while IL-4 make Naive CD4+ T cells highly express STAT6 and GATA3 and differentiated to TH2 cells. Even through T-bet and GATA3 are master regulators for TH1/TH2 cells differentiation. There are many other transcription factors, such as RUNX family proteins, IRF4, Dec2, Gfi1, Hlx, and JunB that can impair TH1/TH2 cells differentiation. In recent years, noncoding RNAs (microRNA and long noncoding RNA) join in the crowd. The leukocytes should migrate to the right place to show their impact. There are some successful strategies, which are revealed to targeting chemokines and their receptors, that have been developed to treat human immune-related diseases.

Keywords Th1/Th2 · Transcription factor · Migration · Epigenetics · MicroRNA · LnRNA

2.1 Introduction

Naïve T cells can differ into different subsets based on the signals they faced. Right and moderate stimulation will lead to precise CD4+ T-cells responses, while incorrect signals cause dysfunctions like auto-immune diseases and allergy.

An erratum to this chapter is available at DOI 10.1007/978-94-017-9487-9_9.

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B. Sun (ed.), *T Helper Cell Differentiation and Their Function*,
Advances in Experimental Medicine and Biology 841,
DOI 10.1007/978-94-017-9487-9_2

Activation of CD4+ T cells takes place in secondary lymphoid organ, and chemokine receptor expression patterns on CD4+ T cells induces them to ingress, stay and egress, which drive them to the right place, at the right time, and receive right signals. The main frame of how chemokines orchestrate has been established in recent years, and many studies showed some critical CCR molecules drive T cells toward where they were needed, which means same T cells subset with different chemokine expression will lead to divergent disease condition. As CD4+ T cells plays the key role in adoptive immune system, chemokine receptors interference by small molecules or antibodies is new approach to disease treatment.

Classic Th cell model tells us every subset develops by producing its unique cytokines and master regulator. But recent researches show that things maybe a little complicated. With new technologies such as ChIP-seq, RNA-seq, and database analysis, potential transcript factors and binding cites were found in different subsets and stage of T cells. These results tell us T cell differentiation is not clear cut from starting point to the end, but struggling from mass of signals and molecules regulation, under subtle controls by master regulator, instead of simply switch on and off. In epigenetic level, the modification of DNA or histone may help to deciding cell fate, by facilitating or blocking transcript factor binding.

2.2 Transcription Factors and Cytokines in Th1/Th2

This Th1/Th2 paradigm was first thought useful for initial categorization of mechanisms involving elimination of microbial pathogens. For instance, Th1 cells are critical for the clearance of many intracellular pathogens, such as *Leishmania major*and [136], while Th2 cells were found to be important for elimination of helminthic parasites, such as *Nippostrongylus brasiliensis* and *Schistosoma mansoni* [134].

For several decades, it has been recognized that CD4 T cells specialize in response to antigens like microbial challenges [109]. The first subsets identified were Th1 and Th2 cells based on the selective production of their cytokines, IFN-γ and IL-4 [156], respectively. The details of the Th1/Th2 transcription factors/cytokines/chemokines production and regulation research will be discussed in this chapter.

2.2.1 A Network of Transcription Factors Determines Th1/Th2

(a) **T-bet**
T-bet is not expressed in naive CD4+ T cells, but is readily induced in response to TCR, IFN-γ and IL-12R signaling pathways. T-bet is regarded as the master regulator for Th1 cell differentiation and IFN-γ production. Selective expression of T-bet accounts for TH1 cell development and for the TH1 cell-specific expression of IFNγ [158]. And T-bet deficient (*Tbx21*$^{-/-}$)

cells produce diminished but measurable amounts of IFN-γ during in vitro culture and in vivo responses to Leishmania major infection [160]. The IFNγ-STAT1-T-bet pathway serves as a powerful amplification mechanism for in vitro Th1 differentiation [1, 93]. STAT4 activation by IL-12 is critical for Th1 responses both in vitro and in vivo [19, 82, 163] and the expression level of STAT4 is higher in Th1 than that in Th2 cells [165]

(b) **GATA3**

The expression of GATA3 is induced by the TH2-cell-inducing cytokine interleukin-4 (IL-4) in a signal transducer and activator of transcription 6 (sTAT6)-dependent manner [122]. The role of GATA3 in Th2 cells is well known as the master regulator to guide the differentiation of Th2. After the ligation of TCR in naïve CD4+ T cell, the mRNA levels of IL-4 and GATA3 are both up-regulated within several hours and 24 h, respectively [60, 178]. GATA3 can facilitate the conversion of the IL4-IL5-IL13 locus to an open conformation, so that the other transcription factors involved in Th2-cell differentiation can access this locus [9, 88, 89, 152]. In addition to the function of promoting in Th2 cells, GATA3 also inhibits the expression of interferon-γ (IFN-γ) and directly transactivates IL5 and IL1. The production of high levels of IL-4 and the autoactivation of GATA3 expression build a positive-feedback loop that further induces the expression of GATA3 and the differentiation of Th2 [121]. Zhu et al. generated mice with a conditional deletion of Gata3 and Gata3-deficient mouse T cell lines and found that both IL-4—dependent and —independent Th2 differentiation was diminished, permitting Th1 differentiation in the absence of Ifng and IL12. They concluded that Gata3 serves as a principal switch in determining Th1-Th2 responses [190].

(c) **RUNX family**

Besides the traditional master regulator T-bet of Th1 [159] with activation of STAT1 by receiving IFNγ [1, 93] together with IL-12 induced activation of STAT4, and GATA3 of Th2 [183] through phosphorylation of STAT6 under IL-4 stimulation [81, 150, 161]. Other transcript factors were found in Th1 and Th2 differentiation, proliferation, and migration. And there results consist of an intact network of how Th1 and Th2 cells are developed and regulated. One example of this is Runx (Runt-related transcript factor) family. There are 3 Runx family members, Runx1, Runx2 and Runx3 [43]. It is showed that binding sites for Runt domain transcription factors are essential for CD4 transcriptional silencer function, and that different Runx family members are required to fulfill unique functions at each stage. Taniuchi and colleagues found that RUNX1 is required for active repression in CD4-negative/CD8-negative thymocytes, whereas RUNX3 is required for establishing epigenetic silencing in cytotoxic lineage thymocytes. Cytotoxic T cells deficient in Runx3, but not helper cells, had defective responses to antigen, suggesting that RUNX proteins have critical functions in lineage specification and homeostasis of CD8-lineage T lymphocytes [162]. Another nature paper found Runx1 are important in CD4 T cell's IL-2 producing processes [120], and also crucially required for normal

hematopoiesis including thymic T-cell development. In this study, several lines of evidence supported a model in which the interaction suppresses IL2 and IFN-gamma production, upregulates Treg cell-associated molecules, and exerts suppressive activity.

(d) **IRF4**

Another important transcript factor is one of IRF4 (IFN regulatory factor family members 4). IRF4 is a transcription factor essential for the development of Th2 cells, IL17—producing Th17 cells, and IL9—producing Th9 cells [153], and implies the potential relations among these three subsets. Negishi and colleagues demonstrated that IRF4 interacted with MYD88 (myeloid differentiation primary response gene 88) in the cytoplasm of human embryonic kidney cells. Mutation and coimmunoprecipitation analysis showed that IRF4 interacted with the TIR/IL1 region of MYD88, and IRF4 is also found inhibiting the interaction of MYD88 with IRF5 (IFN regulatory factor family members 5) Zheng and colleagues showed that in mouse T regulatory cells, high amounts of IRF4, a transcription factor essential for TH2 effector cell differentiation, is dependent on Foxp3 (forkhead box p3) expression. They proposed that IRF4 expression endows T regulatory cells with the ability to suppress Th2 responses. Indeed, ablation of a conditional Irf4 allele in T regulatory cells resulted in selective dysregulation of Th2 responses, IL4-dependent immunoglobulin isotype production, and tissue lesions with pronounced plasma cell infiltration, in contrast to the mononuclear cell-dominated pathology typical of mice lacking T regulatory cells. Zheng concluded that T regulatory cells use components of the transcriptional machinery, promoting a particular type of effector CD4+ T-cell differentiation, to efficiently restrain the corresponding type of the immune response [184].

(e) **STAT family**

Activation of STAT1 by IFN-γ is important for the induction of T-bet during in vitro Th1 differentiation [1, 93]. STAT4 expression is higher in Th1 than in Th2 cells [165]. STAT4 expression is likely to be regulated positively by IFN-γ [50] and negatively by IL-4 and GATA3 [50, 165]. Activated STAT4 can directly induce IFN-γ production and expression of IL-12Rβ2 and T-bet during Th1 differentiation [165, 166]. Low levels of STAT5 activation are sufficient for cell proliferation and survival; however, strong STAT5 signaling is required for Th2 differentiation [33, 185]. STAT5 directly binds to the DNase I hypersensitive sites (HS) II and HSIII in the second intron of the Il4 locus in Th2 but not in Th1 cells [185]. STAT6 is the major signal transducer in IL-4-mediated Th2 differentiation and expansion [81, 150, 161]. In vitro, STAT6 activation is necessary and sufficient for inducing high expression levels of the Th2 master regulator gene, GATA3 [81, 188]. STAT6 may also be important for the amplification of Th2 responses at later stages and/or for the generation of Th2 memory cells in vivo [47].

(f) **Dec2**

Among the key co transcript factors, Dec2 (gene name: basic helix-loop-helix family, member e41; bhlhe41) is a transcriptional regulator that control a wide

variety of biological processes, including cell proliferation and differentiation [8, 80, 115]. The common structures shared among the members of this superfamily are the basic domain, which is required for DNA binding, and the helixloop-helix domain, which is involved in dimerization. Dec2 is cloned by Fujimoto in 2001 [51]. In the same year, Garriga-Canut and colleagues showed that rat Sharp1 binds to the M1 muscarinic acetylcholine receptor and acts as a transcriptional repressor of both TATA-containing and TATA-less promoters. Repression occurs either via the bHLH domain or via a C-terminal domain that is sensitive to the histone deacetylase inhibitor trichostatin A, constructs the foundation of the transcript and binding function of Dec2 [52]. In a science paper, He and colleagues found that heterozygous P385R-mutant transgenic mice showed increased activity and less sleep time compared to wildtype mice. Under sleep deprivation, transgenic mice also showed less compensatory gain in non-REM sleep compared to wild type mice, suggesting a role for Dec2 in sleep homeostasis. There were no differences in circadian rhythm compared to wild type mice. The phenotype was not found in Dec2-null mice, suggesting a dominant effect of the heterozygous P385R mutation and a dominant increase in the quantity of wakefulness. Similar results were found in a Drosophila model [65]. It is surprisingly to find that Dec2 is highly expressed in murine T cells, while Dec1 does not exhibit such a preference, and overexpression of Dec2 leads to higher CD25 (IL-2 receptor α), thus more Th2 differentiation, both in vitro experiments and ex vivo results using Dec2 transgenic mice [94]. In the same year, it is also found that Dec2 is able to induce GATA3 expression, and Dec2 deficiency leads to impaired Th2 responses both in vitro and in vivo. GATA3 also regulates Dec2 expression, suggesting that Dec2 and GATA3 form a positive regulatory feedback loop during Th2 differentiation [179]. Considering that IL-2 is also required in Th2 development [33] just like IL-4 [156], and lack of IL-2/STAT5 signals leads to eliminated Th2 levels, even with normal IL-4 signals and STAT6 phosphorylation [185]. It is also found that IL-2 and STAT5 are necessary for maintain GATA3 expression [63] and regulates IL-4Rα expression, in the very beginning of Th2 cell development [92]. It remains unsolved which mechanism is more significant of Dec2 in Th2 cells, as a co-factor of GATA3, or as a bridge of IL-4 and IL-2 signaling. With more and more finding in cytokines downstream signals crosstalk, it is for sure that more intermediary molecules like Dec2 will be found (Fig. 2.1).

(g) **Other transcript factors**

There are other important transcript factors found in recent years, such as Gfi-1 (Growth factor independent 1) [42, 54, 189, 186], and Ikaros family members [53, 124, 135, 173], c-maf [15, 72, 76], Hlx (H.20 like—homobox) [113], Ets-1 (v-ets avian erythroblastosis virus e26 oncogene homolog 1) as a co-factor of T-bet [58, 108], JunB (v-jun avian sarcoma virus 17 oncogene homolog B) as a member of AP-1 (activator protein 1) family, collaborate with c-maf [90]. Blimp-1 (gene name: pr domain-containing protein 1; prdm1) is induced by

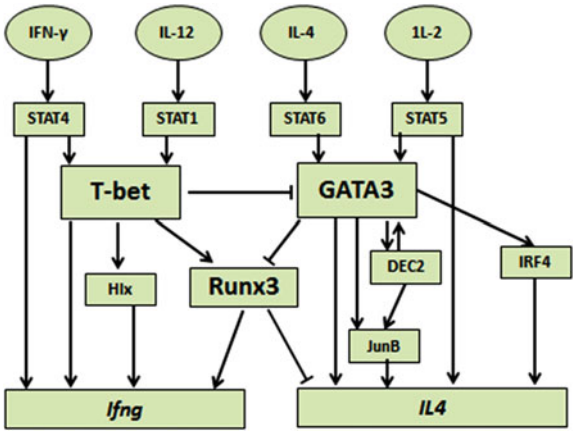

Fig. 2.1 The network of transcription factors in Th1/2 cells. The activation of signal transducer and activator of transcription (STAT) proteins by different cytokines plays a critical role in inducing the expression of the lineage-specific master regulators T-bet (Th1), GATA3 (Th2). The STAT proteins also collaborate with the master regulators and some secondary transcription factors, whose expression is controlled by the master regulators, for the induction of cytokine genes. Positive or negative regulation among these transcription factors occurs at the gene expression level and/or at the protein level through protein–protein interaction, forming a sophisticated transcriptional regulatory network during Th1/2 cell differentiation [191]

IL-2 signaling in CD4 T cells [34, 79, 103], and can directly suppress IFNγ and IL-2 production [27, 105, 104].

With the chip sequence and RNA sequence methods, the binding site and pattern, of transcript factors are widely and systematically identified, GATA3 is the first master regulator in approaching this thoroughly analysis. Zhu found that GATA3 binding exhibited both common and cell-specific patterns among divergent T-cell lineages, and it is surprisingly to find that many genes were either positively or negatively regulated by GATA3 in a cell type-specific manner, suggesting that GATA3-mediated gene regulation depends strongly on existing or not of cofactors in different T cells [172]. As the same researcher found that intergenic long non-coding RNAs are much more significantly changed in different T-cell subsets [69], in the future, this difference of lnRNA environment and other intracellular milieu of divergent T-cell subsets may successfully explain the subtle mechanisms in transcript factor function.

2.2.2 A Network of Cytokines Determines Th1/Th2

The induction of the distinctive patterns of gene expression may be achievable in several ways, but in vitro the major determinants of the differentiated state of the cell are the set of cytokines present during the T-cell receptor (TCR)-mediated

activation process. Several lines of evidence strongly support the notion that the cytokines play a major role in inducing the transcription factors that determine differentiation [60, 112]. Our understanding of this process has evolved over an extended period, and is described in detail below.

One major discovery of Th1/Th2 fate decision is the key role of IL-2. In most CD4 T-cell subsets, IL-2 is just help to proliferate, but in Th2 cells, IL-2 and the downstream STAT5 signals is essential in driving Naïve T cell toward Th2 differentiation [60, 112, 187]. With the finding that Th17 is negatively regulated by IL-2 [32, 170], while Treg need large amount of IL-2 in differentiation and maintenance, without produce any IL-2 [2, 3], and recent data showed Tfh cells also take IL-2 as its critical negative regulation pathway [11, 26].

Th1 cells predominantly produce interferon-γ (IFNγ) and are important for protective immune responses to intracellular viral and bacterial infection. Th1 cells are also responsible for the induction of some autoimmune diseases. In turn, IFNγ is important for the differentiation of IFNγ-producing Th1 cells [93] although IL-12 is also critical for this process [68]. Indeed, in vitro neutralization of IFN-γ will often markedly diminish Th1 development. Although IL-18 is not involved in the differentiation of Th1 cells, it can synergize with IL-12 in inducing IFNγ, implying that IL-18 plays an important role in Th1 responses [139, 180].

Th2 cells mediate host defense against extracellular parasites including helminthes. Similarly, when stimulated by a cognate antigen presented by accessory cells in the presence of IL-4, naive CD4+ T cells differentiate into IL-4-producing Th2 cells [67, 61, 149, 156]. CD25 (IL-2Rα) expression is higher in Th2 cells than in Th1 cells, possibly due to the action of c-Maf [72]. Such higher expression of CD25 may confer hyperresponsiveness to IL-2.

2.3 Cross-regulation Among Transcription Factors and Cytokines During Th1/Th2 Differentiation

During Th cell differentiation toward one lineage, the other lineage fates are usually suppressed. An important cross-regulation during Th differentiation is through repression of transcription factors that are important for lineage determination. For example, GATA3 downregulates expression of STAT4, which is the important factor for mediating IL-12 signaling and Th1 differentiation [165]. GATA-3 deficiency results in spontaneous IFN-γ production, independent of IL-12 and IFN-γ [165]. A constitutively active form of STAT5 inhibits T-bet expression while it also promotes Th2 differentiation [185]. T-bet suppresses GATA-3 function by direct binding of the factors [71].

Th differentiation involves positive feedback by cytokines. The differentiation process also actively involves cross-inhibition of other lineage fates. Mutual suppression between IFN-γ and IL-4 signaling was the takeoff point for studies of

cross-regulation [110, 127]. IL-4 can suppress Th1, Th17, and Treg cell differentiation [189, 186]. TGF-β was also found to suppress both Th1 and Th2 differentiation [56], and both IL-4 and IFN-γinhibit Th17 differentiation [64, 126].

2.4 Migration Signals as Guiding Light

The chemokine system controls CD4 T cell and other leukocyte migration in autoimmune disease, immune responses, virus infection and inflammation, and is implicated plays the key roles in the pathogenesis of many human diseases, and induced several successful strategies, which have been identified to develop drugs targeting chemokines and their receptors, but this has not yet resulted in many new therapeutics, because chemokine system is full of redundancy, pleiotropy, and differences among species, and single changes through chemical drugs will lead to a complex result. But on the other side, our understanding of chemokine biology is continuing to grow and so does drug discoveries. There are lots of findings in exploring the role of chemokines in health and diseases [169].

After differentiation, CD4 T cells need to migrate into focus of infection to exert its function. The research of T-cell migration control obtains lots of harvests, and leads to discovering some drugs and medical methods with significant therapeutic effects. The history of CD4 T-cell migration moves along with corresponding drug discoveries [169]. Critical determinants of the in vivo activities of chemokines in the immune system include their secretion by endothelial cells and together presenting with other extracellular matrix molecules, to recruit corresponding CD4 T helper cells and other immune cells. On the other side, there are cellular uptake via "silent" chemokine receptors (interceptors) leading either to their transcytosis or to degradation. Considering chemokines signals may contradict each other, and different chemokine receptors sometimes share the same chemokine ligand, the total image of T-cell migration regulation is extremely complicated and hard to uncover, represent the complexity of T-cell migration in tissue damage [6, 141]. But in recent years, the mosaic tiles of T-cells migration is on its way to finish.

2.4.1 CCR5, Discovery, Feature and Function

For Th1 cells, CCR5 (C–C chemokine receptor 5) will lead them into inflamed tissues [157]. CCR5 is first cloned in 1996 [144], from a human genomic DNA library based on its similarity to a murine C–C chemokine receptor clone. The human gene, which they designated ChemR13, encodes a 352-amino acid protein (designated CCCKR5 by them) with a calculated molecular mass of 40,600 Da and a potential N-linked glycosylation site. With a set of overlapping lambda clones, they showed that the gene is 17.5 kb from the CMKBR2 gene (CCR2; 601267). The 2 coding regions share 75 % DNA and amino acid sequence identity. One year

later, Mummidi et al. analyzed the genomic structure of CCR5, their conclusion shows CCR5 contains 4 exons, spanning approximately 6 kb, and only 2 introns. There is no intron between exons 2 and 3. Exon 4 contains the open reading frame, the complete 3-prime UTR, and 11 nucleotides of the 5-prime UTR. Transcripts are initiated from 2 distinct promoters, both of which are AT-rich and lack canonical TATA or CAAT motifs; one is upstream of exon 1 and the other downstream, including the 'intronic' region between exons 1 and 3. Complex alternative splicing patterns in the 5-prime UTR and in the 4 exons give rise to multiple CCR5 transcripts. The regulatory sequences and noncoding exons are polymorphic, whereas the protein sequence is not [114].

CCR5 is also found as a coreceptor for the human immunodeficiency virus-1 (HIV-1) in the same year as it was cloned [37, 41]. CMKBR5 (gene symbol of CCR5, also with the short form CKR5) was served as a secondary receptor on CD4 + T lymphocytes for certain strains of human immunodeficiency virus-1 (HIV-1) by Deng et al. and Dragic et al. CMKBR5 and fusin facilitate the fusion of HIV-1 with the plasma membrane of CD4+ cells. Deng et al. [37] found that CMKBR5, and not fusin, promotes entry of the macrophage-tropic viruses believed to be the key pathogenic strains in vivo. A 32-base pair deletion allele (CKR5Delta 32) was identified that is present at a frequency of sim 0.10 in the Caucasian population of the United States. An examination of 1955 patients included among six well-characterized acquired immunodeficiency syndrome (AIDS) cohort studies revealed that 17 deletion homozygotes occurred exclusively among 612 exposed HIV-1 antibody-negative individuals 2.8 % and not at all in the rest of 1343 HIV-1-infected individuals. The frequency of CKR5 deletion heterozygotes was much higher in groups of patients that had survived HIV-1 infection for more than 10 years, and, in some risk groups, twice as frequent as their occurrence in rapid progressors to AIDS. Survival analysis clearly shows that disease progression is slower in CKR5 deletion heterozygotes than in individuals homozygous for the normal CKR5 gene. All this results shows the CKR5Delta 32 deletion may act as a recessive restriction gene against HIV-1 infection and may exert a dominant phenotype of delaying progression to AIDS among infected individuals [36].

In short, CCR5 is found as a Th1 deviation chemokine receptor in 1998 [17]. Since related with HIV-1 infection, a lot of researches focus on this aspect [5, 25, 41, 46, 142, 181], which implies the relationship between virus infection and T-cell migration.

CCR5 is found important in directing T-cell migration from lymph node to disease location in 2001. By using bronchoalveolar lavage and flow cytometry, Campbell et al. proved that T cell migration is controlled by a combination of adhesion receptors and chemokines expressed on vascular endothelium and in the tissue, often in an organ-specific manner. T lymphocytes homing to the lung in both normal and asthmatic subjects express CCR5 and CXCR3 but not CCR9, which is found on T cells homing to intestinal mucosal sites, or selectin p ligand (SELPLG), the high-affinity counter-receptor for P-selectin, which is found on skin-homing T cells. No differences were observed between lung T cells from normal versus

asthmatic subjects. This study provides added support for the concept of a lung-homing pathway separate from other mucosal organs such as the gut and suggests that the chemokine pathways that control T-cell migration [20].

The ligand of CCR5 keeps on in 2003. By using mouse splenic dendritic cells and DCs from Ccr5 −/− mice and Myd88 −/− mice, Aliberti et al. found that *Toxoplasma gondii* stimulated IL-12 production not only through a classic Toll-like receptor/Myd88-dependent mechanism, but also through the release of an 18-kD protein, cyclophilin-18 (C18), that interacted directly with CCR5 on DCs. Cyclosporin A, a major ligand of cyclophilin, or anti-C-18 inhibited IL-12 production in DCs. Aliberti et al. concluded that C18 is a molecular mimic of a CCR5 chemokine ligand [4].

IL-16, which signaled through CD4 on eosinophils in a dose-dependent manner and induced the release of leukotriene C4 (LTC4), as well as eotaxin (CCL11) and RANTES (CCL5), in an autocrine manner, RANTES and eotaxin signaled through their membrane receptor, CCR3, and enhanced eosinophil secretion of LTC4 and IL-4 [12]. IL-16 binds to CD4 and induces a comparable migratory response in CD4-positive T cells. Lynch et al. observed a preferential migratory response in mouse Th1 cells, which express CCR5, but not happened in Th2 cells, which express little CCR5. T cells from CCR5-deficient mice were unable to migrate in response to IL-16. In transfected human osteosarcoma cells, the presence of CCR5 significantly increased IL16 binding activity as compared with CD4 alone; however, IL-16 could not bind CCR5 alone. Lynch et al. [99] concluded that augmentation of IL-16 stimulation by CCR5 plays a role in regulation of Th1 cell recruitment and activation at sites of inflammation.

There are also some clinical researches regarding CCR5. Gulati et al. investigated the expression of chemokine receptor CCR5 in the conjunctival epithelium in 45 patients' cells with dry eye syndromes, a disease of millions of people, primarily women [146], compare with 15 control individuals with normal syndromes. By using 2-color flow cytometry using fluorescein-conjugated anti-CCR5 and phycoerythrin-conjugated anti-CD45 and realtime PCR, they found a significant up-regulation in cell surface expression of CCR5 in patients with either aqueous tear-deficient (e.g., Sjogren syndrome;) or evaporative forms of dry eye syndrome in both protein and mRNA level. The majority of the cells expressing CCR5 were non-bone marrow-derived resident epithelial cells of the conjunctiva, suggesting a role of ocular surface epithelial cells in modulating immunoinflammatory responses in dry eye syndromes. Gulati et al. suggested that chemokine receptor CCR5 or its ligands might serve as useful targets for modulation of these responses [62]. Considering increasing levels of chemokine ligand RANTES with concomitant increase in expression of CCR5 receptor has been described in the lacrimal glands in an animal model of Sjogren syndrome [164]. The expression of CCR5 is very important in the pathogenesis and treatment of dry eyes syndromes.

In 2007, when Th17 cells are started to be considered as a new subsets of CD4+ T cells [22, 35, 85], Sato et al. shows CCR5 is a key marker in distinguishing Th1 cells and Th17 cells in human. By analyzing healthy donors PBMC (Peripheral blood mononuclear cell) with flow cytometry and ELISA, Sato et al. found that

CCR2+CCR5−, but not CCR5+, CD4+ T cells produced large amount of IL17 and little IFNg. Within the CCR2+ population, CCR5+ cells produced IFNg and CCR5-negative cells produced IL-17. Sato et al. concluded that human Th17 cells are CCR2-positive/CCR5-negative. This research provides a simple cell surface marker of human Th1 and Th17 cells.

Recently a research proves CCR5 is an important target in immune responses toward bacterial pathogen *Staphylococcus aureus* that causes significant morbidity and mortality worldwide. *S. aureus* secretes a number of protein products that allow the organism to effectively subvert the host immune system. Such factors include super-antigens, antibody binding proteins, cytolytic peptides, and pore-forming cytotoxins [48]. Alonzo et al. identified the HIV coreceptor CCR5 as a cellular determinant required for cytotoxic targeting of subsets of myeloid cells and T lymphocytes by the *Staphylococcus aureus* leukotoxin ED (LukED). They found that LukED was cytotoxic to a human T-cell line ectopically expressing CCR5 (HUT-R5); whereas another T-cell line (Jurkat), which lacks detectable CCR5, was insensitive [7]. In other respect, when CCR5 levels were reduced in HUT-R5 cells using lentiviral CCR5 shRNA, the cells were protected from LukED-mediated killing. And CCR5 antagonist, maraviroc, Vicriviroc and TAK-779, are found potently blocked LukED killing of CCR5+ cells. Alonzo et al. further demonstrated that cell killing is blocked by CCR5 receptor antagonists, including the HIV drug maraviroc, in a LukED-dependent manner, and direct interaction between LukE with CCR5 is also proved. Remarkably, CCR5-deficient mice are largely resistant to lethal *S. aureus* infection, highlighting the importance of CCR5 targeting in *S. aureus* pathogenesis, suggests that CCR5 could provide much-needed therapeutic alternatives in the treatment of *S. aureus* infections.

2.4.2 CXCR3, Discovery, Feature and Function

Another chemokine receptor preferentially expressed by Th1 cells and is critically involved in Th1 recruitment to inflamed tissue is CXCR3 (cxc motif, receptor 3). Under IFNγ stimulation, tissues will produce CXCL9 (cxc motif, ligand 3), CXCL10 (cxc motif, ligand 10), or CXCL11 (cxc motif, ligand 11), all three are ligand of CXCR3, which could recruit Th1 cells moving toward and infiltrated into inflamed tissue [49, 86, 97], such as lung [20].

As important chemokines mediating migration of immune cells into infected or inflamed tissues to initiate effective immune responses, the chemokine receptor CXCR3 is preferentially expressed by Th1 cells and CD8+ cytotoxic T cells [97, 98, 177] and is critically involved in their recruitment to inflamed tissue. Tissue infiltration of T cells expressing high levels of CXCR3 depends on IFNg induced release of the CXCR3 ligands CXCL9 (also known as monokine induced by gamma-interferon, MIG), CXCL10 (interferon-induced protein of 10 kDa, IP-10), or CXCL11 (interferon-inducible T-cell alpha chemoattractant, I-TAC) [29, 117]. CXCR3 is also expressed on nonlymphoid tissue-homing CD4+CD25+ regulatory

T (Treg) cells [83]. Naïve T cells does not constitutively express CXCR3, but is rapidly upregulated following DC-induced T-cell activation [143].

Like other wide spectrum of receptors, intercellular migration signals is mediated by peptide ligands interacting with specific cell surface receptors in CXCR3. As many peptide-binding receptors, CXCR3 also belong to the G protein-coupled receptor family and exhibit common structural features, including the presence of 7 transmembrane domains and a number of conserved amino acid residues. CXCR3 is the first chemokine receptor identified that is highly induced by T-cell activation. The ORF was first identified in incomplete form in 1995 on a genomic clone isolated by polymerase chain reaction-based homology hybridization. The gene was named GPR9 and was originally mapped incorrectly to human chromosome 8p11.2-12. Marchese et al. used PCR and genomic DNA library screening to clone 2 novel human genes, GPR9 and GPR10, and a rat gene, GPR14. Each of these encodes a G protein-coupled receptor. The receptor encoded by GPR9 was found to share the highest identity with human IL8 receptor type B (IL8RB) (38 % overall and 53 % in the transmembrane regions), followed by IL8RA (36 % overall and 51 % in the transmembrane domains) [102] and map to chromatin 13 by Loetscher et al. [97].

As found in 1996, the human CXCR3 receptor cDNA has an open reading frame of 1104-bp encoding a protein of 368 amino acids with a molecular mass of 40,659 dalton [10]. The sequence includes seven putative transmembrane segments characteristic of G-protein coupled receptors [21]. It shares 40.9 and 40.3 % identical amino acids with the two IL-8 receptors, and 34.2–36.9 % identity with the five known CC chemokine receptors. The CXCL9/CXCL10 receptor is highly expressed in IL-2-activated T lymphocytes, but is not detectable in resting T lymphocytes, B lymphocytes, monocytes and granulocytes [40]. The superfamily of T-cell chemokines is made of an array of chemoattractant proteins that has been divided into 4 branches (C, CC, CXC, and CXXC) on the basis of the relative position of the cysteine residues in the mature protein [118]. Structural variants of chemokines are associated with differences in their ability to regulate the trafficking of immune cells during hematopoiesis and inflammatory responses. Chemokines exert their attractant properties after binding to distinct membrane receptors. Because a single chemokine receptor binds several chemokines, it is often difficult to evaluate the activity of these structures in lymphocyte homing at that time. For instance, CXCL10 and CXCL9, CXC chemokines that are induced by IFN-gamma, bind the CXCR3 receptor and are shown to be specifically chemotactic for activated lymphocytes [97].

The research of CXCR3 activities moves on, trying to figure out the importance of persistence expression of CXCR3. Although FACS analysis demonstrated that 40 % of resting T lymphocytes and low numbers of B cells and natural killer cells expressed CXCR3, Loetscher et al. found that these cells did not have detectable CXCR3 transcripts and did not respond to CXCL9 or CXCL10. However, exposure to IL2 with or without addition of phytohemagglutinin for 10 or more days results in cultures of fully responsive CXCR3. Treatment with anti-CD3 antibodies in the presence or absence of soluble anti-CD28 antibodies was inhibitory [98].

Using Northern blot analysis, Bonecchi and colleagues showed that polarized Th1 cells preferentially express CXCR3 and CCR5 among four CXC (CXCR1-4)

and five CC (CCR1-5) chemokine receptors analyze. In contrast, Th2 cells preferentially express CCR4 and, at least in a subpopulation of Th2 cells, CCR3. Th1s and Th2s selectively migrate in response to the corresponding chemokines. It is an early work systematically compared Th1 and Th2 chemokines receptor regarding their migration [17].

In recent years, new ligand of CXCR3 is still found. The human CXC chemokine platelet factor-4 (CXCL4) is encoded by 2 genes, located on chromosome 4 and probably arose through duplication [44, 57]. The 2 genes are indeed highly related and give rise to mature proteins that differ in only 3 amino acid residues in the carboxylic acid (COOH)–terminal part. Analysis of conditioned media from thrombin-treated platelets revealed that both CXCL4 genes are translated into proteins [154]. Different cell have different splice form of CXCL4. Afterward, tumor cells and smooth muscle cells were identified as alternative cellular sources for CXCL4L1 but not for CXCL4, indicating that not every cell type that produces CXCL4 also releases CXCL4L1 and vice versa [86, 167]. By binding analyses and using CXCR3 blocking antibody with function assay, Struyf and colleagues found that human CXCL4L1, the potent inhibitor of angiogenesis, had lower affinity for heparin and chondroitin sulfate-E than did CXCL4 and that CXCL10 and CXCL4L1 could displace each other on human microvascular endothelial cells. CXCL4L1 bound to both CXCR3A and CXCR3B. Neutralization antibodies to CXCR3 blocked CXCL4L1 antiangiogenic activity, and human CXCL4L1 activity was reduced in mice treated with anti-human CXCR3 or in mice lacking Cxcr3, as assessed by tumor growth and vascularization of Lewis lung carcinoma. Like CXCL4, CXCL4L1 attracted activated T, natural killer, and dendritic cells, but preincubation with CXCL10 and CXCL11, pertussis toxin, or anti-CXCR3 reduced or neutralized this activity. Struyf and colleagues concluded that CXCR3A and CXCR3B are involved in the chemotactic and vascular effects of CXCL4L1, and provide evidence that both PF-4 forms can attract immature DCs in a CXCR3-dependent manner. Moreover, the angiostatic and antitumoral effects of CXCL4L1 in vivo are also mediated through CXCR3 [155].

And some studies found CXCR3 is essential and as a marker in deciding precise cell fate. The regulation of memory CD4+ helper T-cell function, such as polarized cytokine production, remains unclear. Endo and colleagues in 2011 examined expression of cell surface markers to identify functionally distinct subpopulations of mouse memory Th2 cells. They use FACS analysis to demonstrate 4 Th2 subpopulations based on high or low expression levels of CD62L (SELL) and Cxcr3. All 4 subpopulations produced moderate levels of Il4 and Il13, but Th2 cells with low levels of both CD62L and Cxcr3 (Cd62l-lo/Cxcr3-lo cells) selectively produced IL-5. IL-5 production in Cd62l-lo/Cxcr3-lo cells was together with histone H3-K4 methylation, a marker for the permissive conformation of chromatin [175], at the IL5 promoter. DNA microarray analysis and quantitative RT-PCR showed that CD44+ memory Th2 cells expressing IL-5 had lower levels of Eomes and Tbx21 and higher levels of Rora and Pparg than memory Th2 cells lacking Il5 expression. RNA silencing demonstrated that Eomes downregulation was required for Il5 expression and that Eomes had no effect on H3-K4 methylation at the Il5

promoter. Instead Eomes suppressed Gata3 transcriptional activity by inhibiting Gata3 binding to the IL5 promoter. Depletion of Cd62l-lo/Cxcr3-lo cells ameliorated memory Th2 cell-dependent airway inflammation in mice. Endo et al. concluded that IL5 production preferentially occurs in the CD62L-lo/CXCR3-lo subpopulation regulated by EOMES expression and Eomesodermin was shown to interact with the transcription factor GATA3, preventing GATA3 binding to the Il5 promoter. Memory Th2 cell-dependent airway inflammation was attenuated in the absence of the CD62L(lo)CXCR3(lo) population but was enhanced by Eomes-deficient memory Th2 cells. Thus, IL-5 production in memory Th2 cells is regulated by Eomesodermin via the inhibition of GATA3 activity [45].

2.4.3 CCR4, Discovery, Feature and Function

Th2 cells also have specific chemokine receptors, CCR4 (C–C chemokine receptor 5) [107, 129, 145], cloned in 1999 [116], as well as CCR8 [23, 78]. As other C–C receptor family members, CCR4 are a kind of small, mostly basic, structurally related molecules that regulate cell trafficking of various types of leukocytes through interactions with a subset of 7-transmembrane, G protein-coupled receptors [138, 143]. Chemokines also play fundamental roles in the development, homeostasis, and function of the immune system, and they have effects on cells of the central nervous system as well as on endothelial cells involved in angiogenesis or angiostasis. Chemokines are divided into 2 major subfamilies, CXC and CC, based on the arrangement of the first 2 of the 4 conserved cysteine residues; the 2 cysteines are separated by a single amino acid in CXC chemokines and are adjacent in CC chemokines. The International Radiation Hybrid Mapping Consortium mapped the CCR4 gene to chromosome 3 (sts-X85740). Several CCR genes, including CCR1, map to 3p22-p21.

CCR4 is cloned in 1995 By RT-PCR analysis using degenerate oligonucleotide primers corresponding to conserved sequences of CCR1 and the IL8 receptors alpha (IL8RA) on an immature basophilic cell line, followed by screening of a spleen cDNA library, Power and colleagues isolated a cDNA encoding CCR4 [133]. The deduced 360-amino acid CCR4 protein contains 3 potential N-glycosylation sites and multiple potential phosphorylation sites. Northern blot analysis detected a 4.0-kb CCR4 transcript at high levels in thymus and peripheral blood leukocytes and at lower levels in spleen; no expression was detected in prostate, testis, ovary, small intestine, and colon. RT-PCR analysis detected CCR4 expression in stimulated and unstimulated T and B cells, basophils, and monocytes but not in T-cell lines or pulmonary macrophages. Hence in the future, this work will be necessary to characterize postreceptor signaling pathways in order to define the precise function of these receptors in different leukocyte populations and their relevance in inflammatory diseases.

An early report in 1998 showed that polarized Th1 cells preferentially express CXCR3 and CCR5. In contrast, Th2 cells preferentially express CCR4 and, at least

in a subpopulation of Th2 cells, CCR3. It is found selective expression of CCR3 by cells of the Th2 subset. However, the extremely low level of expression, as compared for example with CCR4, may reflect a minority of positive clones in the Th2 population. Consistent with receptor expression, MIP-1α (CCR1 agonist) and MCP-1 (a selective CCR2 agonist) showed comparable chemotactic activity for Th1s and Th2s. In contrast, MDC (19; a selective CCR4 agonist) was at least 10 times more active on Th2s versus Th1s, whereas MIP-1β (CCR5) and IP-10 (CXCR3) were more active on Th1s. Eotaxin (a selective CCR3 agonist) was inactive or weakly active only on Th2s [17].

The ligand of CCR4 in first found in 1997. Thymus and activation-regulated chemokine (TARC) is a previously identified CC chemokine that is expressed constitutively in thymus and transiently in stimulated peripheral blood mononuclear cells. By receptor binding analyses with secreted alkaline phosphatase (SEAP) and used it for specific binding, Imai et al. showed that CCR4 is a specific receptor for TARC-SEAP. Besides that, macrophage-derived chemokine (MDC) is a recently identified member of the CC chemokine family, which contains 37 % identical amino acids similar with TRAC, and Imai et al. [74] showed that CCR4 is a specific receptor for MDC. They confirmed that MDC is also a functional ligand for CCR4. Since MDC and TARC are both expressed in the thymus, one role for these chemokines may be to attract CCR4-bearing thymocytes in the process of T-cell education and differentiation. By northern blot analysis, they detected CCR4 expression in human T-cell lines and peripheral blood T cells, but not in B cells, natural killer cells, monocytes, or granulocytes [75, 73].

2.4.4 S1P Receptor Signals as Treat Target

Among all the chemokine signaling, there is an overwhelming one sphingosine-1-phosphate (S1P), S1P is formed by phosphorylation of sphingosine, a major component of all sphingolipids, the production of S1p depends on a reaction catalysed by two isoforms of sphingosine kinase, SPHK1 and SPHK2, which have distinct and overlapping functions [96, 119]. The expression of SPHK1 is depend on the activation from numerous stimuli, including pro-inflammatory cytokines, and promotes the formation of S1P, while SPHK2 is ubiquitously expressed [100]. The level of S1P is dynamically and tightly regulated by kinase and lyase of S1P, the collaboration of these two enzyme in most tissues keep S1P concentration stable in low [148], while in erythrocytes and platelets, the lack of S1P lyase made these two kind of cells the main source of S1P in the body [125]. This difference in producing and maintaining S1P cause the concentration gradients of S1P in blood and tissues, especially in the part with wound, inflammation or infection, and this gradients is very important in directing immune cells to migrant to where they are needed [24, 128, 130, 137, 147].

The regulation of immune cells movement is precisely controlled by S1P receptor expression [151]. S1P receptor is specifically expressed in divergent

immune cells, in CD4+ T cells, S1Pr1 (sphingosine-1-phosphate receptor 1), and S1Pr4 (sphingosine-1-phosphate receptor 1) are primarily expressed [77, 168]. S1P receptors are all GPCR (G protein–coupled receptors). S1Pr1 and S1pr4 play the key role in driving naive CD4 T-cell egress out of thymus and migrate into secondary lymph node after their maturation [151]. During the activating, S1Pr1 and S1pr4 quickly down regulated under TCR (T-cell receptor), which keeps naive T cells stay in the lymph node, waiting the APC (antigen presenting cell) with the pathogen to activate them [59]. Then S1Pr1 and S1Pr4 re-expressed with their transcript factor KLF2 (kruppel-like factor 2) [174], driving effector T cells to lesion tissues. The mechanism of S1Pr1 and S1Pr4 stay uncovered until recently, Li and colleagues found that ECM1 (extracellular matrix protein 1), which is specifically expressed in Th2 cells, is responsible for KLF2 and S1Pr1 revival in Th2 cells, through interacting with CD122, IL-2 receptor β, and blocking late IL-2 signals [91]. These results showed that Th2 cells are under significant control of IL-2 signals, not only in the early differentiation, but also in the late activation.

S1Pr1 is first named as EDG1 in 1998, in a Science paper [87], Lee and colleagues showed EDG-1 bound S1P with high affinity and high specificity. Overexpression of EDG-1 induced exaggerated cell-cell aggregation, enhanced expression of cadherins, and formation of well-developed adherent junctions in a manner dependent on SPP and the small guanine nucleotide binding protein Rho. 3 years later, another Science paper proved that cell migration toward platelet-derived growth factor (PDGF), which stimulates sphingosine kinase and increases intracellular S1P, was dependent on expression of EDG-1 [66]. With more and more findings, the importance of sphingosine-1-phosphate receptors in both science research and clinical trial will be found, and so do other chemokine receptors.

2.5 New Fields in Th1/Th2 Differentiation

Recent years are the revolutionary period for biological science discoveries, and immunology, both innate immunology and adaptive ones, also takes advantages of these progresses. Several new fields is open in CD4+ T helper cells research, and contributes a lot to the uncovery of total immune system network, and provide important implies in disease pathobiology and drug design. Patients with auto immune disease or allergy disease are already benefit from these discoveries. One example is high resolution profiling of histone methylations in systematically research T-cell epigenetics [13].

2.5.1 Progress in Epigenetic Research of Th1 and Th2 Cells

Histone modification in Naïve T-cell differentiation is found the key point and marker in recent years. The modification locis includes H3K27, H3K4, H3K9,

H3K14, H3K18 [106, 113]. Epigenetic modification functionally relevant changes to the structures of genome that do not involve a change in the DNA nucleotide sequence. The most common phenomena of epigenetic modification are DNA methylation and histone acetylization, these modifications results divergent gene expression and regulation [175]. Gene expression can be controlled through the action of repressor proteins that attach to silencer regions of the DNA. These epigenetic changes may last through cell divisions for the duration of the cell's life, and may also last for multiple generations even though they do not involve changes in the underlying DNA sequence of the organism; instead, nongenetic factors cause the organism's genes to behave differently, which makes epigenetic modification very important in immune systems [16], since immune cell needs quick and complicated regulation of gene expression and repression based on the same DNA nucleotide sequences [175]. Th cell differentiation involves epigenetic modification and chromatin remodeling at specific loci [182]. Epigenetic regulation includes modification of both DNA and histones, including DNA CpG methylation, histone methylation and acetylation, as well as DNase I HS induction. Epigenetic modification and chromatin remodeling play critical roles in determining specific gene expression induced by common transcription factors such as NFAT [101]. Indeed, NFAT binding to the Il4 promoter increases, whereas its binding to the Ifng promoter decreases during Th2 differentiation, possibly owing to opposite epigenetic modifications at these two loci during Th2 differentiation [182]. The large image of epigenetic research of CD4+ T helper cell is that chromatin DNA modification happened in the traditional key loci of master regulator or other important transcript factors, but also leads to much wide rage, some of which do not even have function reports or any transcript products. Considering that most of chromatin DNA sequence is untranscripted, the future of epigenetic research in CD4+ T cell is bright.

2.5.2 MicroRNA in Th1 and Th2 Cells

Most genome of organisms are not exon, and the importance of nontranslate RNA is proved plays important roles in Th1 and Th2 cells. One recent topic of CD4+ T-cell research is MircoRNA (miRNA). MiRNAs are small, approximately 21-nucleotides RNAs [84, 95], usually target 3'UTR region of objective molecule genes, and shows a lot of function in many physiological processes. In the biogenesis of miRNA, Drosha DGCR8 and Dicer take part in cutting the pri-miRNA to pre-miRNA and mature miRNA, MiRNA is lately found quite important in immune cells, such as dendritic cells, macrophages [31], and CD4+ helper and CD8+ cytotoxic T cells. Different miRNAs target universal and unique molecules in T cells, help to regulate different Th cell develop stage. In 2005, a brief report shows a requirement for Dicer in the generation and survival of normal numbers of αβT cells, but not stable shutdown of a developmental stage-specific gene (developmentally regulated gene silencing) in the T-cell lineage [70], and suggest that

Dicer may not be required continually for heterochromatin maintenance in thymocytes. It is remarkable that Dicer appears to be dispensable for CD4/8 lineage commitment and the implementation of lineage-specific gene expression programs [28] Other article in the same year made the mice with conditional knock out Dicer in T-cell lineage, in that case, pre-miRNA can not develop into duplex and mature miRNA. In this study, Muljo and colleagues found fourfold reduction on average in spleen of CD8+T cells and twofold reduction on average in spleen of CD4+ T cells, and there is an overall decrease of CD3+ peripheral T cells, but no changes in B220 + B cells [140]. These results suggest miRNA-deficient CD4+ T cells are lack in their ability to repress IFN-γ production and are predisposed to become Th1 cells. Even after two consecutive rounds of activation under Th2 conditions, 18 % of Dicer-deficient T cells produced IFN-γ but not IL-4, and 15 % of Dicer-deficient cells expressed both IFN-γ and IL-4. The Dicer-deficient Th2 culture behaved very differently: after 5 d in Th1 growth conditions, 87 % of the remaining cells were IFN-γ+IL-4- Th1-like cells, and nearly all of the IL-4cells also expressed IFN-γ. These data reinforce the notion that Dicer is required to repress the Th1 genetic program and raise the possibility that Dicer deficiency may also impair stable commitment to the Th2 lineage. Alternatively, lack of Dicer may render terminal Th2 differentiation so inefficient that a residual pool of uncommitted cells remains after 5 d of culture in Th2 conditions, and these cells expanded preferentially as Th1 cells when switched to Th1 growth conditions [111].

An early report in 2008 prove miR-17-92 is existed in CD4+ T cells, and miR17-92 transgenic mice exert completely disrupted follicular structure in spleen and lymph node, with B cell and T cell mixed, compared with separated B and T-cell zone in WT mice. Moreover, the number of T cells also increased in miR-17-92 TG mice when ages grow up. And cytokine express assay shows IL-4 and IL-10 are higher in TG mice, consisting with the antibody IgG1 and IgG2a styles. Finally, PTEN and Bim are taken as the most possible targets of miR-17-92, but not all of the phenomenal could be explained by that, implies the existence of other targets [176]. This is a typical miRNA research, shows the significant function of this small RNA regulation mechanism, regarding to almost every important aspects of Th cells, including develop, follicular formation, facilitate antibody production, proliferation, and cytokine production [14]. Comparing with protein encoded by gene exon, miRNA exhibits more vague and wide function. One reason for this is the exact mechanism of miRNA is still ambiguous, the sequence analysis of certain miRNA and its potential 3'UTR target only provide an imprecise relations between, which did not give the explanation why other gene expression with coincide mRNA region not affected by this miRNA, and why other miRNA with the same predicted binding site did not have the same function. These problems are not only in immunology research, but also in every aspect miRNA involved in. The miRNA research is just on its beginning; in the future, after precise work mechanism of miRNA is uncovered, the answers, as to how miRNA work in immune systems, can be found.

2.5.3 LnRNA in Th1 and Th2 Cells

The last but not least important field is the found of long noncoding RNA (lnRNA). For a long time, lnRNA is not considered as a key role in physiological processes, but in recent years, it is proved not true [38]. LnRNA lacks of conservation between species, which implies its evolution-trend, and may execute important functions in higher species [131, 132]. But the real estate of lnRNA in immunology is still in mist and far from understood. Even the unique definition and database of lnRNA is waiting to be reached. Even so, some research work has already pulled the lnRNA out of the starting line. In 2011, RNA sequence technologies and relevant computer methods have helped to discovery and define tens of thousands of human lnRNAs [18], which provide the basic database and methods for lnRNA research. Fortunately, with these high throughput ways, the foundation of lnRNome is preliminarily built within a few years, compared with the tens of years for human genome program. Another paper reveals that similar to the way of protein-coding RNAs production, lnRNAs are generated through classic pathways, with similar histone-modification profiles, splicing signals, and exon/intron lengths. On the other side, different from protein-coding RNAs, lncRNAs display a striking preference toward two-exon transcripts, and are predominantly localized in the chromatin and nucleus sites, with a fraction appear to tend to processed into small RNAs. This character implies lnRNAs are much easier to degrade, thus under stronger selective pressure and display levels of selection comparable to protein-coding genes. Comprehensive analysis of their expression in multiple human organs and brain regions shows that lncRNAs are generally lower expressed than protein-coding genes, and display more tissue-specific expression patterns [38, 39]. This tissue specific expression is also confirmed in divergent CD4+ T helper cell subsets [69]. Within the same study, 1,524 genomic regions expressing lincRNAs in 42 samples from T cells at various developmental and differentiation stages and identified, and Hu and colleagues found that the lincRNAs were much higher stage specific or lineage specific, than mRNAs. This work is the foundation of lnRNA research in CD4+ T cells, and serves as a resource for the study of transcriptional regulatory networks during T-cell development and differentiation by comparison of the existing dynamic expression of genes encoding proteins, including transcription factors, cell surface markers and signaling molecules, with the help of these combination. Some findings related lnRNA and epigenetic modification of key cytokines gene loci of CD4+ T helper cells [55]. A series of significant findings of lnRNA and key molecule can be expected in the development, differentiation, and immune responses of T cells (Fig. 2.2).

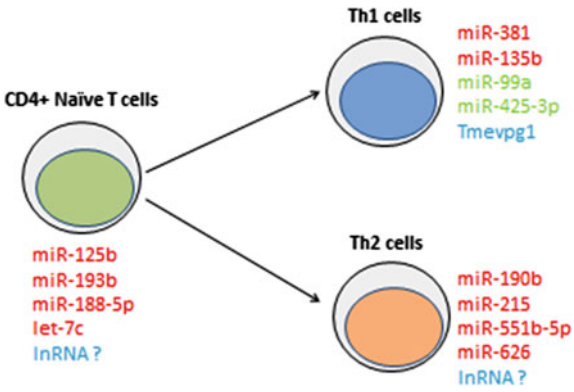

Fig. 2.2 Th1/2 cells differentiation and their micro-/lnRNA signatures. A schematic Th1/2 cells differentiation map is shown and signatures. MicroRNAs in *red* (overexpressed) and *green* (downregulated) are the ones with the strongest subset-specific expression pattern [123]. Tmevpg1 in *blue* is a long intergenic noncoding RNA has the influence on the Th1 cell [30]. More functional lnRNAs are still undiscovered

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Chapter 3
Th1/Th2 Cell's Function in Immune System

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Abstract CD4+ T helper cells regulate appropriate cellular and humoral immune responses to a wide range of pathogens and get involved in many diseases progress. The balance of the earliest determined CD4+ T helper cell subsets, Th1 and Th2, play an important role in allergy and autoimmune diseases. During the research, Animal models in immunology research are necessary and always the powerful tools for the basic scientific research. With the new sequence technologies, the finding of key gene mutation in Th1/Th2 cells has been proved to be related to human diseases. Here, we review four animal models about four key genes in Th1/Th2 cells to introduce the balance between Th1/Th2 cells. Furthermore, the related genetic mutations in human diseases and the new therapies are reviewed in this chapter, which show the importance of Th1/Th2 cells in human diseases further.

Keywords Th1/Th2 · Animal model · Genetic mutation

3.1 Introduction

Th1/Th2 cells are stringently regulated as aberrant cell activity is involved in immunopathologies, such as allergic responses, immunodeficiencies, and lymphomas. The elucidation of the mechanisms that regulate Th1/Th2 cell differentiation, function, and fate should highlight targets for novel therapeutics. With gene-deficient mouse models, it is convenient to define the specific functions of the key genes in Th1/Th2 cell. Indeed, Tbx21-knockout mice exhibit more severe disease after virus infection, and get asthma-like phenotype independent of allergen

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B. Sun (ed.), *T Helper Cell Differentiation and Their Function*,
Advances in Experimental Medicine and Biology 841,
DOI 10.1007/978-94-017-9487-9_3

exposure. In addition, conditional deletion of Gata3 in T cell results in impairment of both Il4-dependent and -independent Th2 differentiation, permitting Th1 differentiation in the absence of Ifng and Il12.

One of the most convincing lines of evidence establishing the importance of Th1/Th2 differentiation process is the existence of monogenic abnormalities in particular differentiation processes or effector functions resulting in disease. A growing set of human disorders in the Th differentiation/function pathways has been identified, which establish the importance of these differentiation processes in humans. In this chapter, we will summarize several key gene mutations in Th1/Th2 differentiation process, including master regulators, STAT family members, and other important transcription factors.

3.2 The Balance Between Th1/Th2 Cells in Allergy and Autoimmune Disease Models

The precise reaction of adaptive immune system protects hosts from many pathogenic and abnormal antigen infections, under the regulation of a very specific way. Animal models in immunology research are necessary, and always determine the level of the work.

3.2.1 Animal Model of T-bet

Finotto et al. [23] generated mice lacking the T-bet gene by targeted disruption. Heterozygous or homozygous knockout mice exhibited airway hyperresponsiveness in response to methacholine. T-bet homozygous knockout mice showed peribronchial and perivenular infiltration with eosinophils and lymphocytes compared with wild-type littermates. T-bet heterozygotes, which display only 50 % reduction of T-bet protein expression, displayed a phenotype very similar to that of mice with a complete absence of T-bet. T-bet heterozygotes and homozygote knockout mice also demonstrated thickening of the airway subbasement membrane collagen layer as well as increased expression of some cytokines. However, the asthma-like phenotype in these mice was independent of allergen exposure, and was not altered by allergen exposure. Finotto et al. concluded that mice with a targeted deletion of the T-bet gene and severe combined immunodeficient mice receiving CD4(+) cells from T-bet knockout mice spontaneously demonstrated multiple physiologic and inflammatory features characteristic of asthma.

By replacing the C terminus of T-bet, which contains the trans-activation domain, with the repression domain of the Drosophila “engrailed” protein, Mullen et al. [48] generated a dominant-negative (DN) T-bet cDNA. Introduction of DN T-bet into cells developing under Th1-inducing conditions, but not into mature Th1 cells, and substantially inhibited their capacity to express Ifng, and resulted in

defective persistence of hypersensitivity site I chromatin within Ifng. Levels of Il12rb2, however, were reduced in both developing and mature Th1 cells by DN T-bet. Screening for homeobox factors expressed in Th1 cells by oligonucleotide array and RT-PCR analysis determined that only Hlx (142995) is expressed at higher levels in Th1 lymphocytes than in Th2 lymphocytes. Hlx appeared at a slower rate than T-bet, and could be induced by T-bet. Ectopic expression in Stat4 −/− Th2-like cells of both T-bet and Hlx allowed maximal and synergistic expression of Ifng in these cells. Introduction of DN T-bet into mature Th1 cells inhibited the expression of Hlx.

Svensson et al. [70] noted that both Il15 −/− mice, which lack NK and NKT cells, and Ifng −/− mice are highly susceptible to genital herpes simplex virus (HSV)-2 infection. CD4-positive T cells and IFN-γ are the most important components of acquired immunity to genital HSV-2 infection, and impaired HSV-2-specific IFN-γ responses in humans correlate with recurrent clinical disease. Svensson et al. vaginally infected T-bet −/− mice with HSV-2. T-bet −/− mice had increased vaginal and spinal cord viral titers, more severe disease, shorter time to death, reduced NK-cell activity, impaired Ifng production, lower specific antibody production, and fewer splenic B cells compared with wild-type mice. These differences were even more marked in mice, first vaccinated with an attenuated HSV-2 strain, and then challenged with virulent HSV-2. However, CD8-positive T cell-mediated cytotoxicity was actually stronger in T-bet −/− mice compared with wild-type mice. Svensson et al. concluded that T-bet is important in both innate defense and for generation of protective Th1 immunity against genital HSV-2 infection, primarily through its control of NK and CD4-positive T-cell function.

Ravindran et al. noted that T-bet-deficient mice resolve infection with the intracellular pathogen Listeria monocytogenes despite having only small numbers of Cd4-positive Ifng-producing T cells. In contrast, they found that challenge of T-bet −/− mice with an attenuated Salmonella strain resulted in death of most mice in less than a month. T-bet −/− mice failed to produce IFN-γ and to switch immunoglobulin isotypes. Spleen cells of infected T-bet −/− mice did not produce IFN-γ, but they did secrete increased levels of IL-10, but not IL-4. Ravindran et al. [61] concluded that CD4-positive T cells expressing T-bet are required for development of Salmonella-specific Th1 cells, regulation of IL-10 production, and resistance to Salmonella infection.

By using immunohistochemical analysis, Wang et al. found expression of T-bet in inflammatory infiltrates of human synovial tissue from patients with rheumatoid arthritis (RA). T-bet −/− mice with the collagen antibody-induced arthritis model of RA had markedly reduced joint inflammation at both early and late time points. Mice lacking both Rag2 and T-bet were resistant to disease. However, adoptive transfer of dendritic cells expressing T-bet reconstituted inflammation in both T-bet −/− and T-bet −/− Rag2 −/− mice. Wang et al. [77] concluded that T-bet has a vital role in DCs that links innate and adaptive immunity to regulate inflammatory responses.

3.2.2 Animal Model of GATA3

GATA3 was initially found with development functions. Lim et al. found that null mutations of Gata3 in mice led to a reduced accumulation of tyrosine hydroxylase (TH) and dopamine beta-hydroxylase (DPH) mRNA, whereas several other sympathetic nervous system (SNS) genes were unaffected. They showed that Th and Dbh deficiencies led to reduced noradrenalin in the SNS, and that noradrenaline deficiency was the proximal cause of death in mutants by feeding catechol intermediates to pregnant dams, thereby partially averting Gata3 mutation-induced lethality. The older, pharmacologically rescued mutants showed abnormalities that could not be detected in untreated mutants. These late embryonic defects included renal hypoplasia and developmental defects in structures derived from cephalic neural crest cells. Thus, Lim et al. [43] showed that Gata3 has a role in the differentiation of multiple cell lineages during embryogenesis.

To elucidate GATA3 function, Pandolfi et al. disrupted the mouse gene by homologous recombination in embryonic stem cells. Mice heterozygous for the Gata3 mutation were found to be fertile and appeared in all respects to be normal, whereas homozygous mutant embryos died between days 11 and 12 postcoitum and displayed massive internal bleeding, marked growth retardation, severe deformities of the brain and spinal cord, and gross aberrations in fetal liver hematopoiesis. The functions of GATA1 and GATA2 had previously been studied by comparable methods. The results in aggregate demonstrated that each GATA-binding protein has a unique and essential function during the development of the mouse embryo. In each case, targeted mutagenesis also revealed surprising roles for each factor, underscoring the power of this experimental approach: GATA1 is essential for erythroid cell development, while disruption of GATA2 indicates a function during very early events in the development of all blood cell lineages [55].

Using microarray analysis, Kaufman et al. identified Gata3 as an induced transcription factor in embryonic day-13 to -18.5 mouse skin. Whole-mount in situ hybridization analysis revealed Gata3 expression in early vibrissae follicles, and later in developing epidermis and in the cone of presumptive inner root sheath (IRS) precursor cells within hair follicles. Examination of pharmacologically rescued Gata3 −/− embryos and grafted Gata3 −/− skin showed aberrations in hair follicle morphogenesis that included not only structural defects in the IRS and hair shaft, but also molecular defects in cell lineage determination. Kaufman et al. [32] concluded that, along with LEF1 and WNTs, GATA3 is at the crossroads of both lymphocyte differentiation and of the IRS versus hair shaft cell fate decision in hair follicle morphogenesis.

Pai et al. [54] generated mice conditionally lacking Gata3 at early (double-negative) and late (double-positive) stages of thymic differentiation. They found that Gata3 was indispensable for thymocytes to pass through beta selection, the process by which T-cell receptor-beta is paired with pre-T-cell receptor-alpha, a requirement for double-negative stage-3 cell survival. Furthermore, Gata3 was required for single-positive Cd4 thymocyte development. Pai et al. concluded that

continued expression of GATA3 is required at multiple stages of thymocyte differentiation.

Zhu et al. generated mice with a conditional deletion of Gata3 and Gata3-deficient mouse T-cell lines and found that both Il4-dependent and -independent Th2 differentiation was diminished, permitting Th1 differentiation in the absence of Ifng and Il12. Deletion of Gata3 from established Th2 cells abolished production of Il5 and Il13, but not of Il4. Mice lacking Gata3 produced Ifng rather than Th2 cytokines in response to infection with Nippostrongylus brasiliensis. Zhu et al. [85] concluded that Gata3 serves as a principal switch in determining Th1-Th2 responses.

Van der Wees et al. analyzed auditory brainstem response thresholds in heterozygous Gata3-knockout mice from 1 to 19 months of age and demonstrated a hearing loss of 30 dB compared to wild-type littermates. No physiologic or morphologic abnormalities were found in the brainstem, cerebral cortex, or the outer or middle ear. However, the cochleae of Gata3 +/− mice showed significant progressive morphologic degeneration starting with the outer hair cells at the apex and ultimately involving all hair cells and supporting cells in the entire cochlea. Van der Wees et al. concluded that hearing loss following GATA3 haploinsufficiency is peripheral in nature and that this defect is detectable from early postnatal development and continues through adulthood [2].

Kouros-Mehr et al. found that Gata3 was the most highly enriched transcription factor in mammary epithelium of pubertal mice. Conditional deletion of Gata3 led to severe defects in mammary development due to failure in terminal end bud formation during puberty. After acute Gata3 loss, adult mice exhibited undifferentiated luminal cell expansion with basement-membrane detachment, which led to caspase-mediated cell death [38].

3.2.3 Animal Model of STAT6

Kuperman et al. developed mice conditionally expressing STAT6 only in the lung epithelium and demonstrated that these mice were protected from all pulmonary effects of IL13, a critical mediator of allergic asthma [15]. Reconstitution of STAT6 only in epithelial cells was sufficient for IL13-induced airway hyperreactivity and mucus production in the absence of inflammation, fibrosis, or other lung pathology [40].

Bour-Jordan et al. [6] showed that T cells from double-knockout mice deficient in Ctla4 and Stat6 were skewed toward a Th2 phenotype in vitro and in vivo by bypassing the need for Stat6. Instead, induction of Gata3 occurred in vitro and Cd4-positive cells migrated to peripheral tissues in vivo. In addition, T-cell receptor cross-linking induced a relative increase of Nfatc1 versus Nfatc2 nuclear translocation and enhanced NF-KB activation compared with Stat6 −/− T cells. Bour-Jordan et al. proposed that CTLA4 regulates T-cell differentiation by controlling the overall strength of the T-cell activation signal, bypassing the cytokine dependency of Th2 differentiation.

Wang et al. noted that BALB/c mice are prone to develop Th2 rather than Th1 responses to antigen and are resistant to experimental myasthenia gravis. However, they found that after immunization with muscle acetylcholine receptor (AChR), BALB/c mice lacking Stat6 were susceptible to EMG and developed more anti-AChR antibodies and complement-fixing anti-AChR antibodies than wild-type or Stat4 −/− mice. Stat6 −/− mouse Cd4-positive T cells proliferated to AChR in a manner comparable to wild-type and Stat4 −/− mice, but Stat6 −/− mice had abundant AChR-specific Ifng-producing Th1 cells that were nearly absent in wild-type and Stat4 −/− mice. Wang et al. [78] concluded that anti-AChR Th1 cells are important in MG pathogenesis.

Chen et al. reported that mice lacking Stat6 were susceptible to virus infection. They found viruses or cytoplasmic nucleic acids trigger STING (also named MITA/ERIS) to recruit STAT6 to the endoplasmic reticulum, leading to STAT6 phosphorylation on Ser(407) by TBK1 and Tyr(641), independent of JAKs. Phosphorylated STAT6 then dimerizes and translocates to the nucleus to induce specific target genes responsible for immune cell homing. Virus-induced STAT6 activation is detected in all cell-types tested, in contrast to the cell-type specific role of STAT6 in cytokine signaling, and Stat6(−/−) mice are susceptible to virus infection. Thus, STAT6 mediates immune signaling in response to both cytokines at the plasma membrane, and virus infection at the endoplasmic reticulum [9].

Rosen et al. investigated the role of Stat6 in oxazolone colitis, a murine model of ulcerative colitis. Colitic wildtype mice had increased Stat6 phosphorylation in epithelial cells, T cells, macrophages, and NKT cells. Mice lacking Stat6 had reduced colitis and decreased induction of the pore-forming tight junction protein Cldn2. Likewise, STAT6 knockdown in human colon epithelial cells reduced CLDN2 induction. Wild-type mice, but not Stat6 −/− mice, had increased mRNA expression of the Th2-inducing cytokines Il33 and thymic stromal lymphopoietin (TSLP). Mesenteric lymph node (MLN) cells from Stat6 −/− mice with colitis exhibited reduced secretion of Il4, Il5, Il13, and Ifng. Il33 augmented secretion of Il5, Il6, Il13, and IFNg from both wild-type and Stat6 −/− MLN cells. Rosen et al. [65] concluded that STAT6 is involved in the pathogenesis of ulcerative colitis and has important roles in altering epithelial barrier function and regulating Th2-inducing cytokine production.

3.2.4 Animal Model of c-Maf

Kim et al. [33, 34] demonstrated that the homozygous null mutant Maf mouse embryo exhibits defective lens formation and microphthalmia.

Ring et al. [63] found that Maf −/− mouse embryos exhibited a slightly foreshortened head and abnormal lens development, and that nearly all died within a few hours of birth. The one surviving animal exhibited microphthalmia, followed by cutaneous closure of the ocular chamber. Fiber cell differentiation and elongation ceased by embryonic day 12.5 in Maf −/− lens, with persistence of a hollow

lens vesicle and absence of alpha-crystallin expression. Cells at the equatorial zone of the lens withdrew from the cell cycle and began to express fiber cell-specific proteins, but they failed to elongate and differentiate normally. After embryonic day 16.5, the mutant vesicle became progressively deformed. Ring et al. identified functional MAF-binding sites in the promoter regions of mouse alpha-A-crystallin (CRYAA), mouse beta-B2 crystallin (CRYBB2), and human beta-A4-crystallin (CRYBA4). They concluded that Maf deficiency causes a profound defect in early maturation of primary and secondary lens fiber cells.

Lyon et al. [45] reported a mouse mutant that, in the heterozygous state, exhibits mild pulverulent cataract named "opaque flecks in lens" (Ofl). The mutant was shown to be allelic with a knockout of Maf. Homozygotes for Ofl and for Maf null mutations were similar except for the addition of renal tubular nephritis in surviving Ofl homozygotes. Sequencing identified the mutation as a 1803G-A transition, leading to an arg291-to-gln (R291Q) substitution in the basic region of the DNA-binding domain. Since mice heterozygous for Maf knockouts showed no cataracts, the authors suggested that the Ofl R291Q mutant protein may have a dominant effect. The mutation also resulted in a selective alteration in DNA binding affinities to target oligonucleotides containing variations in core CRE and TRE elements. The authors hypothesized that arginine-291 may be important for core element binding and suggested that the mutant protein may exert a differential downstream effect among its binding targets.

By ethylnitrosourea (ENU) mutagenesis, Perveen et al. [57] identified a semi-dominant mouse c-Maf mutation, resulting in an asp90-to-val (D90V) substitution at a highly conserved residue within the N-terminal minimal transactivation domain (MTD). The phenotype of D90V homozygotes was isolated cataract. Functional analysis revealed that the D90V mutation results in increased promoter activation and enhances p300 recruitment in a cell type-dependent manner. Perveen et al. observed similar enhancement of p300 interaction with the S50T mutation in the MTD of the NRL gene, which suggests a common mechanism of action.

Wende et al. [80] found that conditional knockout of Maf in mouse DRG cells disrupted the architecture and function of several rapidly adapting mechanoreceptor subtypes. Pacinian corpuscles, specialized to detect high-frequency vibrations, were severely atrophied, with loss of innervating axons. In vitro skin-saphenous nerve preparations of Maf-knockout mice revealed abnormal fire response to mechanical stimuli.

3.3 Human Disease Reports and New Therapies Regarding Th1/Th2 Cells

Because of the significant role in Th1 and Th2 cells in adaptive immune system, patients with genetic mutation with the key genes in Th1 and Th2 development or mutations of molecules in APC which will case impair activation signal transduction leads to serious hereditary disease [53]. Early in 1952, tyrosine kinase

mutation was found in agammaglobulinemia patients, which is the first reported human gene mutation [7]. And with more and more discoveries in both basic immunology research and clinical disease research, the knowledge of these two fields reconfirmed each other's conclusion and implies each other's inspiration [8, 17].

The finding of key gene mutation and the relation with human disease develop with both knowledge growth in medical and immunology field, and most importantly, with the new sequence technologies. We are at the eve of affordable full sequence assay of individuals, so the main finding in sequence still need the hints from classic immunology research, to emphases on the target genes. The first step of target genes narrow down is identify the abnormal gene and the frequency of this mutation, with the help of the database of human genome. Lower than 1 % of frequency is considered significant, and among these genes, the mutation related to immunology functions is the most worthy focused on (Table 3.1). In other conditions, if the full sequence is not available or affordable, we need to stick on the suspected genes, in this case, the research of human gene sequence is a tool to demonstrate what happened in mouse genes regulation.

3.3.1 The Discovery of GATA3 Gene Mutation

GATA3 was found located on chromosome 10p15 by in situ hybridization in 1991 [30], and on mouse chromosome 2 in 1993 [14], 4 years before it was found necessary and sufficient for Th2 cytokine gene expression [84] and related to asthma disease [82].

Terminal deletions of chromosome 10p result in a DiGeorge-like phenotype that includes hypoparathyroidism, heart defects, immune deficiency, deafness, and renal malformations. One region that contributes to this complex phenotype is that for the syndrome of hypoparathyroidism, sensorineural deafness, and renal insufficiency. Van Esch et al. performed deletion-mapping studies in two HDRS patients and defined a critical 200-kb region that contains the GATA3 gene. Search for GATA3 mutations in 3 other HDR probands identified 1 nonsense mutation and two intragenic deletions that predicted a loss of function, as confirmed by absence of DNA binding by the mutant GATA3 protein. These results demonstrated that GATA3 is essential in the embryonic development of the parathyroids, auditory system, and kidneys, and showed that GATA3 haploinsufficiency causes human HDR syndrome [76].

Muroya et al. studied nine Japanese families with HDR syndrome. FISH and microsatellite analysis showed heterozygous deletions including GATA3 in four families. Sequence analysis showed heterozygous novel mutations in three families, including a missense mutation in exon 4, an insertion mutation, and a nonsense mutation in exon 6 [49].

In 10 patients with HDR syndrome from 7 unrelated families, Nesbit et al. identified and characterized 7 mutations in exons 3 through 6 of the GATA3 gene.

Table 3.1 Phenotype of Th1/Th2 specific gene-deficient animal models and related diseases in gene-mutation human patients

Gene	Phenotype in animal model	Related factors	Related human disease	Function
Gata3	Embryonic death; impaired thymocyte and Th2 differentiation	IL-4; IL-5; IL-13	Asthma; HDR syndrome; hypoparathyroidism	Function in the differentiation of multiple cell lineages
Tbx21	Airway hyperresponsiveness; asthma-like phenotype independent of allergen exposure; more severe disease after HSV-2 infection	IFNG; Il12rb2; Hlx	RA; type 1 diabetes	Control the expression of the hallmark Th1 cytokine, interferon-gamma
Stat1		IL-17F; IL-17RA; IFN-α/β, IFN-γ, IFN-λ, IL-27/22	AD CMCD; AD CMC; disseminated fungal infections; IPEX	Function in IL-17 immunity, infection and Th1–-interferon-γ responses
Mst1		Histone H2B; FOXO	B cell and T cell lymphopenia	Controll the death of naive T cells; Crucial for thymic egress and lymphocyte migration
Stat6	Myasthenia gravis; susceptible to virus infection; reduced colitis and decreased induction of Cldn2	AChR; STING; TBK1; CLDN2; IL-33; IgE	Asthma	Mediates immune signaling in response to both cytokines and virus infection; Involve in the pathogenesis of UC; Alter epithelial barrier function and regulate Th2-inducing cytokine production
Stat4		IFNa	SLE	Critical in the dysregulation of the IFNa pathway and SLE susceptibility
c-Maf	Defective lens formation and microphthalmia		Cataract	Function as a DNA-binding factor in early maturation of primary and secondary lens fiber cells

Using electrophoretic mobility shift, dissociation, yeast 2-hybrid, and glutathione S-transferase pull-down assays, Nesbit et al. [51] demonstrated that mutations involving the C-terminal zinc finger (ZnF2) or adjacent basic amino acids result in a loss of DNA binding, but those of the N-terminal zinc finger (ZnF1) either lead to a loss of interaction with specific zinc finger proteins of FOG2 (ZFPM2) or alter DNA-binding affinity.

Hernandez et al. [27] reported a mother and daughter with HDR and female genital tract malformations in whom they identified a deletion in the GATA3 gene.

Chiu et al. sequenced the CASR and GATA3 genes in five unrelated Chinese families with familial hypoparathyroidism. They identified three novel mutations in the GATA3 gene responsible for familial hypoparathyroidism and deafness. Except for a previously described polymorphism, they found no genetic variants in the CASR gene [11].

Ali et al. analyzed the GATA3 gene in 21 HDR probands and 14 patients with isolated hypoparathyroidism (FIH); no mutations were found in the FIH patients, but 13 different heterozygous germline mutations were identified in the HDR probands, including 1 missense, 1 splice site, 3 nonsense, and 8 frameshift mutations. EMSA analysis revealed three classes of GATA3 mutations: those involving of loss of DNA binding due to loss of the C-terminal zinc finger, which represent over 90 % of mutations reported in GATA3; those resulting in reduced DNA-binding affinity; and those that do not alter DNA binding or affinity but likely alter the conformation change that occurs during binding in the DNA major groove, as predicted by three-dimensional modeling [4].

In a 14-year-old boy with neurologic symptoms in addition to the HDR triad of hypoparathyroidism, sensorineural deafness, and renal dysplasia, who did not have any microdeletion in the 22q11.2 or 10p14 regions by FISH analysis, Ferraris et al. identified a heterozygous de novo 2-bp deletion in exon2 of the GATA3 gene. The authors concluded that haploinsufficiency of GATA3 may be responsible for a complex neurologic picture in addition to the known triad of HDR syndrome [21].

In a 29-year-old Portuguese with severe hypoparathyroidism, bilateral mild neurosensory deafness, and agenesis of the vagina and uterus but no kidney abnormalities, Moldovan et al. analyzed the GATA3 gene and identified a heterozygous missense mutation. The authors noted that this patient, along with the mother and daughter with HDR and female genital tract malformations studied by Hernandez et al., seemed to confirm the role of GATA3 in regulating developmental mechanisms of the uterus and vagina [46].

3.3.2 The Discovery of Tbox21 Gene Mutation

TBX21 is a Th1-specific T-box transcription factor that controls the expression of the hallmark Th1 cytokine, interferon-gamma [71]. And cloned in the same year [83]. Tbx21 has six exons and is on chromosome 11D in an area showing homology of synteny with human chromosome 17.

Akahoshi et al. identified a -1993T-C SNP in the promoter region of the TBX21 gene and found that the substitution increases the affinity of an unknown nuclear protein for the binding site in that region, resulting in increased transcriptional activity of the TBX21 gene. There was a significant association between the promoter SNP and aspirin-induced asthma in a Japanese cohort (p = 0.004), with increased risk associated with a C allele (OR = 1.93; 95 % CI, 1.22–3.06). The association was confirmed in additional independent samples from patients with asthma and nasal polyposis regardless of aspirin hypersensitivity (p = 0.008) [3].

Sasaki et al. in 2004 screened for polymorphisms in the T-bet gene and detected two microsatellite repeat polymorphisms located in intron 1 and the 3′-flanking region, and two single nucleotide polymorphisms, including a His33Gln substitution within the coding region. In the Japanese population, polymorphisms in Tbx21 and Th1-related genes have been linked in humans to a greater risk of developing type 1 diabetes, which is Gln-positive phenotype and (CA)14 allele in 3′-flanking region of T-bet. Furthermore, Gln33 T-bet showed a significantly higher transcriptional activity of the IFN-g gene via a dual luciferase reporter assay, suggests the T-bet Gln33 polymorphism, which is present at a greater frequency in Japanese patients with type 1 diabetes, is responsible for more transcription from the IFN-g promoter, and suggests that T-bet-mediated control of IFN-g production is a contributing factor to the pathogenesis of this disease. Sasaki and colleagues study suggests the first evidence of an association between type 1 diabetes and polymorphisms in the T-bet gene, and that variation in T-bet transcriptional activity may play a role in the development of type 1 diabetes, possibly through the effect on IFN-gamma production in Th1 cells [67].

3.3.3 The Discovery of STAT1 Gene Mutation

Of STAT1, by using whole-exome sequencing, a JEM paper in 2011 identified heterozygous germline mutations in STAT1 in 47 patients from 20 kindreds with AD (autosomal dominant) CMCD (Chronic mucocutaneous candidiasis disease) [44], while this disease is used to be taken caused by IL-17F deficiency [36, 59] or AR (autosomal recessive) IL-17RA deficiency, because high titers of neutralizing auto antibodies against IL-17A, IL-17F, and IL-22 are found in CMCD patients [58]. But these genotype phenomena are not common enough to reach the etiology conclusion. Previously described heterozygous STAT1 mutant alleles are loss-of-function [19] and thus AD are easier to get bacterial disease caused by impaired STAT1-dependent immune system responses to IFN-γ. Other loss-of-function STAT1 alleles cause AR predisposition to intracellular bacterial and viral diseases, caused by impaired STAT1-dependent responses to IFN-α/β, IFN-γ, IFN-λ, and IL-27, as the expected results of STAT1 deficiency. One example of this alleles is MSMD-causing loss-of-function STAT1 allele L706S [19] K201N [37] and K211R [39] with the mutation on coiled-coil (CC) domain of STAT1, which plays a key role in unphosphorylated STAT1 dimerization and STAT1 nuclear

dephosphorylation. Loss-of-function STAT1 mutations lead to severe viral and mycobacterial infections [5]. Not only impaired IL-12 and IL-23 signaling, STAT4 deficiency is also reported in STAT1 loss-of-function patients [68].

On the other side, the 12 AD CMCD-inducing STAT1 mutant alleles R274Q in 4 different families are gain-of-function and increase STAT1-dependent cellular responses to these cytokines, and to cytokines that predominantly activate STAT3, such as IL-6 and IL-21. All of these mutations affect the coiled-coil domain and impair the nuclear dephosphorylation of activated STAT1, accounting for their gain-of-function and dominance. Stronger cellular responses to the STAT1-dependent IL-17 inhibitors IFN-α/β, IFN-γ, and IL-27, and stronger STAT1 activation in response to the STAT3-dependent IL-17 inducers IL-6 and IL-21, hinder the development of T cells producing IL-17A, IL-17F, and IL-22. Gain-of-function STAT1 alleles therefore cause AD CMCD by impairing IL-17 immunity [64]. Another study researched 14 patients from five families with autosomal dominant CMC (Chronic mucocutaneous candidiasis), a disease characterized by susceptibility to candida infection of skin, nails, and mucous membranes [35], and poor production of interferon-γ, interleukin-17, and interleukin-22 [59], thus have mucosal antifungal immunity [12]. Th1–interferon-γ responses were defective in patients with autosomal dominant CMC [75], and Th17 responses in these patients were also found with this disorder [20]. With the significant finding in CD4+ T.cells subset in recent years, these patients cellular abnormality suggests that the defect lay within the interleukin-12 receptor and interleukin-23 receptor signaling pathways, and by array-based sequence capture followed by next-generation sequencing, heterozygous missense mutations in the DNA sequence encoding the coiled-coil domain of STAT1 in the patients [74]. Recent reports show that some STAT1 gain-of-function patients even have disseminated fungal infections or an IPEX (immunodysregulation polyendocrinopathy enteropathy X-linked syndrome)-like syndrome [73]. Some patients with disseminated Coccidioides immitis or Histoplasma capsulatum with heterozygous missense mutations in the STAT1 coiled-coil or DNA-binding domains, enhanced STAT1 phosphorylation, delayed dephosphorylation, enhanced DNA binding and transactivation, and enhanced interaction with protein inhibitor of activated STAT1. The mutations caused enhanced IFN-γ-induced gene expression, but we found impaired responses to IFN-γ restimulation, thus with severe, disseminated dimorphic yeast infections [66].

3.3.4 The Discovery of MST1 Gene Mutation

Mammalian sterile 20-like protein kinase 1 (MST1), also known as serine/threonine protein kinase 4 (STK4). MST1 is cloned and named by Creasy and Chernoff in [16]. One year after, Taylor found MST1 have kinase function, and rename it as STK4 [72]. DNA in eukaryotic cells is associated with histone proteins; hence, hallmark properties of apoptosis, such as chromatin condensation, may be regulated by posttranslational histone modifications. Cheung and colleagues reported that

phosphorylation of histone H2B at ser14 correlates with cells undergoing programmed cell death in vertebrates. They identified a 34-kD apoptosis-induced H2B kinase as caspase-cleaved MST1. MST1 could phosphorylate H2B at ser14 in vitro and in vivo, and the onset of H2B ser14 phosphorylation was dependent upon cleavage of MST1 by caspase-3. These data revealed a histone modification uniquely associated with apoptotic chromatin in species ranging from frogs to humans and provided insights into a physiologic substrate for MST1. These data also provided evidence for a potential apoptotic histone code [10]. Using human and other mammalian cells found that MST1 phosphorylated FOXO transcription factors at a site conserved within the forkhead domain of FOXO proteins from mammals to *Caenorhabditis elegans*. Oxidative stress induced MST1-mediated phosphorylation of FOXO3 at ser207, which disrupted interaction of FOXO3 with other proteins, promoted FOXO3 nuclear translocation, and induced cell death. Knockdown of the *C. elegans* MST1 ortholog Cst1 shortened life span and accelerated tissue aging, whereas Cst1 overexpression promoted life span and delayed tissue aging. The Cst1-induced life span extension was dependent on the FOXO ortholog Daf16 [42].

In two consanguineous unrelated Turkish families with four patients affected by combined immunodeficiencies with multiple bacterial and viral infections, autoimmunity, and progressive CD4 and naive CD8 T-cell lymphopenia, Nehme et al. identified putative truncation mutations in the STK4 gene. The patient in the first family was homozygous for an arg117-to-ter substitution. The three affected sibs in the second family were homozygous for a 1-bp deletion that resulted in a frame-shift at residue 368 and a contiguous nonsense codon at residue 369. Parents and healthy sibs in both families were heterozygous for the mutations, indicating autosomal recessive inheritance. RT-PCR analysis showed loss of function and expression of STK4 in patients. Lymphoproliferative responses and lymphocyte survival were also impaired. FOXO1, IL7R, and BCL2 were poorly expressed in patient T cells, whereas FAS expression was upregulated. Nehme et al. [50] concluded that the STK4/FOXO1 pathway has a role in controlling the death of naive T cells.

In three members of a consanguineous Iranian kindred with T- and B-lymphopenia, neutropenia, cardiac malformations, and recurrent bacterial, viral, and fungal infections, warts, and abscesses, Abdollahpour et al. identified a homozygous premature termination mutation in the STK4 gene. Parents and healthy sibs were heterozygous, indicating autosomal recessive inheritance. Western blot analysis showed that patients with homozygous mutations expressed no STK4, whereas heterozygous carriers expressed intermediate levels compared to wildtype homozygous subjects. STK4-deficient lymphocytes and neutrophils exhibited enhanced loss of mitochondrial membrane potential and increased susceptibility to apoptosis [1].

Patients with MST1 deficiency have B cell and T cell lymphopenia, decreased numbers of CD62L+ (also known as L-selectin) CCR7+ central memory T cells, TCRs that show impaired variable-α (Vα) gene usage (which suggests that they have a restricted TCR repertoire), elevated EBV loads and EBV-positive lymphomas In addition, MST1 is crucial for thymic egress and lymphocyte migration [47].

3.3.5 The Discovery of STAT6 Gene Mutation

Quelle et al. [60] identified a number of expressed genes in the signal transducers and activators of a transcription (STAT) family. Human and murine full-length cDNA clones were obtained and sequenced. The sequence of the human cDNA was identical to the sequence published by Hou et al. [28] for the interleukin-4-induced transcription factor (called by them IL4 Stat), while the murine STAT6 amino acid and nucleotide sequences reported by Quelle et al. were 83 and 84 % identical to the human sequences, respectively.

By screening an embryonic lung fibroblast cDNA library with a wildtype STAT6 probe, Patel et al. identified two variant cDNAs, which they termed STAT6b and STAT6c, encoding an N-terminal 110-amino acid truncation and a 27-amino acid deletion in the SH2 domain, respectively. RNase protection analysis detected ubiquitous expression of all three variants with STAT6b expression greatest in spleen and STAT6c expression greatest in lung [56].

In the Chromosomes location research, Copeland et al. [13] found that seven mouse Stat genes map in three clusters, with each cluster located on a different autosome. They suggested that the Stat family arose by a tandem duplication of the ancestral Stat gene, followed by dispersion of the linked loci to different chromosomes. They mapped Stat6 and Stat2 to the distal region of chromosome 10. During an analysis of NAB2, Svaren et al. obtained the sequence adjacent to this gene by PCR of genomic DNA. They found that the STAT6 gene is located unusually close to the NAB2 gene, such that the 3-prime ends of their mRNAs overlap [69]. Since the human NAB2 gene was previously mapped to 12q13.3-q14.1, it is likely that STAT6 maps to the same position. By fluorescence in situ hybridization, Leek et al. [41] mapped STAT6 to 12q13.

Duetsch et al. [18] identified 13 single-nucleotide polymorphisms (SNPs) in STAT6 and tested them for linkage/association with asthma and related traits (total serum IgE level, eosinophil cell count, and SLOPE of the dose-response curve after bronchial challenge) in 108 Caucasian sib-pairs. Neither the SNPs nor a GT repeat in exon 1 showed linkage/association to asthma. A significant association was found between a SNP in intron 18 and an increase in total IgE levels ($P = 0.0070$), as well as an association between allele A4 of the GT repeat polymorphism and an increase in eosinophil cell count ($P = 0.0010$). The authors concluded that rather than contributing to the pathogenesis of asthma, the human STAT6 gene is more likely involved in the development of eosinophilia and changes in total IgE levels [18].

In a case-control association study of 214 white British subjects, Gao et al. demonstrated a significant association with asthma of an allele with a 13-GT repeat sequence in exon 1 of the STAT6 gene (OR, 1.52; 95 % CI, 1.02–2.28; $p = 0.027$), whereas the 16-GT allele showed an inverse association with asthma ($p = 0.018$). Furthermore, individuals with the 13-GT allele had higher IgE levels compared with individuals with the 16-GT allele ($p = 0.004$). Transient transfection assays of different alleles revealed significantly higher transcriptional activity with the 13-GT allele compared to the 16-GT allele in Jurkat, HMC-1, and BEAS-2B cell lines. Gao

et al. [24] suggested that the GT repeat polymorphism of the STAT6 gene contributes to susceptibility to atopic asthma and total serum IgE levels, and that variation in the length of the GT repeat sequence influences the regulation of promoter activity.

Several studies have shown linkage of 12q13-q24 with atopy-related phenotypes. STAT6 is 1 of the candidate genes in this region, because of its involvement in Th2 cell differentiation, recruitment, and effector function. Studying a population-based cross-sectional cohort of 1,407 German adults, Weidinger et al. evaluated 6 polymorphisms of STAT6 for evidence of association with serum IgE levels and atopic disease. One polymorphism in intron 2 showed a significant association with total serum IgE ($p = 0.015$). A STAT6 risk haplotype for elevated IgE showed odds ratios of 1.54 ($p = 0.032$), 1.6 ($p = 0.025$), and 2.54 ($p = 0.007$) for IgE percentiles of 50, 60, and 90 %, respectively [79].

3.3.6 The Discovery of STAT4 Gene Mutation

STAT4 was cloned and located to chromosome 2q32 in 1997 by Yamamoto, and found not ubiquitously, but expressed in specific tissues, including spleen, heart, brain, peripheral blood cells, and testis [81].

Since linkage peaks containing the STAT4 gene had been reported in genome scans of patients with systemic lupus erythematosus, Remmers et al. included the genotyping of three series of patients with SLE and control subjects of European ancestry as part of a large case-control disease-association analysis of a linkage region on chromosome 2q associated with rheumatoid arthritis. They found that a haplotype marked by the STAT4 SNP rs7574865 was strongly associated with SLE [62], being present on 31 % of chromosomes of case patients and 22 % of those of controls ($P = 1.87 \times 10(-9)$; odds ratio for having the risk allele in chromosomes of patients versus those of controls, 1.55). Homozygosity for the risk allele, as compared with absence of the allele, was associated with a more than doubled risk for SLE and a 60 % increased risk for rheumatoid arthritis. Independently, Gateva et al. [25] and Han et al. [26] replicated the association of SLE susceptibility with one STAT4 SNP at Chromosome 2:191964633 (rs7574865).

To identify risk loci for SLE susceptibility, Gateva et al. [25] selected SNPs from 2,466 regions that showed nominal evidence of association with SLE (P less than 0.05) in a genomewide study and genotyped them in an independent sample of 1,963 cases and 4,329 controls. This new cohort replicated the association with STAT4 at rs7574865 (combined P value = $1.4 \times 10(-41)$, OR = 1.57, 95 % confidence interval of 1.49–1.69).

Han et al. performed a genomewide association study of SLE in a Chinese Han population by genotyping 1,047 cases and 1,205 controls using Illumina-Human610-Quad BeadChips and replicating 78 SNPs in 2 additional cohorts (3,152 cases and 7,050 controls). Han et al. found association with the STAT4 gene at rs7574865 (combined P value = $5.17 \times 10(-42)$, odds ratio = 1.51, 95 % confidence interval 1.43–1.61).

By measuring serum IFNa activity and IFNa-induced gene expression in PBMCs from a cohort of 270 SLE patients of different ethnic backgrounds, Kariuki et al. [31] showed that the T allele of the STAT4 SNP rs7574865 was simultaneously associated with both lower serum IFNa activity and greater IFNa-induced gene expression. Although the IRF5 SLE risk genotype was associated with higher serum IFNa activity, the influence of STAT4 was dominant on the sensitivity of PBMCs to serum IFNa. Kariuki et al. concluded that the risk variant of STAT4 is critical in the dysregulation of the IFNa pathway and SLE susceptibility.

3.3.7 The Discovery of c-Maf Gene Mutation

Maf was cloned by Nishizawa et al. [52] in human, and by Ring in mouse [63].

In five affected members of a three-generation family with autosomal dominant juvenile-onset cataract (CTRCT21), Jamieson et al. [29] identified a 1670G-C transversion in the MAF gene, resulting in an arg288-to-pro (R288P) substitution in the basic region of the DNA-binding domain of MAF, predicted to cause an abnormal helical conformation. The cataracts were cortical pulverulent opacities in a lamellar distribution. Nuclear pulverulent opacities were present in two cases. There was later progression with posterior subcapsular opacification that necessitated surgery in adult life. Two of the five affected individuals had microcornea, and one also had bilateral iris colobomas. The mutation was not found in 217 other subjects with a range of eye anomalies or in 496 normal control chromosomes.

Wende et al. [80] found that skin of carriers of the R288P substitution displayed reduced acuity to high-frequency vibration compared with normal controls. In 12 affected members of a three-generation family with congenital cerulean cataract, 6 of whom also had microcornea, Vanita et al. identified heterozygosity for an 890A-G transition in the MAF gene, resulting in the replacement of a highly conserved lys297 with arg (K297R) in a basic region of the DNA-binding domain of the protein. The mutation was not found in 106 unrelated controls.

3.3.8 Other Human Gene Mutation Reports

Very few report of gene mutation specifically block human Th1 cell development, but there are patients with mendelian susceptibility to mycobacterial diseases, and are easy to get clinical disease caused by weakly virulent mycobacterial species in otherwise healthy individuals. Since 1996, disease-causing mutations have been found in five autosomal genes (*IFNGR1, IFNGR2, STAT1, IL12B, IL12BR1*) and one X-linked gene (*NEMO*). These genes display a high degree of allelic heterogeneity, defining at least 13 disorders. Although genetically different, these conditions are immunologically related, as all result in impaired IL-12/23-IFN-gamma-mediated immunity. These atopic mutation shows important role of Th1 cells in anti-batetials immunity [22].

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Chapter 4
Regulatory T-Cell Differentiation and Their Function in Immune Regulation

Miranda Piccioni, Zuojia Chen, Andy Tsun and Bin Li

Abstract Regulatory T-cells (Treg) represent a subset of CD4+ T-cells characterized by high suppressive capacity, which can be generated in the thymus or induced in the periphery. The deleterious phenotype of the Scurfy mouse, which develops an X-linked lymphoproliferative disease resulting from defective T-cell tolerance, clearly demonstrates the importance of Treg cells for the maintenance of immune homeostasis. Although significant progress has been achieved, much information regarding the development, characteristics and function of Treg cells remain lacking. This chapter highlights the most recent discoveries in the field of Treg biology, focusing on the development and role of this cell subset in the maintenance of immune balance.

Keywords Treg · Development · Characteristics · Function

4.1 Introduction

CD4+ regulatory T-cells are composed of two major populations: thymus-derived Treg cells, named as natural Tregs (nTregs), which originate from the thymus, and peripherally derived Treg cells, named as induced Tregs (iTregs), which are generated in the periphery. Both of the nTreg and iTreg cell subsets possess the ability to suppress T-cell proliferation and inflammatory immune responses. Among these Treg cell subsets, there are important differences in their differentiation environment, antigen specificities, and mechanisms of suppression. Although it is still

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Advances in Experimental Medicine and Biology 841,
DOI 10.1007/978-94-017-9487-9_4

elusive regarding the specific function of each Treg cell subset to immune regulation, there is a possibility that nTregs and iTregs have separate roles in the maintenance of immune homeostasis.

Recently, more experimental evidence has revealed distinct mechanisms of Treg suppression in various inflammation settings and tissues, suggesting a classification of Treg cell subphenotypes according to Foxp3 (Forkhead box P3) heterogeneity. A subset of Treg cells was discovered to rely on the transcription factor IRF4, which is also required for Th2 cell differentiation and function [227]. IRF4 associates with Foxp3 and targets a set of genes synergistically to suppress Th2 cell function. The deletion of IRF4 expression in Treg cells results in a selective failure to control Th2 immune responses [227]. Another study found a CXCR3+ Treg subset dependent on T-bet transcription factor, which is essential for regulating Th1 cell activity [97]. T-bet was shown to upregulate CXCR3 expression in Treg cells allowing them to migrate, proliferate, and accumulate at the sites of Th1 responses. Accordingly, Treg cells deficient in T-bet expression have no effect on inhibition of Th1-mediated inflammation [97]. As a key factor in Th17 cell induction, STAT3 also directs Treg cells toward the regulation of Th17 cell responses. Treg cells expressing STAT3 may control Th17-dependent pathology [31]. Similar to these studies, the transcription factor Bcl6, important in T follicular helper cell (Tfh cell) development, was demonstrated to be essential for Treg cell mediated regulation of germinal center responses. CXCR5 can be induced by Bcl6 in Treg cells enabling them to localize in the germinal center. Lack of CXCR5+ Treg cells results in increased germinal center reactions [35, 109]. Thus, in response to various environmental settings, Treg cells may be further defined as T-bet+ FOXP3+ Treg, IRF4+ FOXP3 + Treg, STAT3+ FOXP3+ Treg or Bcl6+ FOXP3+ Treg subsets depending on the selective upregulation or activation of transcription factors associated with Th1, Th2, Th17, and Tfh cells, respectively. FOXP3 may act together with these factors to endow the cells with tailored function needed to suppress corresponding types of CD4+ T-cell responses under particular inflammatory conditions. Treg cells are able to respond to local stimuli and to adopt multiple reactions accordingly, perfectly adapting to the diverse inflammatory settings. Further studies will be needed both to characterize the molecular mechanisms underlying the functional differentiation of Treg cells and to purify and expand the specific Treg subsets.

To extend the study of functional Treg subsets into nonlymphoid tissues, unique tissue-resident Treg populations have been identified and characterized. The documentation of CD4+ FOXP3+ Treg cells in nonlymphoid tissues such as skin, intestine, lung, liver, fat, muscle, placenta, and others, including normal tissues and inflammatory sites [27, 36, 53, 54, 77, 134, 166, 168, 186], clearly indicates the diverse distribution of Treg cells throughout the body and underlines characteristic properties of each tissue-resident Treg subset [26]. The first well-characterized example is represented by a subset of Treg cells residing in visceral adipose tissue (VAT) and regulating insulin metabolism [36, 53]. Although VAT Treg cells exhibit a classical Treg signature, they display a distinct gene profile and TCR repertoire sequences, chemokine receptors and transcription factors, when compared to lymphoid-organ Treg cells. PPARγ (peroxisome proliferator activated

receptor gamma), was identified as a master regulator in the orchestration of VAT Treg cell function and differentiation [36]. Most recently, another unique Treg subset harboring a special transcriptome, a specific TCR repertoire and peculiar function was identified to accumulate in injured skeletal muscle [27]. Muscle Treg cells control muscle inflammation and potentiate muscle repair upon injury. The growth factor Amphiregulin is highly expressed in muscle Treg cells and helps to enhance muscle regeneration [27].

Given the emerging strategies of Treg cell modulation for cancer therapy, research is focusing on the study of tumor associated Treg cells. Similar to other tissue-resident Treg cells, the Treg cells found in tumors exhibit a distinguishing phenotype and are grouped into a different subset. In the tumor environment, Treg cells are capable of promoting angiogenesis, mediating metastasis, and suppressing antitumor immune responses [66, 147, 170, 190]. The understanding of tumor-associated Treg cell biology may help to identify novel targets and methods for the diagnosis and treatment of cancer. The exploration of tissue-resident Treg cell subsets is just at the beginning; many key questions regarding the origin, maintenance or mechanisms of action of these unique Treg cell populations remain to be answered. A strong connection exists between Treg cell localization and function, and the complexity and versatility of Treg cells reveal the need to study and illustrate the role of Treg cells contextually.

4.2 nTreg Cells: Development and Characteristics

The majority of functionally mature CD4+CD25+ regulatory T-cells are produced in the thymus and constitute to a distinct cell lineage in the periphery. Nude mice transferred with thymic suspensions depleted of the CD4+CD25+CD8− fraction show various autoimmune diseases [88]. When compared to CD4+CD25+ regulatory T cells, CD4+CD25+CD8− thymocytes exhibit similar function and phenotype, including high expression level of Foxp3, CTLA-4 (cytotoxic Tlymphocyteantigen 4) and GITR (glucocorticoid-induced TNFR family related gene), suppression toward T-cell proliferation, and are anergic to TCR stimulation in vitro [146, 179, 189]. Foxp3 deficiency also abrogates the CD4+CD25+CD8− population in the thymus [57, 82]. Thus, the thymus is fundamental for Treg cell generation and for the prevention of spontaneous autoimmunity [167].

As a key transcription factor of Treg cells, FOXP3 is indispensable for thymic Treg development. Foxp3 predominantly expresses in both peripheral CD4+CD25+ T-cells and thymic CD4+CD25+CD8− cells, but not in other cell subsets [57, 82]. In Foxp3-transgenic mice, CD4+CD25+ T-cells are increased, whereas Foxp3 deficient mice show spontaneous inflammation, as observed in Scurfy mice. Moreover, Foxp3 deficient bone marrow fails to produce Treg cells [93, 96]. Mutations in the human gene encoding FOXP3 causes the genetic disease IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome), which is associated with autoimmune diseases in multiple endocrine organs [19, 30, 211]. FOXP3 mutations

characterizing IPEX patients abrogate the development of the Treg cells. Whereas depletion of Foxp3 in mice induces the progression of autoimmune diseases [95, 102], transduction of Foxp3 in naïve T-cells upregulates Treg-associated markers including CD25, CTLA-4 and GITR and suppresses the secretion of IL-2, IFN-γ and IL-4 [82]. Thus, the transcription factor Foxp3 is critical in thymic Treg differentiation and is considered the most reliable marker for nTreg cells.

During T-cell development in the thymus, variations in TCR signaling affinity and duration determine a highly diverse population of T-cells. Negative selection eliminates T-cells bearing TCRs with excessive avidity to self-peptide-major histocompatibility complexes (pMHC). Cells escaping negative selection harbor low avidity self TCR-pMHC interactions. The requirement for TCR signaling is also pivotal for nTreg cell lineage commitment in thymic differentiation. Endogenous TCR rearrangement was observed for Treg cell generation in TCR-transgenic mice [81, 136]. T-cells bearing a TCR with low affinity fail to develop into Treg cells [90, 198], suggesting that self-reactivity of Treg cells requires strong TCR-mediated signals. Medullary thymic epithelial cells (mTECs) deficient in MHCII results in a shift from T-cell clonal deletion to Treg cell differentiation [79]. Taken together, thymocytes that are able to differentiate into Treg cells should receive TCR signals of intermediate strength in the range between those that mediate negative and positive selection, to escape deletion and be enriched for Foxp3 induction.

The specificity and diversity of TCRs in Treg cells are critical in thymic Treg cell selection and differentiation. When compared to CD4+ conventional T-cells, the TCR repertoire of Treg cells is a unique set with high diversity but is overlapping to some extent [83]. Usage analysis revealed that Treg cell TCRα sequences exist in a broad variety but only partially overlap with non-Treg cells [142, 143, 213]. TCR transgenic T-cells expressing Treg cell TCRα molecules exhibit increased self-reactivity and undergo homeostatic expansion more rapidly in lymphopenic recipient mice [83]. Treg cells with restricted TCR repertoires are less efficient in inhibiting inflammation [1, 56, 81], suggesting that Treg cells with limited TCRs have impaired protective ability toward autoimmune diseases.

Specific downstream signaling events driven by TCRs are involved in nTreg development. Mice with loss-of-function mutations in Zap70 (zeta-associated protein of 70 kDa) have reduced thymic Treg production [85, 180]. The mutation in LAT (linker of activated T-cells), abolishing its recruitment of PLCγ1 (phospholipase Cγ1), causes complete loss of Treg cells in the thymus [98]. TCR-dependent, DAG-mediated signals by loss of DGKζ promotes the generation of nTreg from thymocytes [173]. In addition to the TCR, co-stimulatory CD28 signaling is also essential for nTreg cell differentiation. Mice fail to produce nTreg cells from thymocytes when deficient in CD28 or its ligands CD80 and CD86 [164, 188]. In TCR transgenic mice, CD28 signaling was also found involved in TCR-dependent Treg cell precursor generation [110]. It is possible that CD28 stimulation contributes to the survival of Treg cell precursors and amplifies TCR signaling to increase efficient FOXP3+ Treg cell progression.

During TCR-CD28 stimulation, several downstream transcription factors including NFκB, NFAT, and AP-1 play critical roles in FOXP3 induction and

nTreg cell maturation. NFAT and AP-1 can bind directly to the promoter region of *Foxp3* [122]. Foxo1 and 3 also bind to the *Foxp3* promoter as well as an intronic regulatory element at the conserved noncoding sequence 2 (CNS2) of the *Foxp3* locus [140]. The *Foxp3* CNS can also interact with CREB-ATF-1 [94]. All these factors help to promote Foxp3 transcription and expression. Mice with mutations in PKC-θ, TAK-1, CARMA1, or IKKβ have reduced numbers of thymic Treg cells [12, 54, 74, 174, 204], suggesting the requirement of NFκB activation in Treg cell selection. The NFκB family member c-Rel is shown to link TCR stimulation and the opening of the *Foxp3* locus. c-Rel homodimer or c-Rel-p50 heterodimer may bind to the conserved noncoding sequence 3 (CNS3) in the *Foxp3* locus, promoting Foxp3 transcription. The deletion of CNS3 or c-Rel causes defective thymic Treg cell development [87, 228]. It is proposed that the binding of c-Rel to CNS3 initiates chromatin remodeling, similar to the mechanism of c-Rel at the IL-2 locus [155]. Another possible model proposed for Treg differentiation is represented by enhanceosome formation. In the *Foxp3* promoter, there exists a Treg-specific enhanceosome, which composes of transcription factors including NFATc2, AP-1 and Smad. During the induction of Treg cells, c-Rel, together with p65, facilitates the formation of the enhanceosome at the promoter and distal enhancers in the *Foxp3* locus to switch on Foxp3 expression upon TCR stimulation [159]. In contrast to NFκB, PI3K-Akt signaling is inhibitory to nTreg cell development. Indeed, overexpression of a constitutively active form of Akt inhibits nTreg cell differentiation, whereas nTreg cell frequency increases with the inactivation of Akt by overexpressing an inactive form of PI3K [149]. Active Akt was demonstrated to phosphorylate Foxo1 and 3a thus inhibiting their association with the *Foxp3* promoter and CNS2 [91]. It seems that TCR-CD28 stimulation regulates nTreg cell development oppositely via NFκB and Akt. Importantly, it has been shown that the acquisition of a Treg cell-type gene expression profile, lineage stability, and full suppressive activity requires a specific CpG-hypomethylation pattern induced by T-cell receptor stimulation. This process, which begins in the thymus and continues in the periphery, can be fully established without a prior expression of Foxp3 at the CD4-single positive stage and represents an essential condition for priming thymocytes toward a regulatory phenotype. It has been shown that a stable hypomethylation pattern at the level of *Foxp3* intron 1 (corresponding to the *Foxp3* conserved noncoding sequence 2, CNS2), *Ctla4* exon2 and *Ikzf4* (encoding Eos) can be induced by TCR stimulation, as well as cytokine treatment (TGF-β or IL-2) and cell proliferation [135]. The CNS2 region seems to be fundamental for Treg commitment, as demonstrated by the reduction in Foxp3 transcription activity after deletion of the *Foxp3* CNS2 locus [161, 228].

Although being indispensable for Treg functionality, Foxp3 appears to intervene quite late during Treg cell development, given its ability to bind to pre-established enhancers in precursor cells, which are previously occupied by Foxp3 co-factors [165]. Interestingly, these pre-established enhancers are also identified as genes characterized by a hypomethylation status in Treg cells [135, 172]. The acquisition of a stable epigenetic pattern is necessary for the subsequent expression of Foxp3 and the maintenance of a stable and specific Treg signature. Indeed, the difficulty of generating highly stable Treg cells in vitro is mainly due to the inability of TGF-β

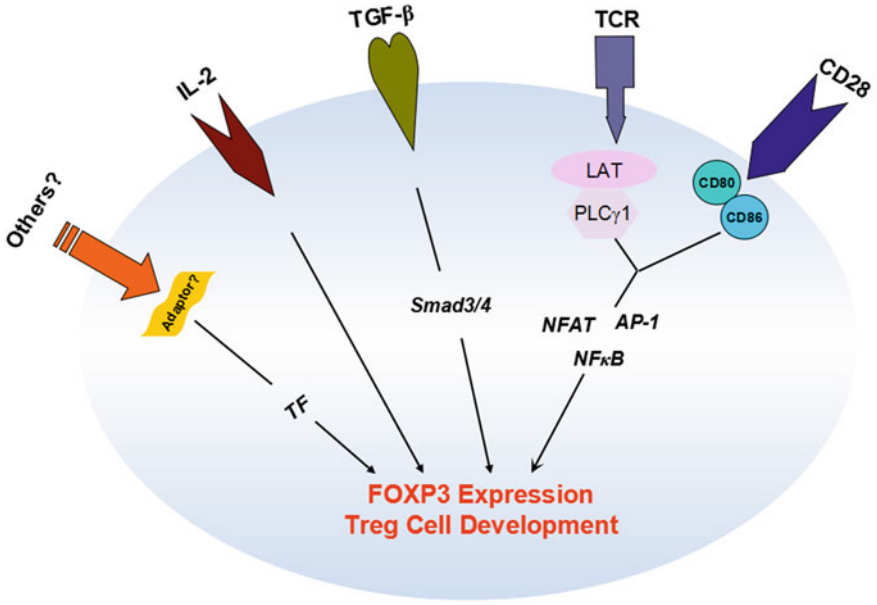

Fig. 4.1 The signaling pathways involved in Treg cell development. TCR and CD28 stimulation is essential for Treg cell development. TGF-β signaling is required for FOXP3 induction and iTreg differentiation. IL-2 pathway is critical for both nTreg and iTreg development. Other important signaling pathways may also help to stablize FOXP3 expression and promote Treg cell development. The key adaptors and transcription factors (TF) are indispensable in the process of Treg cell development

and/or retinoic acids to induce the hypomethylation status at the *Foxp3* CNS2 region that is typical of natural Treg cells [165, 207]. Taken together, the strength and timing of TCR signaling and other molecular events, even preceding Foxp3 expression itself, may be involved in the positive and negative control of nTreg cell fate (Fig. 4.1).

Gene expression is regulated at different levels by a complex network of molecular players. Recent studies have underlined the importance of microRNAs (miRNAs) and long noncoding RNAs (lncRNAs) in the differentiation of Treg cells. miRNAs are small ($\sim$22 nucleotides) noncoding RNAs targeting partially complementary sequences at the 3′-untranslated regions of mRNAs, leading to their degradation or prevention of translation and finally affecting protein expression [14, 73]. The importance of miRNAs in controlling Treg function is demonstrated by the onset of rapid fatal autoimmunity after Foxp3-Cre-mediated Dicer or Drosha ablation [34, 111], as both Dicer and Drosha are RNases involved in miRNA biogenesis [59].

Analysis of the human miRNA signature has revealed that two miRNAs, miR31 and miR21, play a crucial role in FOXP3 expression in natural Treg cells. By binding to the 3′-untranslated region of FOXP3 mRNA, miR31 negatively regulates FOXP3 expression, while miR21 can positively, albeit indirectly, regulate its

expression [158]. The microRNA miR155 is of particular interest, since Foxp3 has been shown to bind to an intron within the DNA sequence encoding the miRNA precursor Bic [125, 226]. During thymic differentiation, Foxp3 drives the expression of miR155, which in turn induces SOCS1 (suppressor of cytokine signaling 1) down-regulation, finally stimulating the Treg proliferative potential and conferring competitive fitness to Treg cells [116]. Attention has also been focused on the role of miR-146a, which allows Treg cells to maintain a proper expression level of STAT1, in order to repress Th1 immune responses while avoiding the differentiation into Th1-like IFNγ-secreting cells [117]. Recently, Liu and colleagues observed lower levels of miR15a and miR16 in cord blood (CB)-derived Treg cells compared to conventional T-cells, and found that over-expression of these two miRNAs led to inhibition of FOXP3 and CTLA4 expression concomitantly with a partial reversal of Treg-mediated suppression in an allogeneic mixed lymphocyte reaction. Interestingly, this also correlated with a reversal in the FOXP3 demethylation status in the CB-derived Treg cells [114]. Compared to miRNAs, the impact of lncRNAs on Treg cells function and differentiation is much less studied, although they are known to play fundamental roles in different cellular processes [86, 92, 115, 206].

LncRNAs are classified as RNA longer than 200 nucleotides that can regulate transcription through different mechanisms, for example by inhibiting the binding of transcription factors to DNA, by affecting the shuttling of transcriptional complexes between subcellular compartments or diverting miRNAs from binding to their target mRNAs [195]. By analyzing lncRNA expression in clinical active Crohn's disease (CD) patients, Qiao and colleagues found an increased expression of the lncRNA DQ786243 compared to clinical inactive CD subjects or healthy controls, and its expression strongly correlated with the expression of CREB (cAMP response element binding protein) [152], which is known to participate in FOXP3 expression [94]. Interestingly, after transfection of DQ786243 in Jurkat T cells, an increase in expression levels of CREB and FOXP3 were observed, as well as an increase of CREB phosphorylation ratio (phosphoCREB/totalCREB) [152]. Although the exact relationship between DQ786243, CREB and FOXP3 remains elusive, this may suggest a role for DQ786243 in Treg function.

4.3 iTreg Cells: Development and Characteristics

Despite the existence of nTreg cells, which develop in the thymus and hold reactivity against self-antigens, another type of regulatory T-cells, namely iTreg, can differentiate in the periphery from naïve CD4+Foxp3^{neg} T-cells, both under homeostatic conditions and in the presence of inflammation, infection and allergy [21, 42]. In vivo, the expansion of a CD25+ T-cell population was demonstrated after Ab-mediated depletion of CD25+ cells in thymectomized BALB/c recipients [104], although some doubts arose about the possible expansion of a residual population of pre-existing CD25+ cells. By using T/B monoclonal mice, which are

totally devoid of Treg cells, it was clearly shown that CD4+CD25+ cells could be induced from CD4+CD25− T-cells after adoptive transfer, upon conditions of homeostatic proliferation. Importantly, the induced T-cell subset holds phenotypical and functional characteristics of Treg cells, e.g., expression of Foxp3, CTLA4, GITR, CD103, anergy, and suppressive capacity [41]. Subsequently, it was shown that, in the absence of nTreg cells, Ag oral administration could induce tolerance in an experimental model of asthma, in concomitance with the appearance of a peripheral antigen-specific CD4+CD25+CD45RB^{low} population expressing Foxp3 and CTLA4 [130]. Interestingly, this conversion of naïve T-cells has also been shown after peripheral injection of foreign antigen upon suboptimal dendritic cell activation [100] and prolonged subcutaneous infusion of low doses of peptide by means of osmotic pumps [4].

The hypothesis of *de novo* induction of Treg cells in the periphery is supported by plenty of in vitro data, which highlight more in depth the mechanisms underlying Treg induction. TGF-β is a pleiotropic cytokine exerting different effects on the immune system, greatly depending on the cytokine microenvironment. Mice expressing a dominant negative TGF-β receptor on CD4+ T-cells are characterized by an autoimmune inflammatory phenotype associated with uncontrolled CD4+ T-cell differentiation into Th1 effector cells [44], demonstrating the importance of this cytokine in maintaining a state of immune tolerance. The first evidence regarding the importance of TGF-β in inducing the differentiation of suppressive cells came in 2002, when Zheng and colleagues showed that upon exposure to this cytokine, CD4+CD25− naïve T-cells could express CD25 and CTLA4, a reduced production of pro-inflammatory cytokines and suppressed autologous antibody production [223]. At that time, Foxp3 was not yet known as the master regulator of Treg cells.

After the landmark discovery of Foxp3 by Sakaguchi et al. [82], Chen and colleagues revealed that induction of CD4+ suppressive cells could be obtained upon TGF-β stimulation [32]. In their system, freshly isolated CD4+CD25− naïve T-cells cultured with anti-CD3 and APCs in the presence of TGF-β showed induction of Foxp3 and CD25 expression, intracellular CTLA4 accumulation and suppression of the proliferation of CD4+CD25− responder T-cells, in association with an increase in surface-active TGF-β. A substantial confirmation to this theory arrived with the generation of a mouse model in which a red fluorescent reporter was knocked-in into the *Foxp3* locus [203], which directly linked TGF-β stimulation of CD4+CD25− naïve T-cells with the induction of Foxp3 expression and the acquisition of a suppressive phenotype. TGF-β stimulation seems to initiate a positive feedback loop involving induction of Foxp3 in association with an increase in Smad3/4 and a decrease in Smad7, respectively positive and negative regulators of TGF-β signaling [178]. This should render CD4+CD25− naïve T-cells highly susceptible to TGF-β-mediated regulatory effects and favor the induction of a Treg phenotype [52].

IL-2 has been shown to play a fundamental role in the maintenance of immune homeostasis, as demonstrated by the development of severe lymphadenopathy and autoimmunity in IL-2-deficient mice [162, 163]. It signals through the high-affinity IL-2R (IL-2 receptor), consisting of three subunits: the α-chain (IL-2Rα, or CD25),

the β-chain (IL-2Rβ, CD122) and the common cytokine receptor γ-chain (CD132) [133]. The fact that Treg cells express high levels of all the three components of the receptor (particularly CD25), clearly underlines the importance of IL-2 signaling for the development and maintenance of a proper regulatory phenotype. Although seeming dispensable for the development of nTreg cells in the thymus [58, 60], IL-2 is critical for the in vitro induction of Treg cells. The use of anti-IL-2 antibodies demonstrated that, by acting in concert with TGF-β, IL-2 plays a crucial role for the induction of Foxp3 and the acquisition of suppressive capacity [225]. Interestingly, CTLA4, known to be fundamental for the activity of Treg cells, was also found to be necessary for the induction of Foxp3 in naïve T-cells in the presence of TGF-β [224], although the mechanism is not fully understood (Fig. 4.1).

A potential weapon against autoimmune diseases could come from the induction of Treg cells by retinoic acid (RA). This molecule, a natural active metabolite of Vitamin A, is a potent modulator of the immune system already known to have beneficial effects in different models of autoimmune and inflammatory diseases [154, 231]. Upon CD3/CD28 stimulation, the capacity of all-trans retinoic acid to assist TGF-β in converting naïve T-cells into Foxp3+ cells with potent suppressive activity has been clearly documented [205]. A more in-depth analysis of the related mechanism showed that the RA-induced increase in the suppressive capacity of the T-cells is accompanied by intense modifications of histones at the post-translational level. In particular, RA treatment enhances histone methylation at CNS2 (conserved non-coding DNA sequence 2) of *Foxp3* and boosts the acetylation of the TSS (transcription start site) of the *Foxp3* gene [118]. The modulation of the epigenetic status by RA could promote Foxp3 induction and help in maintaining the TGFβ-induced suppressive phenotype. However, RA does not modify the methylation pattern of the CpG islands in the CNS3 region of the *Foxp3* locus [118], which has been shown to be fundamental for the stability of Foxp3 in nTreg and in in vivo-induced Treg cells [55, 94]. The high-methylation status observed in the TSDR of in vitro-induced Treg cells [3, 13, 193] could account for the diminished stability of Foxp3 expression and of the suppressive capacity that distinguishes iTreg from nTreg cells.

A key factor influencing the induction of Foxp3+ cells from naïve T-cells is represented by the characteristics of TCR stimulation. Interestingly, the induction of Treg cells is favored in the presence of subimmunogenic conditions of stimulation, particularly by low doses of high-affinity antigens [100, 130]. Indeed, although low affinity antigens can be effective in inducing Foxp3, this effect is only temporary and they cannot guarantee the generation of a persistent population of Foxp3+ cells. The optimal condition seems to be represented by a low density of a high-affinity ligand in order to achieve the induction of a stable Foxp3+ population. Regardless, for the conversion into Foxp3+ cells, a degree of stimulation below the threshold required for the expansion of conventional cells is required [68]. These considerations underline that, besides the cytokine microenvironment, an adequate strength of interaction between the TCR and the MHC/peptide complex plays an irreplaceable role in the induction of Treg cells.

Recently, it has been proposed that, at the beginning of Treg differentiation, a weak TCR stimulation can trigger an increased activation of the Akt-hydrophobic

motif-specific phosphatase PHLPP1 and consequently a transient reduction in the activation of Akt. Therefore, Treg and effector T (Teff) cells may share a similar pattern of differentiation, with slight but significant differences in the timing and intensity in the activation of the PI3K/Akt pathway [50]. The role of the PI3K/Akt pathway in Treg cell induction is still under investigation. Indeed, although some papers underline an antagonizing role for Akt in Treg cell induction [141, 148], the reduced generation of Treg cells upon PDK1 deletion and the enhancement of their generation under stable transgenic expression of Akt clearly state the importance of the PI3K/Akt pathway for Treg cells induction, at least in vitro. Although its role is not fully understood, the PI3K/Akt pathway certainly represents a central node for the fate of iTreg cells requiring further investigation.

Foxp3 is an essential factor for the proper development of Treg cells, but its presence alone is insufficient for the acquisition of a stable Treg phenotype as human naïve T-cells can readily express FOXP3 upon TCR stimulation without acquiring any suppressive capacity [63]. Indeed, many other molecules play a fundamental role for Treg activity and characterize the phenotype of fully active suppressive cells. CTLA4 has a striking effect on T-cell suppressive activity, as demonstrated by the loss of immunological tolerance after delivery of anti-CTLA4 mAbs [29] and the development of fatal lymphoproliferative diseases in CTLA4 deficient mice [192]. Moreover, it acts as a primary element for Treg induction, as it is required for TGF-β to induce Foxp3 and generate suppressor cells [224]. Similarly, CD25, GITR and CD103 are considered canonical markers for identifying suppressor cells when expressed in concomitance with Foxp3. Although being fundamental for their function, these markers cannot be used to distinguish iTreg from nTreg cells, being expressed by both subsets. Recent studies have shown that Nrp1 (CD304) may represent the most reliable marker for distinguishing nTreg from iTreg cells [208, 215]. Weiss and colleagues showed that Foxp3+ Treg cells isolated from secondary lymphoid organs of wild-type mice are comprised of two distinct populations based on the degree of expression of Nrp1. Interestingly, in the Nrp^{high} subset the expression of this marker is associated with high expression of Helios (considered to be a nTreg marker), while the Nrp^{low} subset is characterized by the absence of Helios and high levels of death-associated protein-like 1 (DAPL-1) [208].

Based on the distinction via Nrp1, Yadav and colleagues [216] were able to analyze the TCR repertoire of thymic Tregs and peripheral Tregs, revealing that there exists a limited overlap in the CDR3 amino acid sequences between the two subsets (9.1 %), further emphasizing their different lineage development and specificity while accrediting the hypothesis of Nrp1 as a marker for distinguishing the two subsets. Thus, research on a combination of multiple molecules rather than a single chief marker seems to be the solution for distinguishing nTreg from iTreg cells, but further studies are required to confirm these hypotheses and to unveil the functional significance of the observed phenotypic differences.

Increasing evidence indicates the existence of a strong connection between Treg cells in the periphery and the intestinal microbiota. In particular, the presence of high numbers of Treg cells in the colonic lamina propria [121] and the impairment of their function in germ-free mice [139] clearly defines a main role for intestinal

microorganisms in shaping T-cell development toward a regulatory phenotype. Recently, Honda and colleagues [7] have shown that 17 species of Clostridium synergistically induce the differentiation of a Foxp3+ Treg population likely through the release of bacterial antigens, the production of short chain fatty acids (SCFAs, as acetate, propionate, butyrate, and isobutyrate) and by eliciting the production of TGF-β1 from intestinal epithelial cells. Notably, oral administration of the 17 strains into specific pathogen free (SPF) adult mice had a protective effect on OVA-induced allergic diarrhea and trinitrobenzene sulphonic acid (TNBS)-induced colitis.

Despite recent and quick advances in the field of Treg biology, a more in-depth understanding of the mechanisms of induction, their specificity and markers could help in designing targeted therapies against inflammatory and autoimmune diseases.

4.4 Mechanisms of Suppression

Independently from their origin and development, the main characteristic of regulatory T-cells is certainly their capability to suppress the activation of the immune system. Given that most works aimed at explaining the suppressive potential of Treg cells focus on nTregs, the existence of differences in the mechanisms of action between nTreg and iTreg cells is not yet clear.

Treg cells constitutively express CTLA4, and the importance of this molecule as a key factor for Treg-mediated suppression is demonstrated by the abrogation of Treg protective effect in a murine model of colitis upon CTLA4 blockade [156] and the development of fatal autoimmune diseases in mice after specific ablation of CTLA4 in Treg cells [212]. As it has been demonstrated, Treg cells can induce a decrease in the expression of the co-stimulatory molecules CD80 and CD86 on dendritic cells in a CTLA4-dependent manner, thus reducing the activation and proliferation of naïve T-cells both in vitro and in vivo [137]. Moreover, engagement of CD80/CD86 by CTLA4 also induces the activation of the immunoregulatory pathway of tryptophan catabolism [70] leading to the expression of indoleamine 2,3-dioxygenase (IDO), an enzyme notoriously involved in the generation of suppressive cells in the periphery both under physiological [51] and pathological [43] conditions.

The GITR-related gene is a member of the tumor necrosis factor (TNF) receptor superfamily (TNFRS18), which is highly expressed on CD4+CD25+Foxp3+ nTreg cells and is expressed at low levels in CD4+CD25−Foxp3− effector cells after activation [126, 221]. Although its crucial role for the maintenance of the immune balance is well established, its contribution to Treg function and activity is still under debate, as it was demonstrated that Tregs from both $GITR^{+/+}$ and $GITR^{-/-}$ mice retain their suppressive activity [157]. Engagement of GITR through anti-GITR mAbs was shown to abrogate CD4+CD25+ Treg-mediated suppression, both in vitro and in vivo [126, 179], and GITR-L has been shown to provide CD4+CD25− cells signals in order to render them resistant to CD4+CD25+-mediated

suppression at the beginning of immune responses [184]. Interestingly, GITR co-stimulation can break the anergic state of Tregs inducing hyperproliferation without affecting their regulatory function [106]. Thus, although being fundamental for the activation of Treg cells, GITR involvement in Teff activation and its expression on both subsets [177] does not allow its use as a marker for the identification of Treg cells.

Interestingly, similarly to CTLA4, GITR ligation to its ligand (GITR-L) expressed on plasmacytoid DCs can activate a reverse signaling inducing IDO expression through the noncanonical NFkB pathway [71] thus favoring the induction of a tolerogenic environment. Therefore, expression of CTLA4 and GITR on Treg cells can establish a positive feedback loop able to further sustain the expansion of the Treg population.

Another mechanism through which CD4+CD25+ Treg cells are proposed to exert their suppressive activity is through the production of TGF-β. CD4+CD25+ Treg cells express high levels of bound or soluble TGF-β depending on the type and strength of TCR stimulation [131] and, in both mice and humans, in vitro blockade of TGF-β through recombinant latency-associated peptide of TGF-β1 (rLAP) reverses the inhibitory effects of CD4+CD25+ Treg cells on CD4+CD25− T-cell proliferation. Moreover, in a SCID mouse model of colitis, a protective effect is achieved upon transferring wild-type CD4+CD25+ but not TGF-β1-deficient CD4 +CD25+ T-cells [132]. However, the real weight of TGF-β in controlling the magnitude of regulatory responses is still controversial, as recent works highlighted that deficiency of the TGF-β receptor (TR2) on CD4+ T-cells induces a non-lethal form of colitis without leading to autoimmunity or multi-organ inflammation [181]. However, TGF-β surely has a pivotal role in the maintenance of the immune balance being directly involved in the induction of regulatory T-cells.

Interleukin-10 (IL-10) is a pleiotropic cytokine produced both by the innate and the adaptive branches of the immune system, which has a central role in protecting from infectious, autoimmune and allergic diseases [18, 20, 64, 75, 105]. By generating mice with selective knockout of IL-10 in Foxp3-expressing cells (IL-10 fl/fl x Foxp3-cre), Rubtsov and colleagues demonstrated that, although not being fundamental for the control of systemic autoimmunity, IL-10 is essential in keeping a state of immune tolerance at mucosal surfaces, such as the colon, lungs, and skin [160]. The importance of IL-10 in the control of autoimmunity has been also revealed by a recent paper, which demonstrated that protection from type 1 diabetes (T1D) was related to the presence of Foxp3+ICOS+IL-10-producing T-cells in the pancreatic islets [99]. The abundance of regulatory T-cells at mucosal surfaces of the intestinal tract might be explained, at least in part, by the presence of indigenous Clostridia, which are involved in the induction of Foxp3+ IL-10+ suppressive T-cells [6]. Although Foxp3+ T-cells can secrete IL-10, this is considered a key feature of another subset of suppressive CD4+ T-cells, namely Tr1, which lack Foxp3 and are characterized, both in human and in mouse, by the expression of CD49b and LAG3 [62]. Similarly to Foxp3+ T-cells, induced-Tr1 cells display a low proliferative capacity following TCR stimulation and strong in vitro suppressive function toward allogeneic CD4+ T-cells. Their ability to produce large amounts of IL-10 and small

quantities of TGF-β [72] determines their capacity of inhibiting the proliferation of Teff cells and the production of IL-2 and IFN-γ [2].

IL-35 is a heterodimeric IL-12 family member composed of the Epstein-Barr virus-induced gene 3 (Ebi3) and the IL-12α (p35) subunits, firstly described in 2007 by Vignali and colleagues [37]. Mouse Foxp3+ Treg cells but not resting or activated Teff cells were shown to produce large amounts of IL-35, which was partially responsible of their suppressive activity, since $Ebi3^{-/-}$ and $IL\text{-}12\alpha^{-/-}$ displayed reduced regulatory activity and failed to cure IBD (inflammatory bowel disease) in vivo [37]. CD4+CD25− T-cells can be converted into IL-35-producing cells through IL-35 treatment. Although iTr35 (induced Treg35) cells do not seem to require Foxp3 for their differentiation and function, they are effective in restoring immune homeostasis in $Foxp3^{-/-}$ mice, in preventing the onset of EAE (experimental autoimmune encephalomyelitis) and IBD (inflammatory bowel disease) and in blocking the anti-tumor response after injection of B16 melanoma cells [38]. IL-35 may represent an important and additional mechanism adopted by the immune system for maintaining immune homeostasis, but further studies are required to clarify its role, particularly in humans.

The high levels of expression of CD25 (IL-2R) by regulatory T-cells prompted some research groups to hypothesize a mechanism of "competition" for IL-2, which would deplete IL-2 availability in the microenvironment and initiate a Bim-dependent apoptotic process, finally leading to an inhibition of Teff cell responses [144]. However, discrepancies regarding this model still exist, as results from another group showed, both in vitro and in vivo, that the absence of Bim-mediated apoptosis has no effect on Treg-mediated suppression of Teff cells [187]. Moreover, the specific effects mediated by IL-2 could be strictly dependent on the local microenvironment. For example, upon *Candida albicans* infection, IL-2 consumption by Treg cells boosts Th17-mediated anti-fungal activity, thus leading to the clearance of infection [145].

The role of cAMP (cyclic AMP) in inhibiting the differentiation, proliferation, and the production of Th1-and Th2-associated cytokines is already established [22]. A peculiar way of suppression mediated by nTreg cells is represented, at least in mice, by the conveyance of their intracellular cAMP to CD4+ activated T-cells via the protrusion of gap junctions (GJs), connexin membrane proteins which can vehicle cAMP very efficiently [16, 17, 23]. Suppression through cAMP is cell-contact dependent, thus a close proximity between the donor and the receiving cell is required in order to achieve the transferring of the second messenger. Similarly, CD4+CD25+Foxp3+ T-cells can express CD39 and CD73, ectonucleosidases involved in the production of adenosine (ADO) from exogenous ATP/ADP, which, by binding to G-protein coupled receptors on responder T-cells, lead to an increase in the intracellular levels of cAMP and to the suppression of Teff responses [45]. This mechanism of suppression seems to be strongly represented in the tumor microenvironment, where Treg activity can suppress anti-tumor immunity thus favoring the development of cancer.

One of the most intriguing issues in Treg research is represented by their ability to suppress different types of effector responses. Some clues concerning Treg

plasticity come from recent studies analyzing the expression of chemokine receptors and Th-associated markers in ex vivo human CD4+CD45RO+CD25+CD127^{low}FOXP3+ memory Treg cells [48]. By using this strategy, distinct subsets of Treg cells, each mimicking a specific Th phenotype, have been identified. For example, Th17-like Treg cells represent a suppressive subpopulation of FOXP3+RORγt+IL-17+ T-cells expressing CCR6 and CCR4 but lacking CXCR3 and CCR10, while Th1-like Treg cells, expressing T-bet and IFN-γ, show high levels of expression of CXCR3. While the first population holds all the characteristics to migrate to the sites of Th17-mediated inflammation, the second should be able to specifically suppress Th1-mediate effector responses. Notably, all the Th-like Treg subsets retain suppressive activity and the ability to produce IL-10. What emerges from these observations is that Treg cells are perfectly equipped for adapting to different inflammatory environments and to fine-tuning suppressive responses depending on the local milieu.

4.5 CD4+CD25+Treg Cells in Health and Disease

The GALT (gut-associated lymphoid tissue) represents the largest part of the immune system, in which a strong interplay between the indigenous microflora and immune cells takes place. Indeed, about 10^{12} bacteria/g of stool and approximately 10^{12} lymphoid cells/meter of small intestine are present at this mucosal interface [120, 127]. Colonic Treg cells have been shown to express TCR repertoires specific for the colonic microflora [103], thus sustaining the hypothesis of a role for the commensal bacteria in shaping the immune system. This mechanism appears to be of primary importance for preventing the induction of T effector responses against commensal bacterial antigens, which could lead to the development of inflammatory diseases such as colitis and IBD. Recently, Clostridium clusters IV and XIVa, representing the spore-forming fraction of mouse indigenous microflora, have been shown to induce the expression of Foxp3, ICOS, and IL-10 in CD4+ naïve T-cells via a TGFβ-dependent mechanism and to be protective toward the development of colitis [7]. NMR-based metabolome analysis has revealed that luminal concentrations of SCFAs (short chain fatty acids) positively correlates with the number of Treg cells in the colon. Particularly, butyrate appears to be the main inducer of Treg cells both in vitro and in vivo. Besides promoting the expression of Foxp3, this compound increases genome-wide histone H3 acetylation at both the promoter and at the CNS3 (conserved noncoding sequence 3) [61] and CNS1 (conserved noncoding sequence 1) [5] of the *Foxp3* gene locus, probably enhancing the accessibility of transcriptional regulators to the enhancer elements and the promoter region of the *Foxp3* gene. Similarly, propionate (another SCFA holding histone deacetylase inhibition) potentiates extra-thymic Treg generation both directly and by promoting the ability of dendritic cells to sustain their differentiation [5]. However, the idea that the majority of colonic Treg cells is composed of extra-thymic induced Treg cells has been challenged by recent findings showing that most of the Treg

cells in the colon share TCR repertoires with CD4+Foxp3+ thymocytes and might therefore originate in the thymus [28]. In humans, subjects carrying inflammatory diseases, such as IBD, harbor 25 % fewer bacterial genes compared to healthy individuals [153]. Given the role of microbiota in inducing Treg differentiation, it is becoming clear that dysbiosis of the gastro-intestinal tract might lead to an imbalance of the immune system, negatively affecting tolerance and favoring the development of inflammatory and autoimmune diseases.

The importance of Foxp3+ T-cells in healthy conditions is also demonstrated by the role played by this subset of lymphocytes during pregnancy. At the beginning of this physiologic process, a tolerant environment is needed in order to allow the implantation of the blastocyst in the maternal uterus. The number of circulating Foxp3+ suppressive T-cells increases during pregnancy [183], and extra-thymic generation of fetal alloantigen-specific Treg cells seems to take place at the beginning of pregnancy, at least in mice [166]. By tracking the influx of Treg cells in $Foxp3^{gfp}$ mice by in vivo 2-photon microscopy, Teles and colleagues observed an increase in the number of Treg cells in vaginal fluids during the estrus phase of the cycle, as well as failure of embryo implantation and increasing accumulation of CD8+ T-cells in the uterus upon Treg cell ablation [191]. Thus, the role of CD4 +CD25+Foxp3+ Treg cells as immunomodulators is pivotal not only for maintaining immune homeostasis and protecting the body from autoimmunity and inflammation, but also for allowing the successful occurrence of a fundamental physiologic process as pregnancy.

Autoimmune diseases comprise a large number of pathologies characterized by exacerbated immune responses against self-antigens. Although the mechanisms leading to these kinds of diseases are not fully understood, their multifactorial basis is almost clear. Indeed, both systemic and organ-specific autoimmune diseases seem to rely on genetic, infectious, and environmental predisposing factors [33]. However, the involvement of Treg cells in the development of some of these diseases has been evaluated in depth by many studies.

Type 1 diabetes (T1D) is an inflammatory-autoimmune disease characterized by inflammation and destruction of insulin-producing β cells of the pancreas. A link between T1D and the lack of Treg cells is already clear, as demonstrated by the increased susceptibility to the development of T1D in subjects suffering from IPEX [210]. An altered number of Treg cells does not appear to be the reason for the onset and development of diabetes, since most works have demonstrated that there is no difference between the number of Treg cells between T1D subjects and healthy controls in peripheral blood [25, 151]. Probably, both a decreased suppressive capacity of Treg cells and enhanced resistance to suppression of Teff cells represent the basis of the loss of self-tolerance observed in T1D. The pancreas of pre-diabetic NOD mice is characterized by an increase in the percentage of IFN-γ-producing "exFoxp3" cells, which, besides losing Foxp3 expression, display low levels of CD25 and high levels of CD127 [230]. FOXP3+Treg cells isolated from T1D patients display lower level of expression of GITR compared to healthy counterparts [214], although their suppressive capacity is not impaired. Notably, T1D-Treg cells

are more susceptible to apoptosis [65, 89, 214], which could account for the diminished number of GITR+ suppressive cells observed in T1D subjects.

Systemic lupus erythematosus (SLE) is the prototype of systemic autoimmune diseases. The disease mainly arises from a strong hyperactivation of autoreactive CD4+ T-cells followed by an aberrant expansion of B lymphocytes. Auto-antibodies targeting nuclear antigens are secreted, seriously damaging skin, joints, blood vessels, and the central nervous system (CNS). Glomerulonephritis can also occur, due to deposition of immuno-complexes in the kidney [39, 124]. Some works identified a resistance to suppression of responder T-cells as one of the immune features underlining the progression of SLE [199, 200], but increasing evidence indicates that deficiencies in the number and/or suppressive capacity of CD4+CD25^{high} Treg cells also play a pivotal role in the development of the disease [128, 196]. In mice models of SLE, as in the NZB/NZW F1, the frequency of CD4+CD25+ T-cells is reduced, albeit these cells express Foxp3 and exert suppressive activity [84], pointing out a role for these cells in the development of the autoimmune disease. In autoimmune-prone female NZB/NZW F1 mice, depletion of CD25+ T-cells by the PC61.5 anti-CD25 monoclonal antibody increases the production of IgG2a anti-nuclear antibodies, accelerating the development of glomerulonephritis [76]. In the New Zealand Mixed 2328 (NZM2328) mouse model, adoptive transfer of CD25+ T-cells from asymptomatic NZM2328 adult mice suppress the signs of autoimmunity at the level of prostate, thyroid and lacrimal glands, although it has no effect on the development of glomerulonephritis and sialoadenitis [9]. Conversely, adoptive transfer of purified and ex vivo expanded CD4+CD25+CD62L^{high} thymic-derived Treg cells is beneficial in decreasing the incidence of renal failure in the NZB/NZW F1 lupus-prone mice [171]. Data related to humans are even more contradictory. Indeed, while some studies observed a decrease in the percentage of CD4+CD25+ Treg cells in SLE patients [11, 119, 196], others reported similar [200, 220, 222], or increased levels of CD4+CD25+ Tregs [107, 185, 217] between SLE and healthy subjects. Interestingly, regulatory T-cells from SLE affected subjects poorly respond to RA stimulation in the presence of TGF-β when compared to healthy subjects, suggesting that the TGF-β/RA signaling might be defective in lupus T-cells [182].

In cancer, an increasing amount of pre-clinical and clinical data clearly indicate that the increase in the frequency of Treg cells strongly correlates with a poor prognosis, probably due to the impairment of anti-tumor responses against cancer cells. Indeed, a shorter survival correlates with high numbers of CD4+CD25 +FOXP3+ T-cells within the tumor or the peripheral blood in breast and ovarian cancer [46, 69], hepatocellular carcinoma [108], glioblastoma multiforme [202], renal cell carcinoma [176], myeloid leukemia and lymphoma [218, 219], and melanoma [201]. However, a high number of Treg cells are associated with a favorable prognosis in other type of tumors, particularly in colorectal cancer [101], probably due to a dampening in bacteria-induced inflammation, which can drive the development of this type of cancer [209]. The negative impact of CD4+CD25 +Foxp3+ Treg cells on anti-tumor immunity is shown by the improvement of survival and the reduction of metastasis achieved by blocking Treg function through anti-CD25 antibodies [138]. Whether Treg cells in cancer consist of nTreg

or iTreg cells is still unclear. Indeed, it has been demonstrated that induction of CD4+CD25+ suppressive cells can occur in thymectomized and CD25−depleted tumor-bearing mice, thus excluding mechanisms of recruitment and/or proliferation of pre-existing Treg cells [197], and that this mechanism could be TGF-β-dependent [113]. In ovarian cancer, Treg cells (presumably nTreg cells) have been shown to be recruited to the tumor site in a CCL22-mediated fashion, CCL22 being produced by both tumor cells and tumor-associated macrophages [40]. The finding that TCR repertoires of antigen-experienced Tconv (conventional) and Treg cells in tumor-bearing mice are largely nonoverlapping [78] may suggest that the suppressive cells present in the tumor microenvironment are mostly of thymic origin, as a greater degree of overlap should have been observed if the Treg cells had originated from the CD25− subpopulation. Of course, the two possibilities do not exclude each other, and the most realistic scenario is the concomitant induction of Treg cells as well as the recruitment of nTreg cells in the tumor. This possibility has been explored by Zhou and colleagues, who reported that both the simultaneous expansion of the nTreg population and the *de novo* generation of the Treg population contribute to tumor tolerance [229]. A more in-depth understanding of the mechanisms involved in tumor tolerance will be of primary importance for the development of anti-cancer therapies targeting regulatory T-cells.

4.6 Application of Treg Cells in Therapy: Clinical Perspectives and Technical Challenges

More than 200 clinic trials related to the use of Treg cells for curing different kind of diseases have been registered since 2002, mostly focusing on the treatment of hematological diseases (GvHD) and autoimmune diseases (allergies, atopic dermatitis and Crohn's disease), transplantation (solid organ transplantation and HSCT) and inflammatory diseases (ClinicalTrials.gov Identifiers: NCT01795573, NCT01830673, NCT01988506, NCT02060318, NCT01446484). Given the deleterious role of Treg cells in most type of cancers [10], clinical trials in this field are mainly based on the infusion of Treg-depleted T lymphocytes in order to boost the immune system against the tumor (NCT01513109, NCT00675831).

Graft versus host disease (GvHD) represents the major cause of morbidity and mortality after hematopoietic stem cell transplantation (HSCT) [67]. The first clinical trial aimed to evaluate the effect of Treg treatment in GvHD is quite recent [194] and was conducted on two subjects. Results reported a significant alleviation of symptoms only in the patient characterized by a chronic GvHD, while in the case of a severe grade IV acute GvHD no improvement was achieved. Different Phase I/II clinical trials carried out since that moment, one in the US and the other one in Italy, have demonstrated the efficacy of Treg-based therapies in improving the incidence of GvHD in a cohort of patients previously subjected to HSC transplantation. In the US study, umbilical cord blood (UCB)-derived Treg cells were infused after

nonmyeloablative double UBC-transplantation in 23 patients considered at high risk of developing GvHD [24]. Isolated umbilical Treg cells, which were activated by anti-CD3/CD28 stimulation in the presence of recombinant human IL-2, were infused in the patients at a concentration of 0.1−30 × 105 Kg and were detectable for at least 14 days, peaking at day +2. The 23 Treg-infused patients displayed a significant reduction in the incidence of GvHD compared to 108 identically treated historical controls (43 % vs. 61 %, $p = 0.05$) without showing thereafter any sign of deleterious infection, relapse or early mortality. In the Italian study, 28 patients with high risk of hematological malignancies who underwent HLA-haploidentical HSCT were infused with freshly purified and expanded CD4+CD25+ Tregs followed by Tconv cells to enhance immune reconstitution [47]. Interestingly, only 2 of the 28-treated patients developed acute GvHD, corresponding to the subjects receiving the highest dose of Tconv (2×10^6 Tconv/Kg). The remaining 26 patients did not develop any sign of GvHD, displayed an improved immunity to opportunistic pathogens and the infusion did not weaken the graft-versus-leukemia effect. Notably, no immunosuppressive therapy was administered after HSCT, demonstrating the efficacy and safety of Treg adoptive transfer, at least in the clinical settings described above.

Few studies have focused on Treg-based treatment in solid organ transplantation (SOT). One of these trials is evaluating the induction of liver transplantation tolerance after injection of donor alloantigen-specific CD4+CD25+ Tregs from peripheral blood after liver transplantation (ClinicalTrials.gov Identifier: NCT01624077), while the other is assessing the influence of autologous Treg injection on the prevention of organ rejection in children with kidney transplants (ClinicalTrials.gov Identifier: NCT01446484). However, both trials are still ongoing and more time will be needed in order to obtain sufficient data toward an outcome. The ONE Study consortium (www.onestudy.org), made up of academic and industrial partners, aims to identify the most promising results and regulatory cell products for further improving research and clinical trials in the field of solid organ transplantation. Other clinical trials still under investigation in Italy need to be mentioned for their contribution to the field of Treg-based immunotherapies, although they are focusing on a peculiar type of Treg cells, namely Tr1, producing IL-10 but not expressing FOXP3 [8].

Interesting results from a clinical trial aimed to evaluate the improvement of Type 1 Diabetes (T1D) by treatment with Treg cells have been published [123]. In this study, 10 T1D children were administered with autologous Treg cells ($10–20 \times 10^6$ Tregs/Kg). In this trial, autologous CD3+CD4+CD25^{high}CD127-lin-doublet-Treg cells were sorted using a combined immunomagnetic/fluorescence-activated technique achieving a final purity of ∼100 % and a good manufacturing practice (GMP)-adapted fluorescence-activated cell sorter (FACS) equipped with single-use sample lines in order to avoid any risk of contamination. Sorted cells were activated by anti-CD3/CD28 stimulation in the presence of recombinant human IL-2 and expanded for no more than 2 weeks. Compared to non-treated controls, in the Treg-treated subjects a decrease in the need of exogenous insulin was observed, with two patients completely remitting. Moreover, blood analysis of the Treg-treated patients revealed a decrease in the levels of HbA1c and a long-lasting increase in the percentage of Treg

cells. Despite the number of subjects involved in the study and the short-term of the follow-up place some limits to its strength, this trial proves at least the safety and the feasibility of Treg-cell adoptive therapies. Another clinical trial (ClinicalTrials.gov Identifier: NCT01210664), which is estimated to be completed in 2016, is evaluating the safety and feasibility of intravenous infusion of ex vivo selected and expanded autologous polyclonal Treg cells in T1D patients. From the start of the study, the three cohorts of subjects involved, receiving respectively a dose of cells of 0.05×108, 0.4×10^8 and 3.2×10^8 cells, will be followed over 5 years in order to assess the safety of the Treg immunotherapy. Although the results of these clinical trials are still uncertain, they appear of pivotal importance for further developing Treg-based immunotherapies and applying this kind of approach to other types of diseases. The above reported studies allow researchers to better understand the role of several factors influencing the outcome of these therapies in humans.

The manufacturing of cells prior to their utilization is certainly a critical parameter, and technical issues still need to be overcome in order to further optimize and standardize T-cell-based therapies.

The first steps during cell preparation are the isolation of Treg cells and their expansion. The protocol for the isolation of Treg cells from PBMCs (peripheral blood mononuclear cells) or umbilical cord blood is based on the expression of the surface markers CD4 and CD25 through a two-step activated cell sorting (MACS) process, which has been proposed to be carried out following GMP (good manufacturing practice) procedures [80, 150]. However, as CD25 is also found on conventional T-cells after activation, the combination of CD4+CD25^{high}CD127^{low} is considered the best in order to avoid the risk of contamination with Tconv cells [112]. Moreover, taking into account the expression of CD45RA it is possible to distinguish naïve from antigen-experienced (CD45RA+ vs. CD45RA-) Treg cells [129]. Given the necessity of considering as many surface markers as possible for their isolation, so far the best approach is based on a fluorescence-activated cell sorting (FACS) protocol, albeit no GMP guidelines exist yet for this kind of procedure. Of course, FOXP3 expression needs to be taken into account when assessing the purity of the isolated T-cells, but, obviously, it cannot be used for their separation being FOXP3 an intracellular marker. The subsequent step involves the activation of the Tregs in order to expand them and obtain a number of cells suitable for clinical application. Subsequent stimulation with anti-CD3/CD28-coated beads in the presence of rhIL-2 allows to achieve a good number of cells and is used for polyclonal expansion often in combination with rapamycin, which allows the expansion of suppressive CD4+CD25+FOXP3+ Treg cells while inhibiting the growth of CD4+CD25− cells, highly sensitive to the anti-proliferative effect of this drug [15]. For a schematic representation of Treg isolation and handling for clinical application, please refer to Fig. 4.1.

A big challenge for Treg manufacturing is represented by the possibility to cryopreserve PBMCs or Treg cells before/after expansion, which would allow a better planning of Treg infusion during treatment. However, even small differences during the handling of these cells have shown to heavily affect their viability and biological properties. Some studies have highlighted a decrease in the yield of Treg

cells from cryopreserved PBMCs probably due to an increase in their fragility and susceptibility to apoptosis [49, 175]. Moreover, cryopreservation of PBMCs has shown to significantly affect the subsequent detection of Treg-associated surface markers by FACS [169]. Other strategies are based on the direct cryopreservation of Treg cells, both before and after expansion. For example, Peters and colleagues have developed a clinical grade Treg isolation protocol in accordance with GMP guidelines consisting of a CliniMACS enrichment of CD4+CD25^{high} Treg cells followed by depletion of the CD19+ and CD127+ contaminating cells. After freezing for up to 1 year in liquid nitrogen, the isolated Treg cells, showing a viability of 70–80 %, partially lost their suppressive activity, which could be restored by either expansion with fully HLA-mismatched allogeneic PBMCs or anti-CD3/CD28-coated microbeads. The strategy of freezing already expanded Treg cells from UBC has been adopted in a clinical trial for the treatment of GvHD [24]. In this case, although the viability after thawing was >50 %, an increase in the frequency and absolute number of Treg cells in the peripheral blood of the infused patients was not observed compared to the infusion of freshly isolated cells (Fig. 4.2).

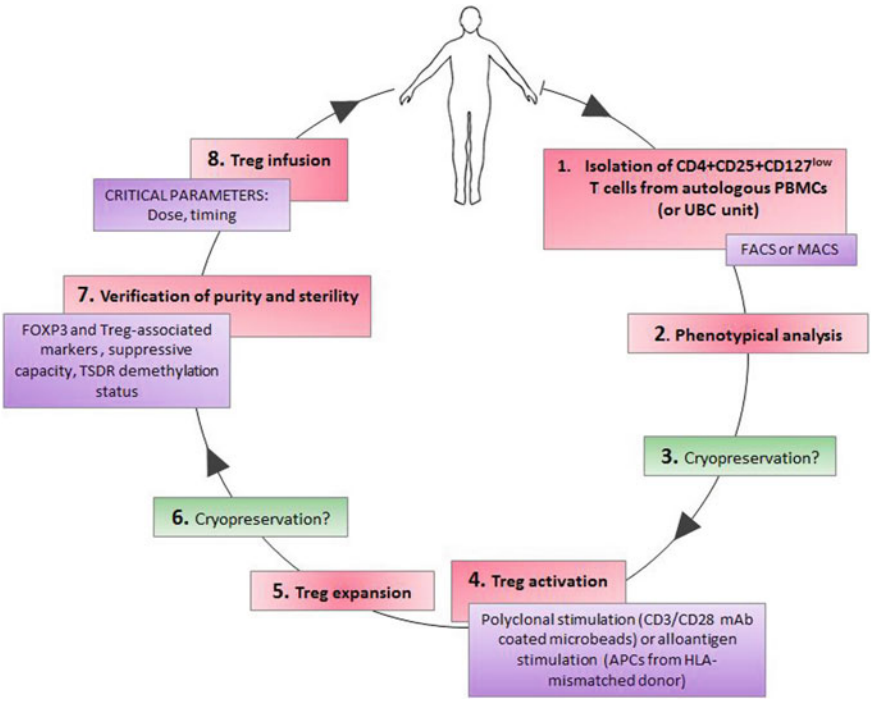

Fig. 4.2 Schematic representation of Treg manipulation for immunotherapy. Regulatory T-cells are isolated from autologous peripheral blood mononuclear cells (PBMCs) or umbilical cord blood (UBC) through MACS or FACS. After phenotypical analysis has been performed, Treg cells are activated and expanded by different kind of strategies. Treg purity, potency, and sterility are checked before infusion into the patient. At different stages, Treg cells might be cryopreserved for a further optimization of the protocol

The examples treated in the first part of this section show that clinical trials in the field of regulatory T-cells are promising. However, technical barriers still exist concerning Treg isolation and manufacturing. For example, improvements in the techniques of cryopreservation would allow an optimization of the timing for clinical infusions and guarantee a better planning of the therapy.

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Chapter 5
Th17 Differentiation and Their Pro-inflammation Function

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Abstract $CD4^+$ T helper cells are classical but constantly reinterpreted T-cell subset, playing critical roles in a diverse range of inflammatory responses or diseases. Depending on the cytokines they release and the immune responses they mediate, $CD4^+$ T cells are classically divided into two major cell populations: Th1 and Th2 cells. However, recent studies challenged this Th1/Th2 paradigm by discovering several T-helper cell subsets with specific differentiation program and functions, including Th17 cells, Treg cells, and Tfh cells. In this chapter, we summarize the current understanding and recent progresses on the Th17 lineage differentiation and its effector impacts on variety of inflammatory responses or disease pathogenesis.

Keywords T-cell differentiation · Th17 cells · IL-17 · IL-22 · Inflammation

5.1 Introduction

Upon various pathogens or injuries challenging, our body initiates both innate and adaptive inflammatory responses to protect us from infection or insult. $CD4^+$ T helper cells are critical components for appropriate establishment of adaptive immune responses. After engagement of T-cell receptor (TCR) and co-stimulatory molecules, naive $CD4^+$ T cells differentiate into different effector T helper cells under the control of distinct cytokines produced by particular pathogens or injuries activated antigen presenting cells (APCs). Over 25 years, Mosmann and Coffman's Th1/Th2 paradigm in $CD4^+$ T helper cells development shapes our view of the landscape of adaptive immunity [189]. Although this paradigm helps us a lot for

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B. Sun (ed.), *T Helper Cell Differentiation and Their Function*,
Advances in Experimental Medicine and Biology 841,
DOI 10.1007/978-94-017-9487-9_5

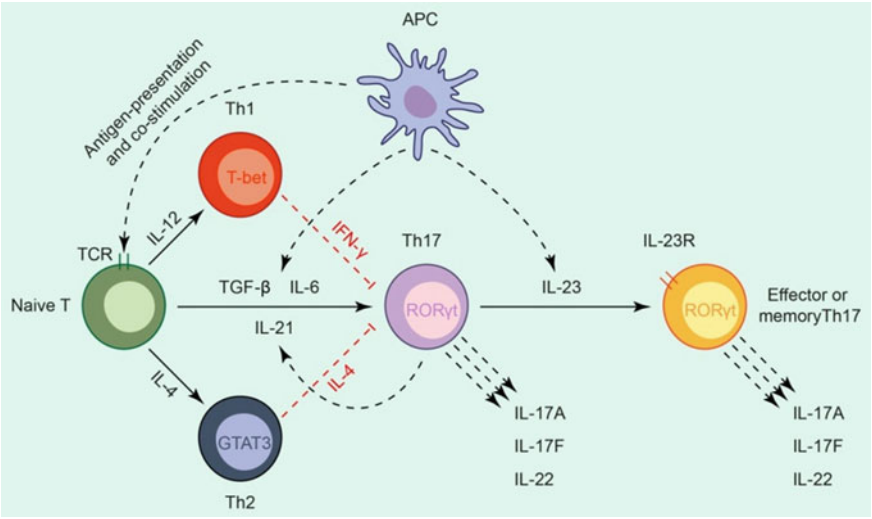

Fig. 5.1 The differentiation of Th17 cells. Naïve T cells can differentiate into three subsets of effector T helper cells under the control of distinct sets of cytokines. After TCR ligation, IL-12 and IL-4 promote Th1 and Th2 differentiation respectively, while the Th17 differentiation is controlled by TGF-β, IL-6, IL-23, IL-1β, and self-secreted IL-21. The differentiation of Th17 cells is inhibited by IFN-γ or IL-4. T-bet (also named Tbx21), GATA3, or RORγt (also known as Rorc) represents the linage-specific transcription factors for Th1, Th2, or Th17, respectively

understanding many aspects of adaptive immunity, some intriguing phenomena cannot be explained until the discovery of a third T helper cells lineage: the Th17 subset, which produces Interleukin-17 (IL-17, also called IL-17A) as its signature cytokine [129] (Fig. 5.1). The function of this newly emerged effector T helper cell subset appears to be distinct from those of Th1 and Th2 subsets and even reinforces certain roles of adaptive immunity system such as host defense and tissue repair responses, which cannot be fully achieved by the Th1/Th2 paradigm [129]. However, Th17 cells are also strong inducers of local tissue inflammation. Persistent and uncontrolled inflammation triggered by Th17 cells becomes a major stimulator in the pathogenesis of many human chronic diseases, which include autoimmune diseases and cancer [129]. The cytokines and the key transcription factors for its differentiation, maintenance, and expansion have been identified. The signaling pathways mediated by IL-17 and the responses mediated by its effector cytokines have also been elucidated.

In this chapter, we review the current understanding of the regulation of Th17 differentiation by the key cytokines and transcription factors and recent progresses of Th17 cell fate commitment and plasticity in both mouse and human. We also discuss the interplay of Th17 cells with other effector T cells such as Th1 and Treg cells. More importantly, Th17 cells produce characterized cytokines like IL-17A,

IL-17F, and IL-22. All those unique cytokines mediate Th17-driven inflammatory responses in both physiological and pathogenic conditions. The signaling pathways and the efforts mediated by those cytokines are also addressed here.

5.2 The Discovery of Th17 Cells

Autoimmune diseases such as multiple sclerosis (MS) and rheumatoid arthritis (RA) are chronic inflammatory diseases. Experimental autoimmune encephalomyelitis (EAE) and collagen-induced arthritis (CIA) mouse models are the most common tools to study those human autoimmune disorders [19, 257]. From a long time, those diseases were associated with uncontrolled self-reactive Th1 responses, since the IFN-γ level was highly correlated with the pathogenesis of EAE and CIA. Blockage of the key Th1 differentiation cytokine IL-12 with antibodies eliminated the progression of EAE and CIA [148, 172]. Rodent genetic evidences demonstrated that both Th1 favorite transcription factors *Tbx21*- and *Stat4*-deficient mice were protected from EAE [17, 35]. All the above evidences indicate that the IFN-γ-producing self-reactive Th1 cells are responsive for the induction of those autoimmune responses. However, this Th1-dominant presumption was intrigued by some experimental contradictions: either the *Ifng*- or its receptor deficient mice were unexpectedly noticed to be susceptible to EAE, rather than resistant to EAE [132, 274]. Similar phenotypes were also observed in both *Il12p35* (one subunit of IL-12)-and *Il12rb2*-deficient mice in EAE model [78, 318]. This paradox raises a question that whether there are unperceived cell populations required for the pathogenesis of EAE. In 2000, IL-12p40 was found to form a novel cytokine IL-23 with a newly identified subunit IL-23p19 [210]. Thus, IL-12p40 is a common subunit for both IL-12 and IL-23, but IL-12p35 and IL-23p19 are the unique subunit for IL-12 and IL-23, respectively. By comparing *Il12p35*-deficient mice with *Il23p19*-deficient mice in EAE or CIA model, researchers found that IL-23 rather than IL-12 was critical for the induction in both models [142, 44, 192]. These results also well explained the phenotype paradox between IL-12 antibodies blockage and *Il12p35*- or its receptor deficient mice in EAE, since blockage strategies targeted IL-12p40 subunit, which also affect the function of IL-23. Following studies showed that IL-23 was crucial for the development of IL-17-producing T helper cells, which was then named as Th17 cells [87, 215].

5.3 Th17 Cell Differentiation

Th17 cells are characterized by production of effector cytokines IL-17 as well as IL-17F, IL-21, and IL-22. Comparing with Th1 and Th2 lineages, Th17 lineage is a more plastic population in its fate determination and its differentiation is controlled by a distinct set of cytokines: after TCR activation and co-simulation, TGF-β and

IL-6, which activate transcription factors Smads and STAT3 respectively, induce the expression of Th17 polarized transcription factor retinoid-related orphan receptor (ROR) γt to initialize Th17 differentiation from naive T cells. IL-21, the cytokine produced by Th17 cells, further promotes this process in a positive feedback manner. After upregulation of IL-23R by the above cytokines in Th17 cells, IL-23 binds to its receptor to drive the terminal differentiation of Th17 cells for their fully function achievement. Other Th17 cell commitment promoting or regulatory cytokines and transcription factors are also identified in recent years, and we will also discuss them in detail as below (Fig. 5.2).

5.3.1 Positive Regulation of Th17 Differentiation by Cytokines

5.3.1.1 TGF-β

TGF-β is a pleiotropic cytokine in T-cell functions and its contribution in Th17 cell differentiation remains contradictory in different conditions [151]. *In vivo* evidences shown that *Tgfb1*-transgenic mice resulted in enhanced generation of Th17 cells and aggressive EAE phenotype when immunized with $MOG_{35–55}$ in CFA [16]. Dominant negative *Tgfbr2*-transgenic mice did not develop the pathological signs of EAE and have no Th17 cells in the spinal cords [282]. T-cell specific *Tgfb1*-deficient mice also did not develop any clinical sign of EAE, and the number of Th17 cells was greatly reduced in those mice [153]. Although Treg derived TGF-β was shown to induce Th17 differentiation *in vitro* [281], but it was not required for Th17 differentiation *in vivo,* since mice with Foxp3-Cre-mediated disruption of TGF-β had similar Th17 cells and comparable EAE pathogenesis when compared with wild-type controls [84]. However, Ox40-Cre-mediated deletion of TGF-β in both activated $CD4^+$ T cells and Tregs resulted in reduced Th17 cell frequencies and ameliorated EAE clinic signs [84], indicating that the production of TGF-β is critical for the pathogenic effector T cells during the organ specific autoimmune diseases. This study also found that, among the activated $CD4^+$ T cells, the Th17 cells themselves produced considerable TGF-β to further promote their functions in an autocrine manner [84].

However, how TGF-β contributes to Th17 cell differentiation remains unclear. TGF-β was reported as an inhibitory cytokine for both Th1 and Th2 cell differentiation by suppressing the transcription factors T-bet and GATA-3, respectively [77, 199]. It was likely that TGF-β promote Th17 differentiation in an indirect way by inhibiting the fate determination of other effector T-cell subsets such as Th1 and Th2 cells. The studies have also shown that TGF-β enhanced Th17 responses by suppressing the transcription factors Eomes and Gfi1, which are critical for Th1 and Th2 related cytokines production [108, 331]. Indeed, comparing with IL-6 which activates STAT3 pathway, TGF-β induced much less expression of RORγt in T

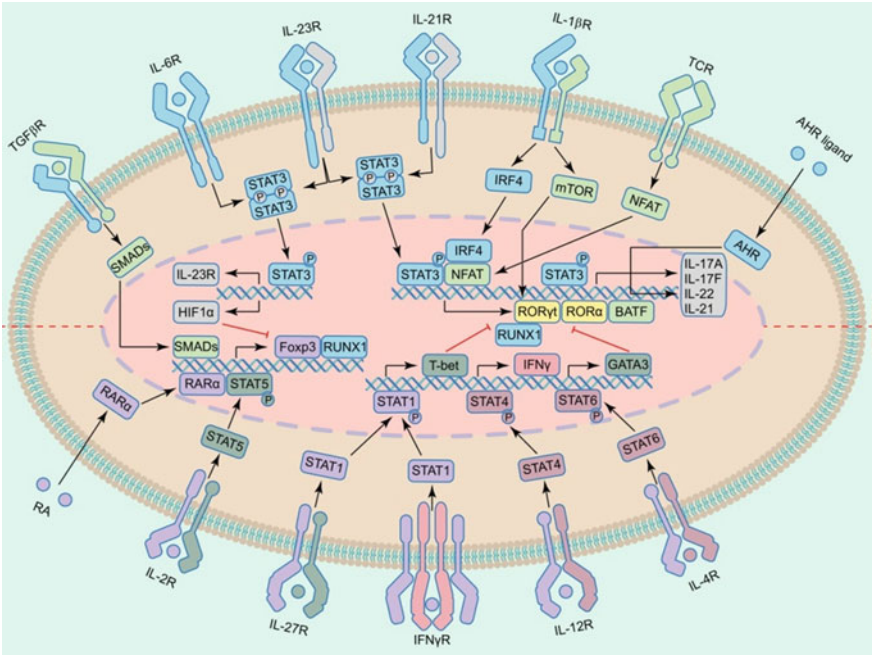

Fig. 5.2 The regulation of Th17 cell differentiation. Upon TCR activation, Th17 cells can be induced in presence of cytokines IL-6, IL-23, and IL-21 that activate STAT3. Activated STAT3 binds to the promoter regions and activates transcription of RORγt and RORα. Transcription factors BATF and IRF4 play a central role in RORγt mediated Th17 cell differentiation. Together with STAT3, RORγt, and RORα activate the expression of Th17 effector cytokines IL-17A, IL-17F, as well as IL-21 and IL-22. IL-6 mediated STAT3 activation also increases the expression of IL-23R, thus promoting the polarizing of Th17 cells. STAT3 activation also induces the expression of HIF1α to inhibit Foxp3 and promotes Th17 differentiation. IL-21 secreted by early Th17 cells functions as a self-amplified autocrine cytokine through IL-21 receptor. IL-1β promotes Th17 polarization by activation of MAPK and Akt/mTOR pathway. IL-1β also induces IRF4 to promote IL-21 secretion. Th17 differentiation is also promoted by activation of aryl hydrocarbon receptors (AHR). TGF-β signals through Smads to limit expression of genes encoding T-bet, Gata3 and other Th1 and Th2-related factors, thus enhancing the Th17 differentiation. TGF-β signaling cooperates with retinoic acid (RA) and IL-2-induced STAT5 activation to promote Foxp3 induction and Treg differentiation. Both Foxp3 and RORγt form complexes with Runx1 and regulate each other reciprocally. IL-12 and IFN-γ, which activate STAT4 and STAT1, respectively, promote Th1 differentiation by induction of T-bet and IFN-γ and then inhibit Th17 polarization. IL-27 also activates STAT1 to upregulate T-bet expression and thus inhibit Th17 development. Similarly, IL-4 signaling through STAT6 to induce GATA3 to suppress Th17 polarization and promotes Th2 differentiation

cells [327]. The IL-6 plus TGF-β produced stronger expression of RORγt than IL-6 alone [327], it is still unclear how TGF-β mechanistically contributes to Th17 cell differentiation. One study found that Th17 cells generated by IL-23, IL-6, and IL-1β rather than those induced by TGF-β and IL-6 caused the pathogenesis of EAE in a transfer model [74]. It seems that Th17 cells generated by TGF-β and IL-6 were not

pathogenic, but they are regulatory Th17 cells which produced high amount of IL-10 [74, 180]. Thus, TGF-β in low concentrations could efficiently initiate the Th17 cell differentiation in cooperated with IL-6, but under this condition, its signaling was insufficient to generate inflammatory Th17 responses [281]. In the contrast, the high concentration of TGF-β favored the generation of induced $FOXP3^+$ regulatory T cells (iTregs) rather than Th17 cells by inhibiting the expression of IL-23R and disrupting the function of RORγt [328], and high concentration TGF-β also inhibited IL-22 expression to generate $IL\text{-}17^+IL\text{-}22^-$ cells [324]. The plastic interplay between Th17 cells and iTregs will be discussed further in the section below.

5.3.1.2 IL-6

IL-6 is also a multiple functional cytokine in immune system and is produced by many immune and stromal cell types [279]. IL-6 binds to its receptor complex IL-6Rα and gp130 to activate STAT1 and STAT3 for downstream biological activities [94]. Its roles in autoimmune diseases have been studied. Several lines of clinic evidences revealed that the patients with MS or RA exhibit higher levels of IL-6 in their cerebrospinal fluid or synovial fluids than normal controls [97, 243]. Previous studies found that *Il6*-deficient mice are protective from CIA and EAE [5, 206, 235] and anti-IL-6 receptor antibodies blockage in mice also suppressed the progression of both CIA and EAE diseases [65, 243]. More importantly, in human, the therapy by Tocilizumab, a humanized anti-IL-6 receptor antibody, had become a novel therapeutic strategy to prevent many autoimmune diseases including RA [200]. The resistance phenotype of *Il6*-deficient mice in the models of EAE and CIA has been poorly explored until different groups identified that IL-6 as a critical differentiation factor for Th17 cell generation. As described above, IL-6 was a strong suppressor of the TGF-β-driven induction of Foxp3 in T cells, instead, IL-6 plus TGF-β induced a unique transcriptional program resulting in the differentiation of Th17 cells [16]. At this condition, TGF-β also induced the expression of IL-6Rα and gp130 indicating that TGF-β is crucial to maintain the responsiveness of T cells to IL-6 [298]. IL-6 then binds to IL-6Rα and gp130 to recruit and phosphorylate the transcription factors STAT3. Deficiency of STAT3 in T cells impaired the induction of RORγt and RORα and consequentially abrogates the generation of Th17 cells [306, 307]. Accordingly, CD4-Cre-mediated STAT3 depletion in T cells protected mice from EAE [88]. Thus, IL-6 is a key switch factor that favors Th17 cell differentiation while suppressing the generation of Tregs.

5.3.1.3 IL-21

IL-21 was firstly described in 2000 by showing that this cytokine has a role in the proliferation of natural killer (NK) cell, B cell, and T-cell populations in different conditions [216]. IL-21 is a member of IL-2 family of cytokines and signals though

the common γ chain of this family and IL-21R [159]. Although it can be produced by several activated CD^+ T cells, IL-21was found to be highly produced in T follicular helper (Tfh) cells and Th17 cells [128, 165, 203, 327]. IL-6 served as a strong inducer of IL-21 [263] though the transcription factor STAT3 but not RORγt [327]. It was found that IL-21 plus TGF-β can also generate Th17 cells *in vitro* as IL-6 plus TGF-β [128, 203, 327], while the relative contribution of IL-6 and IL-21 to Th17 cell differentiation *in vivo* was still controversial. Whereas Th17 can secrete IL-21, it is likely to keep a similar role as IFN-γ and IL-4 in Th1 and Th2 cell development, IL-21 is also a positive feedback amplification factor for Th17 cells. Although IL-21 was induced by IL-6 in Th17 cells, IL-6 plus TGF-β induced Th17 cell differentiation was independent of IL-21 signaling and both *Il21*- and *Il21r*-deficient mice shown similar susceptibility to the control mice in EAE model [42, 249]. Those data suggest that IL-6, rather than IL-21, plays a dominant role in Th17 differentiation in inflammatory conditions where IL-6 is massively produced. While in the absence of inflammation, IL-21 might contribute to the maintaining of precursor pool of Th17 cells, as the memory Th17 cell frequency was reduced in *Il21r*-deficient mice [128]. Thus, it is likely that IL-21 helps to maintain and amplify the pool of Th17 precursors when the level of IL-6 is relative low while under inflammatory conditions when IL-6 is highly produced, IL-21 is dispensable for the differentiation of Th17 cells.

5.3.1.4 IL-23

As discussed above, IL-23 was described as a novel heterodimer cytokine which is composed by IL-23p19 and IL-12p40 subunits in 2000 [210]. Later, researchers found that *Il23p19*-deficient mice, rather than *Il12p35*-deficient mice were protective from the induction of EAE and harbored very few IL-17-producing cells in CNS [44, 142]. The capability of IL-23 in promoting IL-17 production in activated T cells led to a notion that IL-23 was strongly connected with the generation of Th17 cells [2]. Since IL-23R is not expressed on naïve T cells, it is likely that IL-23 is dispensable for the *de novo* generation of Th17 cells. After the discovery of initiation factors for Th17 cell differentiation (IL-6, IL-21, and TGF-β), it became clear that IL-23 was not required for the *de novo* differentiation of Th17 cells but critical for the maintaining and expansion of differentiated Th17 cells. At the present of IL-6, IL-21, and TGF-β, naïve T cells began to differentiate into Th17 cells under the control of transcription factors STAT3 and RORγt, which were critical for the expression of IL-23R in those cells [174, 203, 327]. More recently, one study found that increased sodium chloride concentrations markedly boost the induction of both murine and human Th17 cells and this induction was dependent on NFAT5 and SGK1 [125]. The kinase SGK1 is critical for regulating the expression of IL-23R and thus stabilizing the Th17 cell fate [296]. Increased salt concentration induced its expression, in turn to promote IL-23R expression and enhance Th17 cell-mediated autoimmune responses [296]. Beside to Th17 cells, IL-23 signaling was also crucial for the production of IL-17 or IL-22 in many innate

immune cells [45]. Several genome-wide association studies showed the associations of *Il23r* gene SNPs with Crohn's disease and psoriasis in human [23, 58, 160].

5.3.1.5 IL-1β

The cytokine IL-1β has a broad range of influence on infectious diseases as well as autoimmune disorders [52]. More recently, studies showed that both IL-1β and IL-18 have a role in promoting IL-17 production from Th17 cells [140, 264]. Both cytokines synergized with IL-23 to enhance IL-17 secretion from TCR stimulated T cells [140, 264]. As IL-23, mice lacking IL-1β signaling was resistant to both EAE and CIA induction [169, 264). Following studies showed that IL-1β signaling was required for the early stage of Th17 differentiation by converting $Foxp3^+$ T cells into Th17 cells [36]. After polarization, IL-1β also favored Th17 cells to maintain their own fate [36]. Mechanically, IL-1β signaling promoted Th17 cell function by induction of transcription factor RORγt and IRF4 [36]. The processing of functional IL-1β requires two signals; conversely, inactive pro-IL-1β is produced by TLR signaling in innate immune cells (Signal 1) and then is cleaved by the caspase-1 to become mature and active cytokines (Signal 2) [275]. *Caspase1*- or other inflammasome components such as *Asc*- and *Nlrp3*-deficient mice were all found to be resistant to EAE induction, indicating that they may function though processing of IL-1β [67, 79, 109, 116, 244]. A *Nlrp3* gene mutation, which hyper activated inflammasome, promoted a Th17-dominant responses though uncontrolled production of IL-1β [183]. The inflammasome agonists such as uric acid crystal and extracellular ATP were all reported to promoted Th17 differentiation though inflammasome-derived IL-1β [9, 40].

5.3.1.6 TNFα

TNFα is another important inflammatory cytokine with diverse function in immune system [14]. It was found that both *Il1b*- and *Tnf*-deficient mice were resistant to spontaneous arthritis in SGK mice [90]. In CIA model of DBA/1 mice, antibodies blockage of either IL-1β or TNFα had therapeutical effects on joint pathology [120]. *Tnf*-deficient mice were also protected from EAE induction [111]. Although neither of these cytokines, alone or together, was sufficient for initiation step of Th17 differentiation, TNFα as well as IL-1β was found to amplify Th17 differentiation *in vitro* [281]. Together with IL-1β, the DC cell-derived TNFα promoted IL-6 plus TGF-β directed Th17 differentiation [196, 281]. TNFα or IL-1β can also indirectly promote Th17 development by induction of IL-6. It is likely that cytokines such as inflammatory environments derived IL-1β and TNFα contribute to Th17 differentiation by generating an inflammatory niche which favors its differentiation. As IL-17 is a strong inducer of both TNFα and IL-1β, it is possible for Th17 cells to interact with local or infiltrated cells to set up a positive feedback loop in such an inflammatory niche.

5.3.1.7 IL-17C

IL-17C was primitively described as a novel IL-17 family cytokine, which shared similar proinflammatory effects with IL-17 [105, 150, 302]. Recently, we and others identified IL-17RE, an orphan receptor of IL-17 receptor family, as the functional receptor for IL-17C [28, 224, 253]. IL-17RE was found to be highly expressed on intestinal epithelial cells and critical for IL-17C-mediated mucosal immunity to pathogen infection or colitis [224, 227, 253] (discussed below). While surprisingly, comparing to other $CD4^+$ T cells, Th17 cells also harbored high level of IL-17RE [28], indicating that IL-17C may also contribute to autoimmune response by targeting Th17 cells. The expression of IL-17RE was induced by IL-6 plus TGF-β and was fully upregulated at the present of IL-23 in T cells [28]. Deficient of its ligand, IL-17C, protected mice from EAE induction [28]. IL-17C bound to its receptor IL-17RE in Th17 cells and induced the expression of IκBζ, a nuclear IkappaB family member, to promote the production of IL-17 and Th17 cell response [28].

5.3.2 Positive Transcription Factors

5.3.2.1 RORγt and RORα

The T-bet, GATA3, and Foxp3 represent the lineage-specific transcription factors for Th1, Th2, and Treg cells, respectively. RORγt (also named as Rorc), a splicing variant of RORγ expressed in T cells [93, 181], was found to be the linage-specific transcription factor for Th17 cells [114]. However, in *Rorc*-deficient mice, the frequencies of Th17 cells were not absent but only reduced, indicating that other transcription factors play redundant role in controlling Th17 fate determination. Another ROR family member, RORα, was subsequently identified as a coordinator factor with RORγt to promote Th17-cell differentiation [307]. The deficiency of both RORγt and RORα completely abolished the generation of Th17 cells both *in vitro* and *in vivo* [307]. Both RORγt and RORα were synergistically induced by IL-6 or IL-21 plus low amounts of TGF-β [102, 307]. However, the mechanisms by which RORγt and RORα regulate IL-17 production have not yet been fully elucidated.

5.3.2.2 STAT3

Both IL-6 and IL-21 activate STAT3 in T cells, indicating a master role of STAT3 in Th17 cell programming. T-cell specific deletion of STAT3 impaired Th17 differentiation [306, 307], while retroviral overexpression of a constitutively active STAT3 in T-cells can enhance IL-17 production [179]. The STAT3 directly controlled expression of RORγt, RORα, and IRF4, which were transcriptional factors required for Th17 differentiation [59]. More importantly, STAT3 also bound

directly to the promoters of IL-17A, IL-17F, IL-21, IL-6R, and IL-23R, indicating a direct control of STAT3 on the Th17 differentiation [31, 59, 203]. Patients with Hyper IgE syndrome (HIES) harbored a dominant negative form of STAT3 which led to a serious impairment of Th17 generation in those patients [49, 99, 166, 185, 186, 226]. The mutation of STAT3 and subsequent impairment of IL-17 production may explain the inefficient cleanup of bacterial and fungal infections in those patients.

5.3.2.3 IRF4

IRF4, a transcription factor which has been shown to be critical for the differentiation of the Th1 and Th2 cells [162, 225], was also found to be essential for the development of Th17 cells. *Irf4*-deficient mice were resistant to EAE induction and its deficient-T cells were unable to induce the expression of RORγt and RORα and consequently could not be differentiated into Th17 cells when those cells were treated with TGF-β plus IL-6 or IL-21 [21, 102], while restitution of RORγt and RORα in those cells could partially compensate for the reducing of IL-17 production [102]. In addition, the expression of Foxp3 was increased in *Irf4*-deficient T cells, suggesting the interplay of the Foxp3 and RORγt balance in Treg /Th17-cell differentiation [21]. IRF-4 was negatively regulated by a binding protein named IBP (IRF-4-binding protein), which is shown to play a regulatory role in Th17-cell differentiation [29]. IBP prevented IRF-4 from binding to the transcriptional elements of *Il17* and *Il21* genes and mice lacking IBP developed arthritis-like syndrome and enhanced Th17 responses [29].

5.3.3 Negative Regulation of Th17 Differentiation

5.3.3.1 STAT5

STAT5 is an essential downstream transcription factor of IL-2 signaling, which is critical for the survival of T cells. Genetic abolishment or antibody neutralization of IL-2 promoted differentiation of the Th17 cells *in vivo* [144]. This inhibition effect of IL-2 on Th17 differentiation was independently of Foxp3 and RORγt [308]. Whereas STAT3 is a critical positive regulator of RORγt and Th17 development, disruption of STAT5 led to increased Th17 cell development, probably due to the loss of IL-2-mediated inhibitory effect [144]. This suppressive effect of STAT5 on the differentiation of Th17 might be due to its competition with STAT3 to bind to the same locus sites encoding *Il17* [308].

5.3.3.2 Gfi1

Gfi1 was highly expressed in Th1 and Th2 cells and its expression was controlled by IFN-γ/STAT1 and IL-4/STAT6 pathways. TGF-β, which is critical for either Th17 or Tregs differentiation, suppressed Gfi1 expression [107]. Over-expression of Gfi1 strongly repressed IL-17A expression in both human EL4 T-cell line and primary T cells. This suppressive effect was mainly due to the blockage of RORγt recruitment to the promoter region of IL-17A. In contrast, *Gfi1*-deficient T cells produced more IL-17A than wild-type T cells under Th17 favored differentiation conditions [107]. However, the influences of Gfi1 on iTregs were not as obvious as on Th17 cells [107].

5.3.3.3 LXR

Liver X receptors (LXRs) are nuclear receptors which are originally involved in cholesterol homeostasis and are activated by endogenous oxysterols or oxidized cholesterol derivatives [323]. Recently, LXR was found as a negative regulator for Th17 differentiation. LXR suppressed Th17 responses by promoting the transcription factors Srebp-1 to bind to the *Il17* promoter region, and thus interfering with the AhR-mediated *Il17* transcription [46]. Over-expression of LXR inhibited mouse Th17 differentiation and *Lxr*-deficiency mice were more susceptive to EAE induction than wild-type mice [46].

5.3.3.4 TCF1

T-cell factor 1 (TCF-1) is a transcription factor to activate canonical Wnt pathway and is crucial for normal T cell development [254]. TCF1 was showed to regulate differentiation and maintenance of memory $CD8^+$ cells [329]. Recently, it was also found that TCF1 suppressed IL-17 expression by directly binding to the regulatory region of *Il17* and thereby repressing its transcription [312]. Induction of IL-17 in TCF1 depletion T cells was coupled with up-regulation of RORγt and STAT3 in those cells [168]. Moreover, *Tcf1*-deficient Th17 cells expressed increased levels of IL-7Rα, which potentially enhanced Th17 cell survival [312]. Accordingly, *Tcf1*-deficient mice showed exacerbated EAE phenotype [168, 312]. TCF1 might also repress Th17 responses by induction of Eomes [329].

5.3.3.5 Eomes

Cytotoxic activities of T cells were noticed under controlling of T box transcription factors including T-bet and Eomes through up-regulation of perforin, FasL and granzyme B [63, 223]. Eomes interacted with GATA3 to prevent its binding to IL-5 promoter in memory Th2 cells or functioned together with T-bet to mediate

generation of IFN-γ producing $CD8^+$ cells [62, 76, 207]. In Th17 cells, ectopic expression of Eomes inhibited Th17 cell differentiation through directly binding to the promoter region of *Rorc* and *Il17*. Depletion of Eomes expression could substitute for TGF-β in induction of Th17 cell differentiation [108].

5.3.3.6 NFIL3

NFIL3, also called E4BP4, is a transcription factor with basic leucine zipper structure which regulates multiple immune responses [171]. Recently, it was found that this transcription factor suppressed Th17 cell differentiation though directly binding and inhibiting the *Rorc* promoter [313]. More interestingly, NFIL3 was negatively regulated by the transcription factor REV-ERBα, which is critical for circadian clock pathways [187]. Consequently, Th17 lineage frequency varied diurnally and was altered in *Rev-erbα*-deficient mice [313]. Disruption of light cycle elevated intestinal Th17 cell level and increased susceptibility to intestinal inflammatory disorders [313].

5.3.4 Negative Regulation of mTOR in Th17 Differentiation

The mTOR signaling is critical for regulating organism growth and homeostasis [143]. Recently, mTOR signaling is found to represent a crucial regulator of T-cell differentiation [95]. The mTOR signaling pathways have two distinct complexes: mTORC1 and mTORC2. The conditional depletion of Rictor, a key component of mTORC2, impaired the differentiation of Th1, Th2, and Tregs but not Th17 cells [146], while mTORC1 signaling was found to selectively regulate Th17 differentiation [51]. Recently, it was found that IL-1β signaling induced the activation of JNK and mTOR kinases in Th17 cells. IL-1β-induced Th17 proliferation was impaired in *Mtor*-deficient Th17 cells, indicating the critical role of mTOR activation in Th17 development [82]. Meanwhile, the study also found a negative regulator of IL-1β and TLR signaling, named SIGIRR, was induced during Th17 cell development and SIGIRR inhibited Th17 differentiation and expansion by suppression of IL-1β signaling. Comparing to wild-type Th17 cells, *Sigirr*-deficient Th17 cells shown increased IL-1β-induced JNK and mTOR activation [82]. IL-1β-induced mTOR activation was dependent on AKT, which was constitutively suppressed by GSK3α medicated phosphorylation. Thus, GSK3α severed as a brake to prevent over-expansion of Th17 cells. Upon IL-1β stimulation, a kinase IKKi was found to phosphorylate GSK3α and in turn to release the negative regulation of GSK3α on mTOR activation in Th17 cells [81].

5.3.5 MicroRNA in Th17 Differentiation

MicroRNAs (miRNAs) are about 19–24 nucleotide (nt) single-stranded RNA molecules that post-transcriptionally regulate the expression of genes [333]. In mammals, it was suggested that nearly 85% of microRNA-mediated decay of mRNA [83]. More than 600 different microRNAs were expressed in T cells and many of them were critical regulators for the differentiation stage and activation status of different T-cell populations [124]. As helper T-cell subsets are critical for autoimmune pathogenesis and proper host defense responses, the regulation of lineage commitment by microRNAs are studied to a greater extent. T-cell specific deletion of Dicer, a critical enzyme for microRNA biogenesis resulted in reduced T cells proportion in the thymus or periphery lymph tissues [39, 191]. Recent studies also showed that specific miRNAs are involved in regulation of certain types of T help cell development. T-cell deficiency of miR-155 led the activated $CD4^+$ T cells to favorite a Th2 bias under neutral conditions *in vitro* [230, 270]. It is noticed that miR-155 inhibited the function of Th2 transcriptional factor c-Maf to promote the differentiation of Th1 cells [230]. However, the Th1 differentiation was negatively regulated by miR-29. MiR-29 was reported to suppress Th1 differentiation by targeting the Th2 favored transcription factor T-bet and Eomes or the cytokine IFN-γ [167, 247, 255]. Beside to Th1 and Th2 cells, the microRNAs which regulate Th17 cells function have also been identified. A recent study showed that miR-326 targeted Est-1, a negative regulator of Th17 differentiation, to promote Th17 cell expansion and Th17 related autoimmune pathogenesis [56]. Likewise, miR-301a promoted Th17 differentiation by inhibiting PIAS3, which negatively regulated IL-6-IL-23-STAT3 cascades [195]. MiR-155 also enhanced STAT signaling through suppression of SOCS1 and this might explained the depressed Th17 differentiation and ameliorated EAE phenotype in *Mir155*-deficient mice [194, 310]. More recently, miR-21 was showed to promote Th17 differentiation by disrupting Smad-7, a negative regulator for TGF-β signaling. Like MiR-155, *Mir21*-deficient mice also protected from EAE induction [75]. Meanwhile, microRNA let-7a was recently found to be a negative regulator of Th17 differentiation by suppressing IL-6 secretion in a mouse model of Con A-induced hepatitis [320].

5.4 Interplay Between Th17 Cells and Other T Helper Cells

5.4.1 Interplay Between Th17 and Th1, Th2 Cells

The effector T-helper cell subsets was noticed as distinct and terminal differentiated lineages by studies showing that, once naive T cells have been programmed into Th1 or Th2 cells, these polarities could not be reversed even when new polarizing conditions were reintroduced [193]. The discoveries of specific master transcription factors, T-bet for Th1 while GATA3 for Th2 differentiation, had also improved this

lineage model. Furthermore, several studies showed that Th1 and Th2 antagonized each other to favor their own stable terminally differentiated phenotype [53, 198, 266]. As the crosstalk between Th1 and Th2 cells, cross regulation may also exist between Th17 and Th1 or Th2 cells. Either IFN-γ or IL-4 inhibited Th17 differentiation and IL-17 induction [87]. In addition, the IL-12 family cytokine IL-27, which induced T-bet and IL-12Rβ2 expression to promote Th1 responses inhibited Th17 cell differentiation through activation of STAT1 [13, 258]. Loss of T-bet in T cells strongly promoted IL-17 expression in Th17 cells [178, 309]. As discussed above, IL-4 could induce Gfi-1 to favor optimal Th2 cell differentiation [332] and suppressed Th17 or iTreg cells differentiation [331]. Thus, both Th1 and Th2 favored signaling are inhibitors for Th17 development, while the effects of Th17 derived cytokines on Th1 and Th2 differentiation are still need to be addressed.

Th17 cells are distinct T-helper cell subset and are not derived from the Th1 lineage, since those cells lacking T-bet or Stat4 also expressed RORγt and developed into Th17 cells under Th17 polarizing condition [114]. However, under both homeostatic and inflammatory conditions, IFN-γ^{+}IL-17^{+} cells can be easily detected, suggesting that intricacy is existed between the Th1 and Th17 cell differentiation. *In vitro* polarizing Th17 cells could lose both IL-17 and IL-17F expression when IL-6 and TGF-β stimulation was withdrawn [149]. T cells cultured in Th17 polarizing conditions still failed to keep IL-17 and IL-17F expression and could be converted to Th1 or Th2 cells when those cells were stimulated with IL-12 or IL-4, respectively [147, 149]. *In vitro* polarized Th17 cells failed to keep their IL-17-producing memory when transferred into mice and many of them converted into IFN-γ-producing Th1 cells under a colitis condition [147]. Similar phenomenon was also noticed when Th17 cells were introduced into NOD-SCID mice [15]. In contrast, *in vivo* memory Th17 cells with $CD4^{+}CD62L^{low}$ marker appeared more resistant to conversion [149], suggesting *in vivo* generated Th17 cells are a stable and distinct lineage of T helper cells.

As described above, Th17 cells polarized by TGF-β and IL-6, but without IL-23, were shown to express IL-10 [180]. Since Th17 cells are abundant in the lamina propria of intestine even in steady state, this containment is critical to control overactivated Th17 cells in gut. Upon infection or injury, these Th17 cells can easily become inflammatory Th17 cells when other Th17 polarizing cytokines are released by the local environment, and these cells can also rapidly convert into Th1-like cells for intracellular pathogens cleanup. As Th17-mediated inflammatory responses also cause serious tissue destruction, these pathogenic Th17 cells may be appropriately and timely re-shifted toward protective IL-10-producing Th17 cells when dangerous signals are removed.

5.4.2 Interplay Between Th17 and Treg Cells

TGF-β signaling was essential for formation of immune tolerance, in part due to its capability for induction of peripheral iTreg cells and for maintenance of thymus

derived nTreg cells [30, 152, 175]. However, at lower concentration, TGF-β was also critical for induction of proinflammatory Th17 cells [16, 174, 281]. Mice deficient in TGF-β failed to develop ethier $Foxp3^+$ Treg or Th17 cells and had uncontrolled autoimmune disorders which was caused by aggressive Th1 cells responses [152, 282], indicating a close relationship between Treg and Th17 cell development program in against Th1 differentiation. After TCR ligation, TGF-β could induce both Foxp3 and RORγt expression in $CD4^+$ T cells [328]. These $Foxp3^+$ $ROR\gamma t^+$ T cells were found both in murine and human and noticed as a transitional state for Treg and Th17 cells [284, 328]. In small intestine, the $Foxp3^+$ $ROR\gamma t^+$ cells produced less IL-17 than $Foxp3^-ROR\gamma t^+$ cells [328]. In agreeing with this, Foxp3 abolition led to a notable increase in IL-17 production without affecting RORγt expression [70], indicating that Foxp3 may antagonize RORγt function to favor Treg development. Indeed, Foxp3 suppressed Th17 responses by directly interacting with both RORγt and RORα [57, 307, 328]. $Foxp3^-ROR\gamma t^+$ T cells induced by TGF-β do not produce much IL-17 and have the dual potential to develop into either Treg or Th17 cells depending on the different inflammatory environment. As described, a proinflammatory environment (IL-6, IL-23, IL-1, and IL-21) plus low concentration or TGF-β inhibited Foxp3 function while further enhanced RORγt expression in favor of Th17 differentiation. In contrast, high concentration TGF-β further firmed Foxp3 expression and thus promoted Treg differentiation rather than Th17 cells [328]. This Treg favored shift was further enhanced by IL-2 and retinoic acid (RA) signaling, both of which were suppressors for RORγt but enhancers for Foxp3 expression [41, 144, 190, 261]. As mentioned before, several transcription factors have been noticed to modulate the Foxp3 and RORγt balance during $CD4^+$ T-cell differentiation. Runx1 was found to cooperate with RORγt to promote Th17 cell differentiation [317]. Additionally, in Treg cells, Runx1 and Foxp3 were also indicated to from a complex which was required for inhibition of IL-2 and IFN-γ expression and Treg suppressive activity [209]. Thus, Runx1 is required for governing potential plasticity in $Foxp3^+$ $ROR\gamma t^+$ T cells. IRF4 is critical for Th17 cell differentiation and its absence led to increased Foxp3 expression, reduced RORγt expression, and thus loss of IL-17 secretion [21]. Stat3, a transcription factor that was commonly activated by IL-6, IL-21, or IL-23, was also crucial for Th17 cell differentiation by binding to the promoter regions of *Il17* and *Il17f* [31]. These proinflammatory cytokines were found to inhibit TGF-β-induced Foxp3 expression in a Stat3-dependent manner [306, 327].

5.4.3 *Epigenetic Regulation Between Th17 and Other T Helper Cells*

Epigenetic regulation is critical for controlling gene expression through changing chromatin structure, histone or DNA modifications, and small noncoding RNAs expression and T helper cells are also under control of these regulatory strategies

[8, 184, 290]. Recent studies by using chromatin immunoprecipitation on gene array chips (ChIP-chip) and high-throughput sequencing (ChIP-Seq) found several histone modification and DNA methylation changes accompanying with $CD4^+$ T-cell differentiation. Trimethylation of histone H3 lysine 4 (H3K4me3) was a permissive mark, while H3 lysine 27 (H3K27me3) was a silence mark to be found at the promoters and the enhancers of different subset specific genes [289]. In Th1 cells, H3K4me3 mark was found at the *Ifng* locus while H3K27me3 mark was found at the *Il4* and *Il17* loci. In Th2 cells, however, *Il4* locus was modified with H3K4me3 while *Ifng* and *Il17* loci were modified with H3K27me3. Notably, the gene *Tbx21* and *Gata3* showed a bivalent status in Th2 and Th1 cells respectively, indicating cell lineage interconversion may occur between these two types of cells [133, 289]. However, *Rorc* locus was suppressed in both Th1 and Th2 cells, indicating that these two types of cells can hardly reprogrammed into Th17 cells. Consistent with the high plasticity of Th17 cells, the gene *Tbx21* and *Gata3* all showed a bivalent status in Th17 cells, indicating that Th17 cells can easily transmit into Th1- or Th2-like cells under appropriate conditions [133]. *Tbx21*, *Gata3*, or *Rorc* sites were all found to be bivalent modified in Treg cells, suggesting that, like Th17 subset, Tregs are also highly dynamic cell population. However, nTreg cells derived from thymus and iTreg cells derived from peripheral showed different reprogramming propensity. In iTreg cells, the *Il17* locus was silenced by H3K27me3 modification whereas H3K4me3 was found at the *Rorc* locus, agreeing with the notion that TGF-β induced RORγt expression in T cells, but the differentiation of Th17 cells was inhibited by Foxp3 [289, 328]. However, both H3K4me3 and H3K27me3 bivalently existed at the *Rorc* locus in nTreg cells, allowing for the generation of $Foxp3^+ROR\gamma t^+$ cells [289]. When nTreg cells were subjected to Th1- or Th17-polarizing culture conditions, a remarkable number of $IFN\text{-}\gamma^+Foxp3^+$ or $IL\text{-}17^+Foxp3^+$ cells appeared without affecting the expression of Foxp3 [289, 300, 305]. However, TGF-β-induced Treg cells easily lost Foxp3 expression and gained IL-17 expression when these cells were cultured in a Th17 cell-favoring condition [305].

5.5 Human Th17 Differentiation

Sooner after the discovery of cytokines and transcription factors that promote mouse Th17 cell differentiation, efforts were made to induce the differentiation of human Th17 cells. As mouse Th17 cells, human Th17 cells also highly expressed the master transcription factors RORC2, the human homologue to RORγ t [7, 291], and forced expression of RORC2 in cord blood derived T helper cells induced IL-17A, IL-17F, and IL-26, but not IL-22 expression [173]. The functional roles of other Th17-related transcription factors in human Th17 cell development are not fully understood. It was reported that transduction of RORA in human cord blood cells led to increased IL-17 expression [173], indicating that this human homologue to RORα also cooperates with RORC2 to induce Th17 cells as in mice. STAT3 was

also found to be important for the development of human Th17 cells. Patients with hyper-IgE sydrome carried dominant negative mutations in STAT3 gene and T cells obtained from these patients fail to develop into Th17 cells *in vitro* due to the lacking of IL-6-induced STAT3 activation and subsequently RORC2 expression [99, 166, 186]. AHR was also expressed in human Th17 cells [280], but it is still not clear whether it contributes to these cells development.

The cytokines required for the differentiation of human Th17 cells appeared similar to mouse Th17 cells, including IL-6, IL-23, IL-1, and IL-21, however, there were some debates on the necessity of TGF-β in human Th17 differentiation [129]. Several studies found that TGF-β was not required for human Th17 cells differentiation [1, 291]. But one study argued that these cells used above were not totally equal to naïve T cells in mouse. Other studies reported that the human naive cord blood T cells were dependent on TGF-β for their differentiation into Th17 cells. TGF-β was required for induction of RORC, but excess TGF-β inhibited its expression and function [173, 283, 303].

Given the complexity of TGF-β for human Th17 differentiation, the requirement of TGF-β in mouse Th17 differentiation has been re-checked. *Tbx21*- and Stat6-deficient T cells could differentiate into Th17 cell when stimulated with IL-6 alone, even in the absence of TGF-β [48], indicating that TGF-β indirectly regulated IL-17 production by inhibition of factors that required for other cell fates [145]. In addition, the IL-23R was induced in the absence of TGF-β, and IL-23 addition could further induce this receptor expression. In addition, the combination of IL-6, IL-23, and IL-1 was sufficient to induce IL-17 production in a TGF-β independent way [74]. Consistently, Th17 cells were also found in the gut of *Tgfb1*-deficient mice [74, 221].

5.6 Th17 Effective Cytokines

5.6.1 IL-17A and IL-17F

5.6.1.1 IL-17 Family Cytokines

Th17 cells secret characteristic cytokines IL-17A (also called IL-17) and IL-17F. IL-17 family also contains other four cytokines IL-17B, IL-17C, IL-17D, and IL-17E (also named IL-25), which are homologous to the fundamental member IL-17A by bioinformatics analysis. The IL-17 family of receptors, which contains IL-17RA, IL-17RB, IL-17RC, IL-17RD, and IL-17RE, are signal transmembrane receptors with unique structure architecture among the members. All receptor members harbor two fibronectin III-like domains in their extracellular region and a SEF/IL-17R (SEFIR) domain in their intracellular part [68]. IL-17RA (also called IL-17R) has been long known as the receptor of IL-17. However, recent findings indicate that IL-17RA may sever as a common receptor for all IL-17 family cytokines. Other receptors like IL-17RB, IL-17RC, or IL-17RE have been

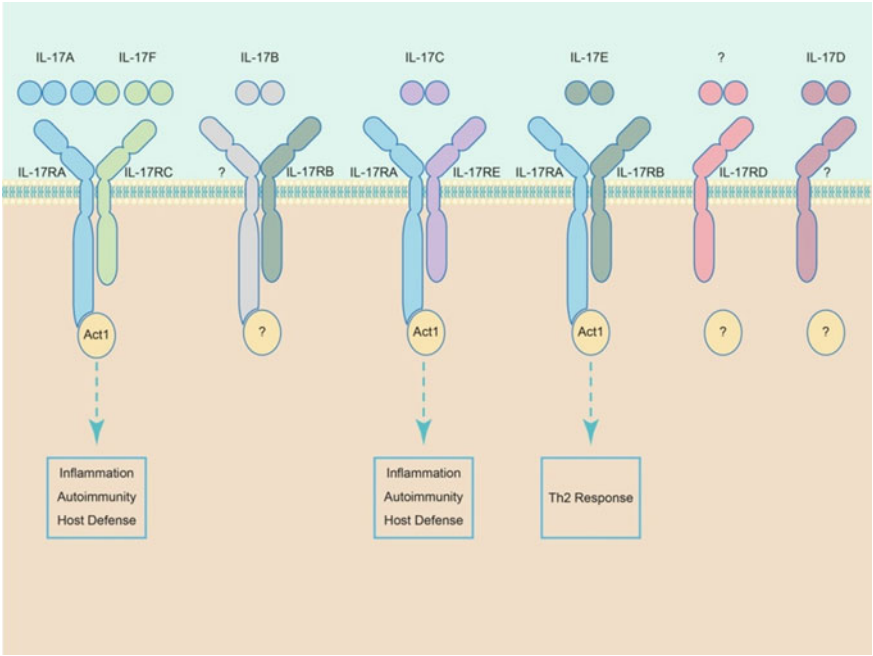

Fig. 5.3 The IL-17 family cytokines and their receptors. IL-17 family cytokines contain six members: IL-17A to IL17F, while the receptor family has five members: IL-17RA to IL-17RE. The most studied members, IL-17A and IL-17F, form homo- or hetero-dimer to bind IL-17RA and IL-17RC receptor complex and activate downstream signaling for host defense, autoimmune diseases and other inflammation responses. Although IL-17B was found as a ligand of IL-17RB, the *in vivo* functional evidences for this pair are still largely unclear. IL-17C binds to IL-17RA and IL-17RE receptor complex to trigger downstream signaling for host defense and autoimmune diseases. IL-17E associated with IL-17RA and IL-17RB receptor complex to mediate Th2 responses. Neither the receptor for IL-17D nor the legend for IL-17RD has been found. The adaptor protein Act1 has been considered as a key adaptor in IL-17A, IL-17F, IL-17C, as well as IL-17E mediated signaling

discovered as specific receptors for IL-17E, IL-17A, as well as IL-17F or IL-17C, respectively. IL-17RD (also called Sef), a negative regulator of FGF signaling, has recently been identified to have a role in IL-17A signaling [182, 231] (Fig. 5.3).

In murine, IL-17F shares the highest homology in amino acid sequence to IL-17 (about 50%), while IL-17B, IL-17C, IL-17D, and IL-17E share 16–30% sequence identity to IL-17 [115]. The high similarity of IL-17 and IL-17F may be responsible for the similar pro-inflammatory outcomes they mediate. IL-17 and IL-17F form homo/hetero-dimer to bind to their receptors, IL-17RA and IL-17RC, and therefore activate NF-κB, MAPKs, and C/EBPs signaling pathways which consequently upregulate the expression of proinflammatory genes [336]. IL-17 also cooperates with other inflammatory cytokines such as TNFα to synergistically induce certain chemokines expression by stabilizing their mRNAs [336]. Recently, we also found

IL-17 enhanced NF-κB activation though an alternative way by downregulating the expression of microRNA-23b in residential cells of several autoimmune diseases [335].

As discussed above, IL-17 and IL-17F are considered to be predominantly produced in the Th17 cells. The differentiation of Th17 cells is controlled by a unique set of cytokines (TGF-β, IL-6, IL-1β, IL-23, and IL-21) and transcription factors (RORγt, RORα, STAT3, IRF4, BATF, AHR, Runx1, and IκBξ). However, beside to Th17 cells, a subset of $CD8^+$ T cells named Tc17 cells can also produce those two cytokines. In addition, several innate immune cells have also been found to release IL-17 and IL-17F. These cells, which include γδT cells, iNKT cells, NK cells, LTi cells, and neutrophils, are noticed as major sources for innate IL-17 and IL-17F. Furthermore, nonlymphoid cells such as intestinal Paneth cells and colonic epithelial cells may also have the capability to produce IL-17 or IL-17F [45, 115]. The rapid releasing of IL-17 or IL-17F by those cells may contribute to appropriate immune responses against many pathogens.

IL-17E (also called IL-25) functions distinctly different from IL-17 and IL-17F. IL-17E promotes Th2 cell responses by inducing the expression of IL-4, IL-5, and IL-13. *Il25*-transgenic mice or mice treated with ectopic IL-17E showed increased releasing of Th2 factors along with eosinophilia [105, 123, 213, 268]. In contrast, due to reduced Th2 response, *Il25*-deficient mice displayed impaired Th2 responses, thus were more susceptible to parasite infection but were resistant to allergic responses in lung [11, 64, 212, 268, 322]. Either immune cells (macrophages, dendritic cells, mast cells, eosinophils, basophils, and T cells) or nonimmune cells (epithelial cells and Paneth cells) could release IL-17E under certain conditions. As its producing cells, IL-17E can target diverse cell types such as Th2, Th9, NKT, monocytes, macrophages, non-B non-T (NBNT), and epithelial cells [115]. IL-17E is activated downstream signaling through the IL-17RA and IL-17RB receptor complex [228]. The adaptor protein Act1 was also found to mediate IL-17E signaling [38, 121, 265]. Recently, one study showed that intestinal commercial bacteria induced IL-17E production from intestinal epithelia cells and IL-17E in turn inhibited Th17 expansion by suppression of IL-23 production in macrophages [314]. In addition, *Il25*-deficient mice were susceptible to EAE induction [126], indicating a negative role of IL-17E in controlling Th17 responses.

Although the biological functions of IL-17B and IL-17D remain largely unknown [252], recent findings have begun to uncover the functional roles of IL-17C. We and others demonstrated that IL-17C was the ligand for the orphan receptor IL-17RE and delivered its signal through IL-17RA and IL-17RE complex [28, 224, 253]. IL-17C is specifically induced in epithelial cells and keratinocytes by pathogens or inflammatory cytokines and acted as an autocrine cytokine on those cells. Similar to IL-17, IL-17C activates common signaling pathways including NF-κB and MAPKs cascades [224, 253]. IL-17C also targeted Th17 cells for promoting IL-17 production [28]. *In vivo* studies from *Il17c*- or *Il17re*-deficient mice demonstrated that this pathway was critical for protection against intestinal pathogens as well as the progression of several autoimmune diseases including psoriasis, IBD and MS [28, 224, 253].

5.6.1.2 IL-17A and IL-17F Medicated Signaling

Positive regulator

Soon after it was cloned from activated murine T lymphocyte hybridoma cDNA library [232], the downstream signaling pathways of IL-17A were revealed. Typically, IL-17A activates NF-κB, MAPKs, and C/EBPs cascades to upregulate expression of inflammatory genes [251]. IL-17A also synergizes with TNFα to induce gene expression through stabilization of TNFα-induced mRNAs [251]. Other studies also found that it can activate JAK-PI3K and JAK-STAT pathways, although detailed downstream molecular mechanisms were still poorly understood [101, 234]. Recently, we found that IL-17A downregulated microRNA-23b to promote inflammatory responses in tissue resident cells [335]. IL-17RA was initially noticed as the receptor for *Il17a*- and *Il17ra*-deficient fibroblasts have no response to IL-17A stimulation [273, 304, 311]. Later, IL-17RC was found as a receptor component for IL-17A signaling by interacting with IL-17RA as well as IL-17A. Deficiency of IL-17RC in mice impaired IL-17A induced downstream gene expression [304]. Thus, upon IL-17A ligation, IL-17RA binds to IL-17RC to form a heterodimeric receptor complex to initiate intracellular signaling pathways. The receptor proximal signaling mechanisms have been uncovered recently (Fig. 5.4).

TRAF6 was firstly found as a positive adaptor in IL-17A signaling, and it was essential for IL-17A-induced activation of NF-κB and JNK pathways and expression of downstream genes such as IL-6 [240]. However, the intercellular region of IL-17RA did not contain any predicted TRAF6 binding motif and TRAF6 was found not responsible for IL-17A-induced mRNA stabilization pathway, suggesting that additional adaptors upstream of TRAF6 might exist for both TRAF6 dependent or independent pathways [89].

By bioinformatic searching, all IL-17R family receptors were found to harbor an intracellular SEFs and IL-17Rs (SEFIR) domain. Furthermore, this SEFIR domain structure also was also noticed in a cytosolic protein called Act1 (also known as CIKS) [202]. After IL-17 stimulation, IL-17R recruited Act1 to its intracellular region through SEFIR-SEFIR domain interaction [27, 220]. Act1 then recruited TRAF6 to IL-17R complex. Act1 was not only a simple adaptor for downstream molecules recruitment, but also was noticed as a U-box like E3 ligase, which mediated Lys63-linked ubiquitination of TRAF6 through recruitment of the Ubc13-Uev1A E2 complex [157]. We recently found that IL-17A downregulated the expression of microRNA-23b (miR-23b) through NF-κB pathway and subsequently releasing its suppression effect on TAB2 and TAB3 to further amplify NF-κB activation [335]. Thus, IL-17A can trigger a positive feedback loop through removing the suppression of miR-23b on NF-κB activation. Since the SEFIR domains are required for the interaction between Act1 and IL-17R, a detailed domain mapping study found that SEFIR domains contain a CC' loop structure and this structure was responsible for the interaction of adaptor and receptor [158]. A mimicking decoy peptide of Act1 CC' loop inhibited IL-17 induced inflammatory responses [158]. Though the SEFIR domain is important for IL-17A signaling

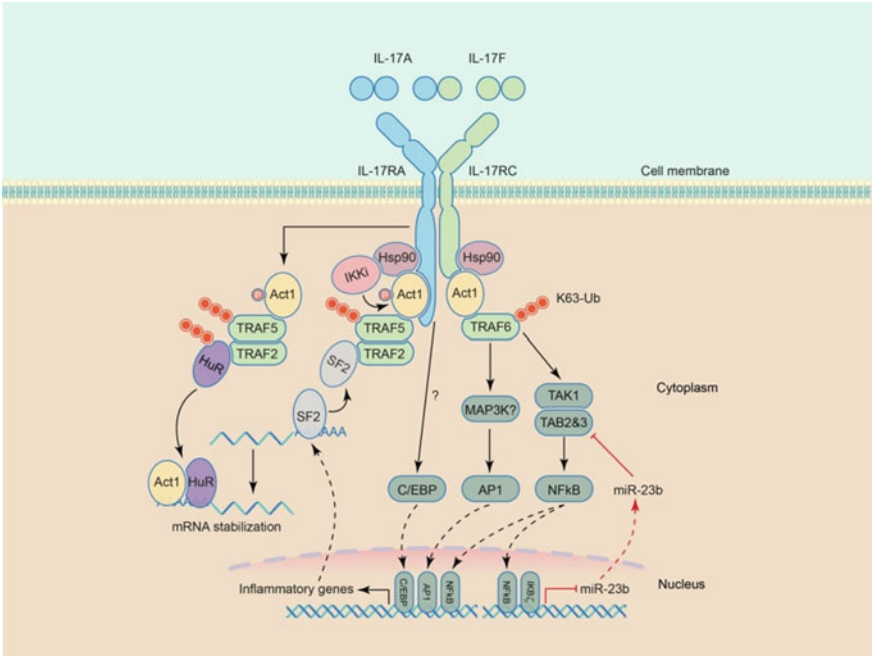

Fig. 5.4 The activation of IL-17 signaling. After IL-17A or IL-17F binding to the receptors, Act1 is recruited to the IL-17RA and IL-17RC receptor complex. Act1 then recruits and poly-ubiquitinates TRAF6 for the activation of NF-κB and JNK pathways. IL-17A-induced NF-κB signaling also suppresses the miR-23b expression and therefore releases its inhibition on TAB2 and TAB3 complex to amplify NF-κB activation. Act1 is also critical for IL-17A induced activation of C/EBP pathway. In addition, Act1 also recruits TRAF2 and TRAF5 to mediate mRNA stabilization pathway. Normally SF2 is bound to certain mRNAs for their degradation. Upon IL-17A challenging, Act1/TRAF5/TRAF2 complex recruits SF2 form mRNA and thus protect them from degradation. The phosphorylation of Act1 at Ser-311 by the kinase IKKi is crucial for the interaction of Act1 with TRAF2 or TRAF5. Meanwhile, Act1 also recruits and ubiquitinates an ARE-binding protein HuR through TARF2 and TRAF5. Act1/HuR complex then bind to the SF2 free mRNA to stabilize them. Hsp90 has been shown to be required for IL-17A signaling by promoting the function of Act1

transduction and inflammatory gene production, other studies also found that, beside to SEFIR domain, the C-terminal region and "TIR-like loop" (TILL) motif of IL-17RA, the cytoplasmic tail region of IL-17RC and the N-terminal domain of Act1, were also critical for IL-17A-mediated signaling [98, 170, 208, 248].

Act1 but not TRAF6 was required for IL-17A-mediated mRNA stabilization of KC induced by TNFα [89], indicating that Act1 mediates either TRAF6 dependent or independent pathways for IL-17A signaling. Recently, the inducible kinase IKKi (also called IKKε) was found for the Act1-mediated mRNA stabilization pathway. IKKi was showed to bind to Act1 upon IL-17 stimulation in mouse fibroblasts. Deficiency of IKKi in airway epithelial cells did not influence IL-17 induced NF-κB activation, but largely impaired IL-17-mediated KC mRNA stabilization in those

cells [22]. IKKi directly phosphorylated Act1 at residue Ser-311 after IL-17A stimulation and the phosphorylation of this site was critical for IL-17A-mediated mRNA stabilization of KC but not for NF-κB activation [22]. Another study showed that The Ser-311 phosphorylation of Act1 was required for recruiting TRAF2 and TRAF5 to generate an Act1-TRAF2-TRAF5 complex. IL-17A induced formation of Act1-TRAF2/TRAF5 complex further recruit mRNA splicing factor 2 (SF2) to prevent its binding and cleavaging of KC mRNA [262]. Besides, Act1 also recruited and ubiquitinated an ARE-binding protein HuR to stabilize the mRNA which released from SF2 [96]. More importantly, deficiency of HuR in epithelium resulted in impaired IL-17A-mediated inflammation in lung, agreeing with the essential role of Act1 in epithelium of lung [96]. More recently, upon IL-17A exposure, heat shock protein 90 (Hsp90) was found to be associated with Act1 and this interaction was critical for the binding of Act1 with other signaling molecules [285]. Inhibitors of Hsp90 prevented the signaling complex formation and in turn inhibited the activation of downstream signaling pathways [285]. Interestingly, Hsp90 lost a psoriasis associated Act1 mutant D10N [60, 104, 256] and this mutation failed to activate downstream signaling mediated by IL-17A [285]. To date, Act1 cooperates with Hsp90 to generate a receptor proximal anchor platform to assemble two distinct IL-17A-mediated cascades: (1) TRAF6-dependent pathway and (2) IKKi-TRAF2-TRAF5-dependent pathway.

IL-17F shares the same receptor set IL-17RA-IL-17RC with IL-17A for downstream signaling. Surface plasmon resonance (SPR) analysis found that human IL-17RA had a higher binding affinity to human IL-17A while human IL-17RC preferred to bind to human IL-17F [138, 295]. Although the binding affinities of those two cytokines are different, they mediated inflammatory responses completely required either IL-17RA or IL-17RC [98, 100, 273, 295, 304], suggesting their mediated downstream signaling all through the IL-17RA and IL-17RC heterodimer complex. Similar to IL-17A, IL-17F also needed Act1 and TRAF6 for downstream signaling [304] and induced NF-κB, MAPKs, and C/EBP activation in different cell types [122, 304, 330].

Negative regulator

As IL-17A exhibits a broad influence on driving inflammatory responses, its signaling is under strict control to prevent harmful persistent inflammation (Fig. 5.5). One early study found that IL-17A activated the kinases GSK-3β and ERK to phosphorylate C/EBPβ at Thr188 and Thr179 in its regulatory 2 domain, and this dual-phosphorylation in turn inhibited IL-17A induced expression of proinflammatory genes [245].

TRAF3 was a critical adaptor for TLRs and RIG-I induced type I interferon production in antiviral responses or was a negative regulator for CD40 or BAFF induced noncanonical NF-κB activation [33, 85, 92, 131, 204, 276, 299]. We recently identified that TRAF3 was an important negative regulator for IL-17A signaling [334]. Forced expression of TRAF3 in cells prevented IL-17A-mediated signaling activation and downstream cytokine production, while silencing of TRAF3 enhanced the activation of NF-κB and MAPKs pathways as well as expression of downstream genes. TRAF3 was found to directly bind to IL-17R for

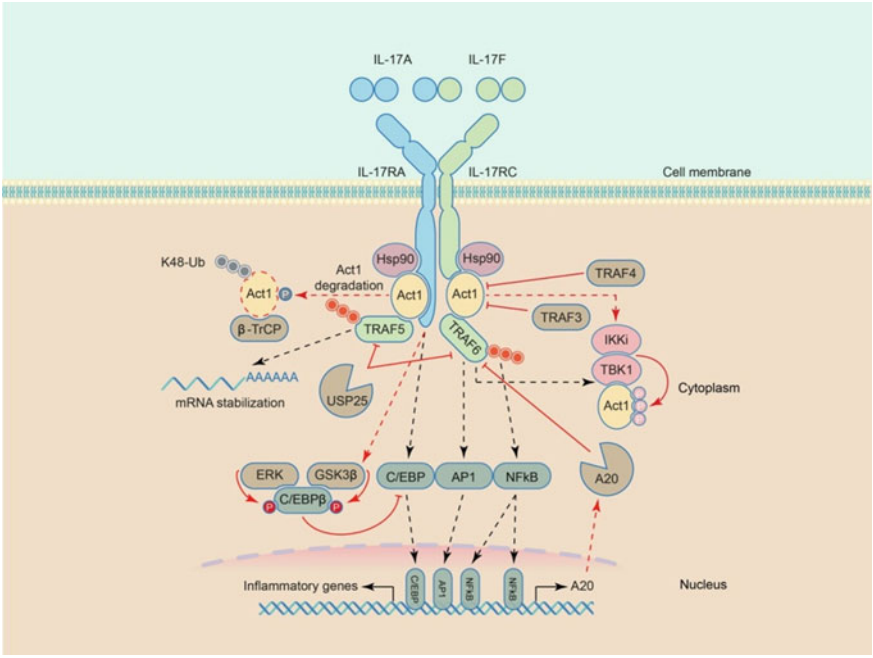

Fig. 5.5 The regulation of IL-17 signaling. Once IL-17 signaling is activated, several mechanisms are adopted to prevent over activation of the signaling. Upon activation, TRAF3 is recruited to IL-17RA and IL-17RC complex to interfere the formation of IL-17R/Act1/TRAF6 signaling complex, while TRAF4 binds to Act1 to disrupt the Act1/TRAF6 signaling complex formation. Although IKKi is required for IL-17A mediated mRNA stabilization pathway, IKKi and TBK1 also triple-phosphorylate Act1 to disrupt the interaction between Act1 and TRAF6 to suppress activation of NF-κB in a TRAF6-dependent negative feedback manner. IL-17A also activates ERK and GSK3β to phosphorylate C/EBPβ and thus suppresses C/EBP activation. USP25, a deubiquitinase, directly removes both TRAF5 and TRAF6 ubiquitination to inhibit IL-17-induced signaling. Another deubiquitinase A20 specifically removes the ubiquitination of TRAF6 to suppress IL-17-mediated NF-κB activation. With persistent IL-17A exposure, Act1 is phosphorylated and then ubiquitinated and degraded by $SCF^{\beta\text{-}TrCP}$ E3 ubiquitin ligase complex, therefore avoiding over activation of the signaling

disrupting the interaction complex of IL-17R, Act1, and TRAF6, thus suppressed IL-17 signaling activity. Transgenic TRAF3 in mice inhibited the IL-17 induced inflammatory responses and subsequently controlled EAE progression [100]. Thus, TRAF3 was identified as the first receptor proximal negative regulator of IL-17A signaling.

Similar to TRAF3, TRAF4 was lately identified to negatively control IL-17A signaling. Upon IL-17A treatment, deficiency of TRAF4 in cells led to remarkable exacerbated activation of IL-17A signaling and increased expression of chemokines. Genetic depletion of TRAF4 caused earlier onset of Th17 cell induced EAE

pathogenesis. TRAF4 suppressed IL-17A signaling by disruption the binding between Act1 and TRAF6 [316]. Therefore, in an alternative way, TRAF4 also interferes with the formation of positive signaling complex for controlling IL-17A signaling.

Ubiquitin-proteasome pathway mediated Protein degradation is a widely used strategy to desensitized specific signaling by erasing key signaling adaptors [197]. We recently found that Act1 protein level significant decreased upon IL-17A persistent stimulation, without affecting its mRNA level. Mechanically, Act1 was degraded in a Lys48-linked polyubiquitination manner by the $SCF^{\beta\text{-}TrCP\text{-}1/2}$ E3 ubiquitin ligase complex, which was the same for degradation of the inhibitor of nuclear factorκBα (IκBα), indicating that both negative and positive signaling events of IL-17 is controlled by the same degradation complex [246]. The degradation of Act1 eventually led to dismissing of downstream pathway activation and consequently desensitized cells to IL-17 exposure.

As described, IKKi phosphorylated Act1 at Ser-311 for IL-17A-mediated mRNA stabilization pathway [22]. Recently, we also found that both IKK-related kinases IKKi and TBK1 were recruited to Act1 upon IL-17A stimulation [222]. Both kinases phosphorylated Act1 on three serine sites different from Ser-311 and phosphorylation of those sites intertered with the association of Act1 with TRAF6 and subsequently suppressed IL-17A induced NF-κB activation. Notably, TRAF6 itself was needed for the activation of TBK1 and Act1 [222]. Therefore, in addition to its positive role in IL-17A signaling by phosphoryting Act1 at Ser-311, IKKi also displayed a redundant role with TBK1 though phosphorylation of Act1 on other Serine sites to control the signaling.

Since Act1 is an E3 ligase for TRAF6 to promote the signaling of IL-17A, the deubiquitinating enzyme (DUB) for negative regulation may exist. Recently, the ubiquitin-specific protease USP25 was found as a negative regulator of IL-17A signaling [326]. Overexpression of this deubiquitinating enzyme inhibited IL-17A induced downstream signaling. By contrast, deficiency of USP25 in MEFs or primary lung epithelial cells led to enhanced IL-17A-mediated responses [326]. Genetic depletion of USP25 in mice consequently enhanced IL-17A induced respiratory inflammation and exacerbated EAE induction [326]. USP25 was found to directly remove TRAF5 and TRAF6 ubiquitination induced by IL-17A and subsequently erase the signals for both TRAF6 dependent and independent pathways [326]. More recently, A20, another deubiquitinating enzyme, was identified as a negative regulator of IL-17A induced NF-κB and MAPK activation [69]. Notably, A20 itself was induced by IL-17A exposure and in turn formed a negative feedback control for IL-17A signaling. Deficiency of A20 in cells led to exacerbated IL-17A induced inflammatory gene expression [69]. Mechanically, IL-17R signaling directly recruited A20 to the distal domain of the receptor and therefore erased the ubiquitination of TRAF6 and also the activation of TRAF6 dependent cascades [69].

5.6.2 Th17 Effector Cytokines in Inflammatory Diseases

5.6.2.1 Th17 Effector Cytokines in Host Defense Against Infection

Th17 cells are found to be critical for host defense responses by releasing its effector cytokines IL-17A, IL-17F, and IL-22. The secreted IL-17A, IL-17F, or IL-22 in turn induces expression of host defense molecules in epithelial cells or keratinocytes to protect host from specific pathogens. Deficiency of IL-17A and IL-17F in mice caused increased susceptibility to the infection of extracellular pathogens, including *Klebsiella pneumonia*, *Citrobacter rodentium*, and *Staphylococcus aureus* [10, 110]. It was found that, an intestinal commensal bacterium, segmented filamentous bacteria (SFB), preferentially induced gut Th17 differentiation in steady state. Mice without SFB led to reduced Th17 proportion in intestine and were more susceptible to *Citrobacter rodentium* infection [113]. However, another study demonstrated that it was *Il22*-deficient mice but not *Il17rc* (a specific receptor for IL-17A and IL-17F)-deficient mice were susceptible to the infection [325]. IL-22 is a critical cytokine for mucosal immunity. It could directly promote epithelia proliferation to keep the epithelia barrier integrity during the infection of pathogens. IL-22 also acted alone or in synergy with other cytokines, such as IL-17A, IL-17F, IL-17C, or TNFα, to induce the expression of antimicrobial proteins such as S100A-proteins, β-defensins, or RegIII family proteins in host defense responses [10, 155, 253, 293, 294, 325]. In addition, IL-22 also induced the expression of multiple mucus proteins in globet cells, which are important for mucosal damage protection [260]. Furthermore, as IL-17A and IL-17F, this cytokine also stimulated the production of numerous inflammatory chemokines and cytokines production during infection [10, 155, 293]. Upon *Klebsiella pneumoniae* infection, neutralizing IL-22 led to remarkable bacterial dissemination in the lung and spleen [10]. Comparing with wild-type mice, the survival rate of infected animals treated with anti-IL-22 decreased significantly [10]. In this study, Th17 derived IL-22 acted in synergy with IL-17 to promote lung epithelia proliferation. The combination of IL-22 with IL-17 also induced several pro-inflammatory cytokines and chemokines or antimicrobial peptides expression in lung epithelial cells [10]. Another study also found that, comparing with wild-type mice, *Il22*-deficient mice died quickly after *Klebsiella pneumoniae* infection [301]. As described above, after *Citrobacter rodentium* infection, deficiency of IL-22 in mice led to increased intestinal damage, bacterial load and mortality [325].

In addition to extracellular pathogens, IL-17A or IL-22 also protected mice from intracellular bacterial and fungal infection. Upon *Francisella tularensis* infection, either neutralization or genetic depletion of IL-17A increased the bacterial burden in mice [156]. It was found that IL-17A, but not IL-17F or IL-22, was responsible for IL-12 production from dendritic cells and promoted Th1 activity during the infection [156]. IL-22, however, was found to protect *Il12p35*-deficient mice from liver necrosis upon *Salmonella enterica* serovar Enteritidis infection. Neutralization of IL-22 in *Il12p35*-deficient mice led to increased cell necrosis in the *Salmonella*

enterica serovar Enteritidis infected liver, indicating a protective role of IL-22 against this pathogen [239]. Th17 differentiation was strongly induced by an oral opportunistic fungus, *Candida albicans* [233]. Deficiency of IL-17A, but not IL-17F in mice, remarkably increased the susceptibility to oral candidiasis, indicating that IL-17A is critical against this pathogen-mediated pathogenesis [233]. In addition, in *Il17ra*-deficient mice, without IL-17A and IL-17F signaling, neutralization of IL-22 in mice increased *Candida albicans* burden in stomach [50]. Similarly, increased fungal burden in the kidney and stomach was found in *Il22*-deficient mice when these mice were intravenously infected with *Candida albicans* [50]. Mice intragastrically infected with *C. albicans* showed upregulated IL-22 in the stomach while several antimicrobial peptides such as S100A8, S100A9, RegIIIβ, and RegIIIγ were all decreased in *Il22*-deficient mice in this model [50], indicating that IL-22 protects against pathogen infections through inducing antibacterial genes in the epithelium.

5.6.2.2 Th17 Effector Cytokines in Autoimmune Diseases

Autoimmune diseases, such as MS and RA, have long been noticed as Th1, Th2, or B cells related diseases. Recently growing evidences showed that the Th17 subset and its unique cytokines IL-17A, IL-17F, and IL-22 display board effects on the pathogenesis of MS and RA as well as many other autoimmune diseases including inflammatory bowel disease, psoriasis, systemic lupus erythematosus, and type 1 diabetes mellitus [336].

MS is a central nervous system autoimmune disease characterized with loss of protective myelin sheaths around the brain and spinal cord axons. It was found that the expression of IL-17A as well as its producing T cells was highly elevated in the brain lesion regions of MS patients [161, 277]. Deficiency of IL-23p19, a specific cytokine subunit critical for Th17 cell differentiation, protected mice from EAE [142]. Genetic depletion of either IL-17A or its specific receptor IL-17RC suppressed the induction of EAE in mice [100, 127, 304]. However, it was found that IL-17F was not required for the initiation stage of EAE but the later stage of this disease seems decreased in *Il17f*-deficient mice [304]. One study identified that *Il22ra2* is a MS risk gene [18]. However, another study showed that *Il22*-deficient mice were still susceptible to EAE induction, suggesting that IL-22 appears not required for EAE [134].

RA is a systemic autoimmune disease which is characterized by symmetric inflammation that causes progressive joint cartilage destruction. Comparing with healthy volunteers and osteoarthritis patients, patients with RA showed remarkable increased IL-17A level in their rheumatoid synovium [130, 337]. In experimental murine models of RA, neutralizing of IL-17A ameliorated, while forced expression of IL-17A aggravated diseases progression [163, 164]. *Il17a*-deficient mice were protected from either collagen induced arthritis (CIA) or spontaneous autoimmune arthritis developed in *Il1rn*-deficient mice [110, 196]. The contribution of IL-17F to the arthritis pathogenesis was found to be extremely limited [110]. Increased IL-22

in serum was also found in human patients of RA, and its expression level was shown to correlate with disease activity [47]. In the CIA model, IL-22 level was increased and deficiency of IL-22 protected mice from CIA induction [71]. Blockade of IL-22 in *Il1rn*-deficient mice significantly reduced the inflammation and bone erosion [176]. However, another study revealed that mice treated with IL-22 prior to the onset of CIA had delayed progression of CIA and neutralization of IL-10 abrogated IL-22 protective effect [236]. These studies indicated that the role of IL-22 in RA still needs to be further characterized.

Inflammatory bowel diseases (IBD) is a chronic autoimmune intestinal inflammatory disease, which is caused by abnormal innate or/and adaptive immune responses. IBD can be classified into two major types, Crohn disease (CD) and ulcerative colitis (UC). IL-17A level was found to be upregulated in either CD or UC patients specimens [66, 259]. The expression of IL-17F was also higher in CD patients compared with UC patients [241]. Genome-wide association study (GWAS) data also identified IL-23R, STAT3, CCR6, as well as Act1, which were either critical for Th17 differentiation or IL-17 signaling, were IBD-associated genes [12, 37, 58, 286]. However, in murine models of IBD, the roles of IL-17A were still controversial. Antibody blockage of IL-17A or genetic depletion of IL-17A in mice caused exacerbated dextran sodium sulfate (DSS) induced colitis, indicating that IL-17A has a protective role of in this model [205, 304]. In contrast, comparing with wild-type mice, *Il17a*-deficient mice were protected from DSS induced colitis in another study [112]. The inconformity of those studies may largely due to the differences of gut microbiota among different facilities. In a parallel study of IL-17A, IL-17F was identified to enhance the inflammation of intestine as *Il17f*-deficient mice were protected from colitis induced by DSS [304]. Increased IL-22 expression has also been found in IBD patients [6, 20] and its serum level was correlated with disease severity [237]. The expression of its receptor IL22RA1 was also increased in UC patients tissues [117, 242]. Both antibody blockade of IL-22 function and genetic depletion of IL-22 or STAT3 in mice led to increased severe colitis, body weight loss and histological score, indicating IL-22 has a protective function in this model [218, 260, 315]. By contrast, IL-22 gene delivery reduced local intestine inflammation and induced the expression of mucus-associated proteins from goblet cells [260].

Psoriasis is a chronic skin immune disorder characterized by hyperplasia of dermis which is probably due to dysregulated interaction between immune cells and keratinocytes. Both IL-17A and IL-17F were remarkably upregulated in psoriatic skin biopsies from psoriasis patients [118, 119, 291]. As GWAS studies in IBD, similar approach also identified *Act1* and *Il23r* as psoriasis-associated genes in psoriasis specimens [23, 60, 104, 256]. In mice, skin intradermal delivery of IL-23 could cause a psoriasis-like symptoms and the skin hyperplasia was meliorated in either *Il17a*- or *Il22*-deficient mice [229]. IL-17A cooperated with other cytokines like TNFα, IFN-γ, or IL-22 to induce the expression of inflammatory genes and antibacterial molecules in human keratinocytes [4, 34, 86, 155, 269]. IL-22 has also been found as a critical mediator in the psoriatic pathology. Increased serum IL-22 level was found in psoriatic patients and showed correlation with the disease

severity [294]. IL-22-transgenic mice had acanthosis and hypogranularity symptoms which resembles psoriasis. These mice were born with stiff and shiny skin and then died quickly after broth [292]. Consistently, IL-23-mediated dermal inflammation was decreased in *Il22*-deficient mice [324]. Similarly, in an autoimmune psoriasis model, neutralization of IL-22 in wild-type mice showed either no development or very mild development of the disease [278]. Like IL-17A, IL-22 also could synergize with several other cytokines to promote the progression of psoriasis [211].

IL-17A is also found to be involved in the pathogenesis of other autoimmune diseases like SLE and T1DM. It was noticed that IL-17A was highly expressed in both SLE patients-derived double negative (DN) T cells and MRL/lpr mice [43, 321]. In a mouse model of SLE, comparing with *Fcgr2b*-deficient mice, recent study identified that *Il17a* and *Fcgr2b*-double deficient mice were resistance to the development of lupus [219]. IL-17A could activate inflammatory genes and autoantibodies production in peripheral blood mononuclear cells (PBMC) isolated from lupus patients [55]. These studies suggest that IL-17 is critical for the pathogenesis of SLE. Although the function of IL-17A in human T1DM progression remains unclear, in murine model of T1DM, IL-17A was noticed to aggravate autoimmune diabetes progression. Antibody blockage of IL-17A ameliorated autoimmune diabetes pathogenesis in non-obese diabetic (NOD) mice [61]. However, deficiency of IL-17A in NOD mice led to comparable hyperglycemia when compared with control NOD mice [127]. It was also noticed that plasma IL-22 levels decreased and correlated with disease severity in SLE patients [32]. However, the role of IL-22 in lupus still needs to be further studied.

As IL-17A signaling is critical for mutiple autoimmune diseases progression, the usage of blocking antibodies against IL-17 or IL-17RA to treat different autoimmune diseases such as RA or psoriasis has shown promising outcomes in clinical trials [72, 73, 103, 214]. Two humanized IL-17A antibodies, AIN457 and LY2439821, were in phase II trials for RA, MS, and psoriasis therapy and were shown no strong adverse safety concerns [73, 103]. A fully human monoclonal antibody to IL-17RA, Brodalumab, was developed to block IL-17R-mediated signaling and was undergoing in phase II trials for psoriasis and RA [214]. As IL-17RA is a common receptor subunit shared by other members of IL-17 family, the strategy for targeting IL-17RC, which is also critical for IL-17A-mediated signaling, maybe more specific.

5.6.3 Th17 Effector Cytokines in Allergy

Allergic diseases such as allergic asthma and atopic dermatitis (AD) are common chronic inflammatory diseases, which are characterized by infiltration of eosinophil, mast cells, and T cells. It was found that both IL-17A and IL-17F were remarkably

increased in bronchial specimens from asthma patients [3, 54, 188]. Enforced expression of both IL-17A and IL-17F led to increased infiltration of neutrophil, but no eosinophil in lung [105, 304]. In OVA-induced airway allergic model, IL-17A was noticed to promote while IL-17F suppressed the allergic responses [110, 196, 304]. However, IL-17F was also showed not required for OVA induced neutrophilia in lung [110]. It was found that host dust mite induced airway inflammation was also dependent on IL-17A [139]. More recently, IL-17A, but not IL-17F, was found to promote airway hyper-responsiveness by directly enhancing the contraction of airway smooth muscle [137]. However, IL-22 has been found to play a protective role in allergic diseases. Increased IL-22 expression was found in both atopic dermatitis (AD) patients and asthmatic patients [201, 217]. In a mouse asthma model, neutralization of IL-22 increased the eosinophil recruitment into the lung [267]. One group showed that IL-22 inhibited antigen-induced airway inflammation by decreasing IL-25 production in lung epithelia cell [267]. Another group showed that IL-22 attenuated allergic responses by inhibiting DC functions [238].

5.6.4 Th17 Effector Cytokines in Cancer

Th17 cells had been implicated to associate with cancer development in human, although its roles in tumor progression are still in controversy between different studies. In hepatocellular carcinoma or colorectal cancer specimens, patients characterizing with high level of IL-17A expression correlated with a poor prognosis [272, 319]. By contrast, patients with ovarian cancer had poor prognosis when the infiltrated IL-17A producing cells numbers and IL-17A expression level were low [135], indicating that the influences of IL-17A on tumorigenesis are largely dependent on the type and stage of tumors.

Several studies by using *Il17a*-deficient mice implicated that IL-17A mainly has a pro-tumorigenic role among different tumor models. Subcutaneous transplantation of bladder carcinoma cell line MB49 or melanoma cell line B16 in *Il17a*-deficient mice led to delayed growth rate of those cells [287]. In either spontaneous or chemical-induced colon cancer models, disruption of IL-17A in mice caused reduced intestinal tumor development in those mice [26, 106]. More recently, it was noticed that either *Il23p19*- or its receptor deficient mice on an *Apc*-CRC background displayed reduced tumorigenesis in intestine, agreeing with the early studies with *Il17a*-deficient mice [80]. Enterotoxigenic *Bacteroides fragilis* (ETBF), an intestinal commensal bacteria, was found to promote intestinal tumorigenesis in $Apc^{min/+}$ mice through promoting Th17 responses [297]. As described, this hematopoietic IL-23-Th17-IL-17A axis was found to mediate microbiota-driven tumor growth. However, we also found that IL-17C contributed to this process in a different way. Unlike IL-17A, which was produced by Th17 cells, IL-17C was specifically secreted by IECs [250]. Furthermore, IL-23 and IL-17A were shown to

be critical for tumor cell growth but not survival during intestinal tumorigenesis [80, 250]. By contrast, IL-17C signaling was critical for IEC survival but not proliferation during the early stage of intestinal tumorigenesis [250]. Although IL-17C has been reported to promote Th17 differentiation [28], we found that the intestinal local resident cells rather than infiltrated hematopoietic cells such as Th17 were critical for IL-17C-mediated tumorigenesis, suggesting that IL-17C-mediated IEC survival instead of Th17 cell differentiation is critical for promoting intestinal tumorigenesis [250]. Both IL-17A and IL-23 were also found to promote other cancers such as cutaneous chemical carcinogenesis, as *Il17a*-, *Il17ra*-, or *Il23p19*-deficient mice developed remarkably decreased skin tumors [91, 141, 288].

As mentioned above, Th17 cells were also correlated with ameliorated prognosis in some studies. Similarly, in some mouse models, those IL-17A producing cells were also found to suppress tumor progression. One study showed that Th17 transplantation inhibited tumor growth through the activation of anti-tumor $CD8^+$ cells [177]. Although there were studies showed that IL-17A enhanced tumor metastasis by showing that deficiency of IL-17A in mice reduced lung metastasis [24, 154, 287], other studies found controversial results by showing that IL-17A ablation led to increased lung metastasis [136, 177]. Thus, it is likely that the pro- or anti-tumor property of IL-17A is mainly dependent on the different tumor types and stages. The role of IL-17F in tumor development remains unclear. Similar to IL-17A, IL-17F was noticed to be critical for intestinal tumorigenesis in $Apc^{min/+}$ mice [25]. By contrast, IL-17F played a protective role in in chemical induced colon cancer model [271].

5.7 Conclusion and Perspective

The discovery of Th17 cells as a distinct T helper cell subset refreshes our understanding of both adaptive immunity and its related immune disorders. Th17 effector cytokines, IL-17A, IL-17F, and IL-22, show unique proinflammatory properties comparing to the typical cytokines of either Th1 or Th2 subsets. Recent progresses of Th17 differentiation and plasticity also expand our knowledge about T-cell development and complexity between different subpopulations. Recent advances in uncovering the molecular mechanisms of signal transduction of Th17 cytokines IL-17A, IL-17F, and IL-22, coupling with largely expanded understanding of their roles in human inflammatory diseases as well as in the relevant mouse models, provide great opportunities to develop new targets and therapeutic strategies. Further investigation of the programs of Th17 differentiation and the signaling mediated by Th17 effector cytokines will benefit for the understanding and therapy of human inflammatory diseases.

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Chapter 6
Tfh Cell Differentiation and Their Function in Promoting B-Cell Responses

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Abstract Follicular helper T cells (Tfh) are a newly defined helper T-cell subset that is specialized in facilitating B-cell responses. These cells have a unique tissue localization pattern and a distinct transcriptional program suited for the B-cell helper function. Co-opting of the follicular program affords regulatory T cells, NK T cells, and γδ T cells with opportunities to participate in the regulation of humoral immunity. Abnormal Tfh development and function can lead to immunodeficiencies, autoimmune inflammation, and tumors. Detailed understanding of Tfh cell differentiation and function in animal models and the human system promises better strategies toward vaccine development and therapies for inflammatory diseases.

Keywords Germinal center · Follicular helper T cells · B cells · High affinity antibody

6.1 Introduction

Human follicular helper T cells (Tfh) were initially described in 2000 as a population of activated T cells in tonsils marked by heightened expression of CXCR5, localization in the B-cell follicle and germinal center (GC), and an enhanced ability to support immunoglobulin production when co-cultivated with B cells [22, 106, 179]. In addition to CXCR5, Tfh cells were found to highly express ICOS, and the signaling lymphocyte activation molecule (SLAM)-associated protein (SAP). T-cell deficiency in any of these molecules leads to impaired GC formation and defective antibody production [6, 31, 44, 76, 79, 98, 143, 191]. However, it has been debated as to whether Tfh cells represent a separate lineage or subset of effector T cells in parallel to the more established Th1, Th2, Th17 or Treg cells [223], mainly due to a

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B. Sun (ed.), *T Helper Cell Differentiation and Their Function*,
Advances in Experimental Medicine and Biology 841,
DOI 10.1007/978-94-017-9487-9_6

lack of unique transcription factors and effector cytokines associated with Tfh functions. In 2009, three groups simultaneously identified Bcl6 as the master transcriptional regulator for Tfh cells [95, 158, 218]. Interleukin 21 (IL-21) has subsequently been demonstrated as a key Tfh cytokine for the in vivo B-cell helper functions [123, 157, 169]. These studies together strongly support Tfh cells as a separate effector lineage. On the other hand, Tfh cells also share features and developmental pathways with Th1, Th2, Th17, and Treg cells. For example, Tfh cells can secret moderate levels of canonical Th2 and Th17 cytokines [15, 111, 174, 219]. Tfh cells can develop from Th2 or $Foxp3^+$ cells under certain conditions [194, 220], making it still work in progress to precisely define the relationship between the Tfh cells and the other Th subsets. While a definitive answer to such a basic question is beyond our reach for now, this chapter is intended to give a general overview of the Tfh development, function, regulation, and implications in diseases.

6.2 Tfh Cells in the Anatomical Context of a T-dependent B-Cell Response In Vivo

6.2.1 Initial T-Cell Activation in the T-Cell Zone

Tightly regulated series of cellular interactions are critical for Tfh cell development and help delivery to B cells. Naïve CD4+ T cells crawl on fibroblastic reticular cell (FRC) network in T-cell zones. They express high levels of CCR7, while the FRC network produces the ligands for CCR7–CCL21 and CCL19. The chemokine gradient extends from the T-cell zone to the T–B border, a region where resting B cells can travel in their normal migratory life (the T–B border defined here includes the inter-follicular region). Via chemotactic response induced by CCL21 and CCL19 and close interaction with FRC, T cells entry to paracortex and are limited to move within this region [73, 134, 216]. In parallel, naïve B cells highly express CXCR5 and follicular dendritic cells (FDC) in follicle express the cognate ligand CXCL13 [56, 72, 152]. B cells crawl on FDC and are restricted to migrate within follicles [9]. Stromal network expressed chemokines not only attract and restrict cell migration through chemotaxis, but also enhance their motility through haptokinesis [164, 186].

Resting T cells follow a “random walk” and scan for cognate antigens presented on dendritic cells (DCs) [122, 147]. T cells in LN move in an amoeboid manner at high speed, about 10–12 μm/min [21, 148, 187], and form some dynamic interactions with DC. The duration of these interactions are at least influenced by T-cell receptor (TCR) recognition of low affinity self-MHC class II ligands [141]. Steady-state DCs are rather sessile than motile, but they actively move their dendrites. The DC dendrites probing and T lymphocyte migration result in efficient DC and T-cell interaction ($\sim$5,000 T cells/h) [122, 147]. Rich DC distribution in T-cell zone, fast speed random T-cell migration, and high T-DC interaction frequency, ensure that any given naive T cell in the T-cell zone will find its specific antigen-bearing DC

during a 12–24 h passage through a LN. However, rather than employing this crucial scanning process as the only safeguard, the adaptive immune system take advantage of several additional means to optimize the encounter. Stromal networks serve as a superexcellent anchorage scaffold. By imaging adoptively transferred, SNARF-1-labeled naive T cells in bone marrow chimeric animals in which GFP was expressed by all nonhematopoietic nucleated cells, T cells were found to crawl on a three-dimensional FRC network on which DC also reside [9]. Thus, the T-DC encounter frequency was largely increased.

Circulating naive T cells exit from HEV through openings and migrate directly onto the FRC-DC network surrounding the HEV. Given the dense package of DC on the FRC network, most of the visualized T cells on this network are in contact with DCs. In vitro data demonstrate that DC support CCL21 induced T-cell motility in an ICAM-1-dependent manner. In addition, CCL21 and ICAM-1 colocalization was detected on DC surface, which suggests that DC may enhance T-cell motility through their surface decorated CCL21 and ICAM-1 in vivo [103]. Finally, naïve T cells enter the LN via HEV, which locates at the paracortical ridge, where newly arriving migratory DCs accommodate [10]. By this means, newly arriving T cells and matured DC gather in the same LN compartment, again facilitating imperative cellular interactions (Fig. 6.1).

6.2.2 T-, B-Cell Migration and Interactions at the T–B Border

T cell priming by antigen-bearing DC in LN occurs in three distinct phases [145, 146]. Within hours of antigen encounter, CD4+ T cells down-regulate CCR7 and up-regulate CXCR5, and move to T-B border in response to FDC expressed CXCL13 [79, 106, 179]. High levels of CCR7 expression impede T-cell migration to follicles. Down-regulation of CCR7 or expression of CXCR5 seems sufficient for T cell positioning at T–B border [76, 79]. In the absence of CXCR5, the GC is impaired and Tfh frequency is reduced [6, 76, 79, 98].

In parallel to the activation of T cells, B cells activated by soluble antigens perfusing into the follicle or by cell-bound antigen presented by DCs around HEV in the extra-follicular region rapidly up-regulate CCR7 and move to T–B border [173]. The T–B borders as well as interfollicular zones are sites where T cells and B cells first interact [61, 105, 165]. The B cell-dependent phase of Tfh differentiation occurs at these regions. It is visualized through intravital microscopy that cognate T and B cells form long-lived interaction at T–B border and interfollicular region [105]. These regions are considered as the sites where Tfh differentiation initiates defined by Bcl6 up-regulation [105]. It is important to note that CXCR5 expression was also recognized as a defining feature of Tfh commitment [202]. However, new evidence suggests that CXCR5 expression on CD4+ T cells is Bcl6-independent in the early phase of immune response [128, 129] and ASCL2 is identified as another transcription factor driving Tfh differentiation [127]. Also, considering early Th1

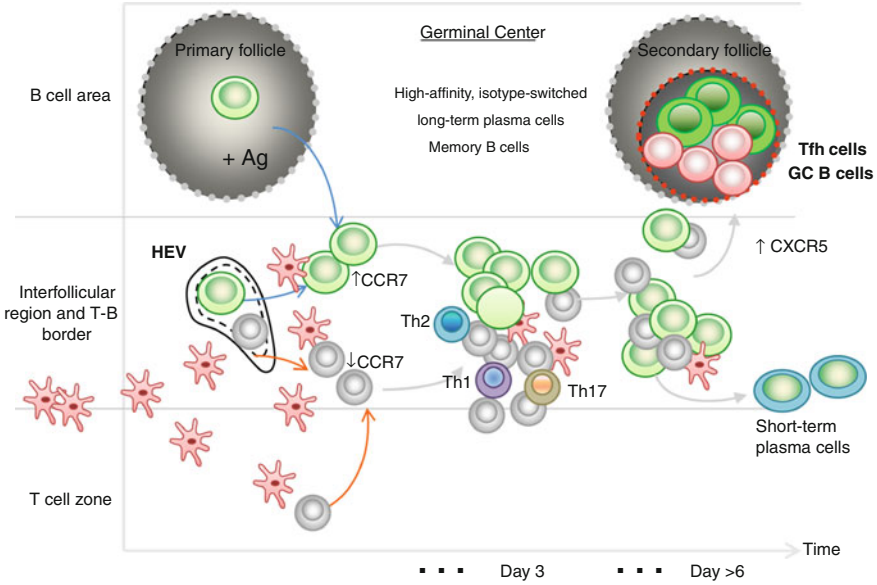

Fig. 6.1 Spatiotemporal dynamics of a primary T-dependent B cell response. Upon immune challenge, DCs carry antigen into the lymph node and present to both T and B cells. B cells can directly recognize free antigen or acquire antigen from other cells such as macrophages (not depicted). Antigen-triggered T and B cells are concentrated at the T–B border and interfollicular region earlier during the response. Receiving differentiation cues from DCs and other cells in the microenvironment, T cells begin to gain effector potentials of different lineages. Some of these T cells interact with cognate B cells and deliver help signals necessary for GC formation. Some of the activated B cells develop into short-term plasma cells, while some other B cells return to the follicle together with CXCR5-expressing T cells to form the GC. Within the GC, B cells isotype-switch, hypermutate and undergo affinity-based selection by T cells to become long-lived plasma cells and memory B cells

development passes through a Tfh-like stage [154], the very earliest stage of Tfh commitment remains to be further defined. Whereas long-lasting T-B interactions have been visualized at the T–B border, precise signals that T cells exchange with B cells at this location are yet to be fully characterized.

6.2.3 Follicular Recruitment of Activated T Cells

After the T–B border phase, the early committed Tfh cells further migrate into follicles. Although CXCR5 expression is sufficient for CD4+ T cell positioning to the T–B border, it is not sufficient for re-positioning T cells into the follicle [5, 79], indicating additional factors involved in this process. ICOS is a classic CD28 family co-stimulatory molecule up-regulated on activated T cells and highly expressed by Tfh cells [185]. It has long been appreciated for its co-stimulatory function [175]

and is demonstrated to be essential for a productive T-dependent B-cell response. A new mechanism of ICOS-mediated Tfh development has recently been discovered. ICOS-deficient T cells are found to be defective in follicular recruitment even when CXCR5 is overexpressed. At the T–B border, bystander B cells, rather than antigen-presenting DCs or cognate B cells, provide the necessary ICOSL for incoming T cells. The ICOSL-ICOS signaling directly drives persistent T-cell motility by a PI3K-dependent mechanism [215]. It is interesting to note that optimal T-cell migration only becomes dependent on ICOS signaling at the T–B border, likely also in the follicle, but entirely dispensable in the T-cell zone. The combined requirement of CXCR5 and ICOS for follicular T-cell seeding perhaps serves as a protective mechanism to avoid the inherent danger of mixing activated T cells into large numbers of follicular B cells that may contain self-reactive receptors. This notion is consistent with the autoimmune syndrome that is associated with ICOS dysregulation [50, 201].

6.2.4 Tfh Cells Inside the GC

Within the follicles, two related Tfh populations are identifiable: Tfh and GC-Tfh, or pre-Tfh and Tfh, according to different nomenclatures. Tfh cells are CXCR5 +Bcl6+CD4 T cells with elevated ICOS and programmed death 1 (PD-1) expression. GC-Tfh cells are further differentiated Tfh cells with the highest levels of Bcl6 and CXCR5 expression. The signature of GC-Tfh is Bcl6++CXCR5++ [79, 219] in mouse and Bcl6+CXCR5hi (vs. CXCR5int) in human tonsil [138, 172]. The highest expression levels of CXCR5 and PD-1 are most frequently used as surrogate markers for GC-Tfh cells in mice [218], while CXCR5 expression combined with high levels of ICOS used in humans [172]. GC-Tfh cells are considered as a further polarized state of Tfh cells.

The Tfh development requires ongoing antigen presentation from APCs. Whether B cells are necessary for Tfh formation depends on the antigen availability. In the presence of excessive antigen, B cells are not required for Tfh cell development, but they become necessary when antigen is limiting. SAP has a critical role in supporting T cell–B cell adhesive interactions but is not essential for sustained CD4 T cell adhesion to DCs [28, 171]. It has been reported that early development of Tfh cells is normal but their functions compromised in the absence of SAP [101, 171], while defects in late Tfh development were noted without SAP expression [125]. The apparent rescue of SAP-deficient cells for Tfh development by antigen boost suggests that the requirement for B cells to support Tfh development relies on their ongoing antigen presentation to T cells rather than delivery of unique signals [48]. At the GC stage, when antigen is limiting, B cells would be expected to have an advantage over other APCs in the acquisition and presentation of antigen due to their high-affinity BCR to efficiently capture antigen and their easy access to antigen deposited as immune complexes on FDCs [189].

Within follicle and GC, CXCL13-producing FDCs attract both CXCR5-expressing Tfh and B cells and also serve as the 3-D scaffold for their migration,

thus promoting efficient T-B encounter and antigen-specific interaction. Tfh cells may interact with FDCs and directly receive differentiation signals. For example, IL-6 is required for Tfh development [157], and can be produced by FDC in GC [114]. IL-6 production late in an immune response promotes Tfh development and/or maintenance [77]. Therefore, FDCs likely contribute to a microenvironment that is conducive for Tfh development by cytokine production and possibly even by contact-dependent mechanisms.

Regardless of whether unique B-cell signaling is dispensable for Tfh development, the T–B interaction is required for Tfh function to deliver help signals to B cells. Thus, although generation of cells of the Tfh phenotype can be boosted with increasing amounts of antigen, SAP-deficient CD4+ cells remains unable to support normal B cells responses as measured by either GC formation or antigen-specific antibody production [44, 46, 84, 137].

It is currently unclear as to whether GC-Tfh cells are terminally differentiated, or cycle between GC-Tfh and Tfh states, or can further differentiate into memory Tfh cells. The proposal that GC-Tfh cells are terminally differentiated and destined to die within GC is based on observations that GC-Tfh cells express a very high level of PD-1 [79, 125, 172, 219] and are susceptible to apoptosis in vitro [172]. Constant cycling between GC-Tfh and Tfh states remains a possible scenario [183].

6.3 Tfh-Associated Molecules, B Cell Help, and Bcl6-Mediated Regulation of Tfh Cells

6.3.1 Tfh-Associated Molecules

Tfh cells have unique gene expression signatures. In addition to CXCR5, Tfh cells also express high level of PD-1 and ICOS [131, 219], which interact with PD-1L1/PD-1L2 and ICOSL, respectively [66, 79, 172]. PD-1 is an inhibitory receptor playing a key role in T-cell tolerance, and high levels of PD-1 on Tfh cells may be a result of constant TCR activation [13]. PD-1 KO and PD-1L1/PD-1L2 double KO mice exhibit increased Tfh development after protein immunization [67]. ICOS-ICOSL signaling during naïve T cell priming by DCs [87] and subsequent T-cell interactions with B cells promote Tfh differentiation [19, 82, 215] and maintain Tfh cells in the GC [2, 59]. Highly expressed on activated T cells, CD40L is the only ligand of CD40, which is indispensible for GC formation [195, 196]. CD40-CD40L signaling exchange during Tfh-GC B-cell interactions regulates Bcl6 expression by inducing NF-κB and IRF4 [113, 212, 213]. Feedback signaling from CD40 to CD40L is crucial for T cell priming and further differentiation [69, 212]. OX40 is a member of TNF receptor family, playing an important role for activated T-cell survival [127]. As reported to induce CXCR5 mRNA expression [55], OX40 may be involved in promoting T cell migration towards the T–B border [139, 206, 207]. OX40-OX40L signaling also enhances plasma cell Ig secretion but may not affect GC maintenance [127, 203, 206].

Besides the surface ligands and receptors as mentioned above, other molecules participate in the T-cell-dependent GC reaction process. SLAM-associated protein (SAP) is identified as the gene whose loss of function is responsible for the X-linked lymphoproliferative (XLP) disease [170, 178]. SAP contains an SH2 domain that can bind to the cytoplasmic tail of SLAM family numbers such as SLAM, CD84, Ly108 and Ly9 [91, 155, 214]. SLAM family members have at least one immunoreceptor tyrosine-based switch motifs (ITSMs) on their cytoplasmic tails [53], and through which SLAM family members bind SAP with high-affinity [53, 182]. Binding enables SAP to block the recruitment of the SH2 domain–containing protein tyrosine phosphatase, SHP-2, to the phosphorylated tyrosine in SLAM family members tails [83, 178, 182]. Together with SLAM, SAP is recruited to the immunological synapse formed between T cells and antigen presenting cells [30, 83, 144]. In SAP-deficient mice, primed T cells can express Tfh markers earlier on, but severe defects in the GC formation ensue, as discussed above [44, 116]. IL-21 is another Tfh-associated molecule that is more important for Tfh function than for their formation. Although autocrine IL-21 may enhance Tfh development [157, 204], other T-cell subsets such as Th17 cells can express IL-21 [38, 108, 167]. In IL-21 or IL-21R knockout mice, the magnitude of the GC response is not severely affected [123, 167, 225], while IL-21 promotes plasma cell differentiation [166, 167] (Fig. 6.2).

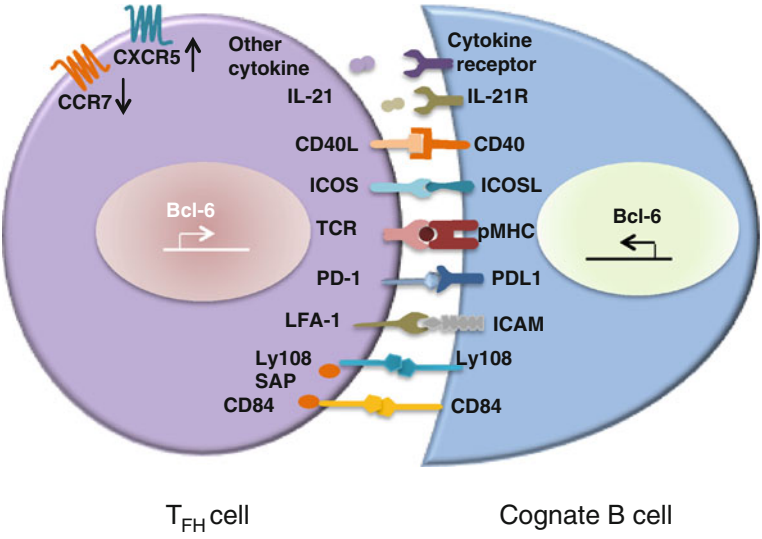

Fig. 6.2 Molecules involved in mediating T_{FH} functions. Chemokine receptors CXCR5 and CCR7 control follicular T-cell recruitment and promote cognate T-B cell co-localization. ICOS directly regulates this process as well. Various molecules modulate the strength of physical T–B interactions and the efficiency with which surface-bound (i.e., CD40L) and soluble helper-signal factors (i.e., IL-21) are delivered to cognate B cells. Bcl-6 is required for activated T cells to develop into T_{FH} cells and for cognate B cells to differentiate into the GC state

6.3.2 Contact-Dependent B Cell Help by Tfh Cells

CD40L is one of the most important contact-dependent help factors that Tfh cells deliver to cognate B cells, as when CD40L or CD40 is deficient, no GC will develop [57, 75]. Using two-photon microscopy, Okada et al. [165] first observed physical interactions between antigen specific T and B cells in vivo as long-lasting mobile conjugates. Such tight synaptic connection ensures T cells deliver help specifically to antigen-presenting B cells. Using SAP deficiency as an example, CD40L expression is not reduced in SAP deficient T cells, but GC cannot form, presumably due to a lack of stable synapse for CD40L delivery [171]. Several molecules from the CD28 superfamily are involved in the T-B synapse, reside in the cSMAC, and are crucial for T–B interactions. These include CD28-B7, ICOS-ICOSL and PD-1-PD-L [181]. ICOS, first reported in 1999, is not expressed on naive T cells, but induced rapidly after T-cells activation [90, 217], is highly expressed on Tfh cells. It is well known that ICOS is essential for Tfh cell formation. ICOS deficiency could reduce the proliferation of T cells; influence the development of Th2 cells and production of cytokines and high affinity antibody [143, 181]. Tfh and GC B cells was obviously reduced or even disappeared in ICOS deficient mice [2, 36, 157, 191]. Mouse with Roquin mutation, which is the inhibitory gene of ICOS, was observed having excessive number of Tfh cells, and suffering severe autoimmune disease [50, 201]. ICOSL (B7RP-1) is the only ligand of ICOS known at presents, and is constitutively expressed on B cells [188, 217]. ICOSL deficiency could influence T-cell dependent GC formation [140, 157, 160]. ICOS-ICOSL interactions are important for both T and B cell partners [140]. Studies of ICOS-deficient mice indicate that ICOS signaling may up-regulate CXCR5 expression by inducing Bcl6 and thereby influence Tfh differentiation [2, 36, 52, 63, 85, 157, 191]. However, CXCR5-deficient T cells could also develop into GC-localized Tfh cells to promote the GC B-cell response [6, 79]. SLAM family proteins belong to the CD2 superfamily and can be localized to the T–B synapse [29]. SLAM family proteins recruit the SAP adaptor, and when SAP is deficient, T cells cannot normally interact with the cognate B cells [171]. This is in part because Ly108 recruits protein phosphatase SHP-1 through its cytoplasmic tail [99] and targets the TCR complex through both its cytoplasmic tail and transmembrane domain (our unpublished data). Another SLAM family member CD84 may also participate in regulation of cognate T–B interactions. CD84-deficient T cells have a reduced ability to interact with B cells in vitro and in vivo, and CD84-deficient mice exhibit decreased GC formation following protein immunization [28], although no such difference was observed in a model of acute viral infection [99], suggesting the contribution by CD84 is more quantitatively than qualitatively necessary.

T–B interactions operate in the GC, with CD40L still one of the most important signals [7, 35, 75, 130]. PD-1, also a member of CD28 co-stimulatory family, is highly expressed on Tfh cells [51, 79]. Its ligands PD-L1 and PD-L2 are expressed on GC B cells [67]. PD-1 could affect the dynamic formation of immunological

synapse between T and B cells through blocking TCR signaling pathway [54], and may regulate how Tfh cells and B cells exchange signals [104]. The inhibitory signals that PD-1 and its ligands deliver help to maintain a balance between the need for T-cell activation and the risk of immunopathology resulting from over-activated T cells [88]. Mice deficient in PD-1 or PD-L1 generate fewer long-lived plasma cells, suggesting that PD-1 probably regulates selection and survival events in the GC [67]. SLAM-mediated IL-4 production is a particular feature of GC-located Tfh cells, possibly due to the high level of SAP expression [109, 219]. CD84 and Ly108 are both up-regulated in Tfh and GC B cells, and they may execute functions unique to the GC stage [28].

6.3.3 Tfh Control by Bcl6 and Other Transcription Factors

Bcl6, a BTB-POZ (bric-a-bric, tramtrack, broad complex–poxvirus zinc finger) family of transcriptional repressors member, originally identified as the master regulator of GC B cells by controlling cell cycle, DNA damage response, and signal transduction [113], is found to be crucial for Tfh development as well [95, 158, 218]. In the absence of Bcl6, hardly any cells of the Tfh phenotype can be found following immunization or viral infection. Terminally differentiated CD4 T cell subsets express high levels of Blimp-1, while Tfh cells express a high level of Bcl6, with Blimp-1 and Bcl6 antagonizing each other [43, 126, 142]. T cells that are forced to constitutively express Blimp-1 fail to differentiate into Tfh cells, giving rise to the notion that primed T cells may be determined to Tfh or non-Tfh, depending on the Bcl6 expression [43, 53, 95]. Bcl6 functions in part by repressing master transcriptional regulators for other T-cell subsets, such as T-bet for Th1, GATA3 for Th2, and RORγt for Th17 [158].

Following immunization or virus infection, enhanced Bcl6 expression could be detected in 2–4 day in dividing T cells [36, 105, 112]. T cells activated in vitro upregulate Bcl6 within 30 min [58]. The increase in Bcl6 expression requires DC priming but not B cell-mediated antigen presentation [65, 105]. CXCR5 is upregulated with a slightly slower kinetics than Bcl6 [112, 128]. The CXCR5+ T cells down-regulate CCR7 and then migrate into the follicle [5, 79]. IL-6 and IL-12 secreted by follicular B cells may promote further Bcl6 up-regulation. In contrast to IL-6 and IL-12, IL-2 potently inhibits GC reaction by repressing Tfh development through STAT5 activation and Blimp-1 upregulation [11, 94, 161].

Although Bcl6 is considered as the key transcription factor for Tfh cells, how Bcl6 regulates Tfh development and function remain unclear. In human T cells, retrovirally enforced expression of Bcl6 leads to up-regulation of CXCR5, PD-1, SAP, ICOS and CD40L [115], implying that Bcl6 programs all functional modules characteristic of Tfh cells. It is not yet clear if these molecules are direct targets of Bcl6-mediated transcriptional regulation. Yu et al. [218] showed that Bcl6 may suppress microRNA species, particularly those of the mir-17–92 cluster that may suppress CXCR5. New evidence indicates, however, mir-17–92 does not suppress

CXCR5; These microRNA species actually promote Tfh development and GC formation in part by suppressing phosphotase phpp2, which antagonizes PI3K signaling downstream of ICOS and PI3K [14, 102]. It has also been shown that enforced Bcl6 expression suppresses production of Th1/2/17 cytokines and related transcription factors such as T-bet, GATA-3 and ROR-γt [218]. Biochemical analysis indeed demonstrates Bcl6 is recruited to the IFN-γ locus and physically interacts with T-bet [162, 163]. Similar evidence is also available for Th2 and Th17 loci [132, 150]. Therefore, Bcl6 may program Tfh development by suppress T-cell differentiation into those other Th fates.

Blimp-1, an antagonist of Bcl6, is significantly suppressed in Tfh cells but not in other Th lineages [43]. Studies of CD8+ T cells reveal that Blimp-1 is associated with terminal effector differentiation and inhibits CD8+ memory T cells formation [100]. In CD4+ T cells, over-expression of Blimp-1 reduces Tfh cells development in vivo and Blimp-1 deficient mice exhibit excessive Tfh expansion [95]. According to the expression kinetics of Bcl6, Tfh commitment may be initiated at inter-follicular region after DC priming. Because IL-2 can trigger a STAT5-dependent process to up-regulate Blimp-1 expression, which then in turn suppresses Bcl6 and drive terminal effectors T-cell differentiation [94, 161], Blimp-1^{lo} T cells are potential Tfh precursors that may further up-regulate Bcl6 and be driven into the follicle away from the T-B border rich in IL-2.

In addition to Bcl6 and Blimp-1, a variety of other transcription factors also participate in the regulation of Tfh development and function. In mice, T cell-specific STAT3 deletion reduces Tfh formation [157, 158], and STAT3 mutations in human also lead to defects in Tfh generation, possibly as a result of reduced production of IL-6, IL-12, and IL-21 [8]. The c-Maf regulates production of IL-21 [81, 115] and IL-4 [15, 159, 224] in T cells and may thereby impinge on Tfh development and function. On the other hand, STAT5 can suppress Tfh in addition to Th17 differentiation [40, 94, 161]. BATF and IRF4 are also required for normal Tfh development, although underlying mechanisms are not fully clear [17, 18, 92]. Ascl-2, a novel basic-helix-loop-helix transcription factor, has recently been found to control early CXCR5 upregulation and initial Tfh commitment [127].

Current evidence indicates Tfh cells are epigenetically flexible. Lysine 4 trimethylation of histone H3 (H3K4me3) marks permissive active chromatin conformations, whereas H3K27 trimethylation (H2K27me3) marks repressive chromatin modifications. Tfh cells sorted from immunized animals or developed in culture exhibit both H3K4me3 and H3K27me3 histone marks at T-bet, GATA-3 and ROR loci, although they display mainly negative markings at IFNγ, IL-4, and IL-17 cytokine loci [132, 211]. On the other hand, following viral infection or protein immunization, sorted Tfh and non-Tfh cells preferentially give rise to progenies of respective types upon secondary challenge [37, 74, 128], suggesting additional epigenetic mechanisms imprinting these preferences are yet to be discovered.

6.4 Other Follicular T Cells that Regulate the T-Dependent B-Cell Response

6.4.1 Follicular Regulatory T (Tfr) Cells

Suppression by regulatory T cells (Treg) serves as a vital mechanism of negative regulation of immune response [96]. The fact that Treg cells can migrate to follicles upon T-cell activation and suppress T dependent GC reaction has been appreciated [121] even before the concept of Tfh cells was well accepted. However, their unique features were not well studied until recently [39, 124]. These follicular regulatory T (Tfr) cells were identified as a subset of Treg cells expressing CXCR5 and Bcl6 that localize to the GCs in mice and humans. They share phenotypic characteristic with both Tfh and Treg and their development depends on Bcl6, SAP, CD28 and B cells. Mice deficient in PD-1 and its ligand PD-L1 harbor more abundant Tfr cells in the lymph node and these Tfr cells exhibit enhanced suppressive ability [176]. Consistent with their suppressive function, Tfr cells lack expression of CD40L, IL-4 and IL-21. Tfr cells are not iTreg cells that can be induced from conventional naive T cells but derived from thymic Foxp3+ precursors. In mice deficient in Tfr cells during an immune response, an outgrowth of non–antigen-specific B cells in GCs leads to fewer antigen-specific cells and attenuated humoral responses. Therefore, similar to other specialized Treg subsets, Tfr migrate into GCs to exert their immunoregulatory functions. Their suppressive mechanisms remain largely unknown.

6.4.2 Follicular NKT (NKTfh) Cells

Natural killer T (NKT) cells represent a subset of T lymphocytes and compose 1–2 % of the lymphocyte population in the murine spleen and average 0.1–0.2 % of T cells in peripheral blood of human [16, 71, 118]. A subset of NKT cells, termed invariant NKT cells (iNKT), express a semi-invariant TCR that uniquely recognizes glycolipid antigens (typically α-GalCer) presented by the MHC I-like molecule CD1d [64]. These cells have been implicated in controlling infectious diseases, allergy, asthma, autoimmunity, and tumors [97]. Without exposure to pathogens, iNKT cells exhibit a wide tissue distribution, while migrating to the splenic marginal zone within a few hours of activation and can be found in GCs 3 days later [205].

Human iNKT cells can efficiently promote autologous naive or memory B-cell proliferation in vitro, and can induce immunoglobulin production [60]. Their B-cell helper ability can take place in a cognate or noncognate manner. Studies with CD1d-/- and Jα18-/- mice demonstrate that iNKT cells can provide cognate help to lipid antigen-presenting B cells and that the help signals involve CD40L, B7-1/B7-2, and IFN-α but not IL-4 [117]. Similar to Tfh cells, iNKT cells can form stable conjugates with B cells presenting glycolipid antigen and induce the formation of

early GCs [33, 110]. These iNKT cells highly express CXCR5 and PD-1, and their helper activities require Bcl6 and IL-21. On the other hand, αGalCer treatment can enhance the B-cell response even when B cells do not express CD1d, as far as CD40 expression on B cells is intact. Activated iNKT cells may stimulate other antigen-presenting cells that in turn activate Th cells and execute helper functions for B cells [193]. An important distinction between B cell responses helped by iNKT cells and CD4 Tfh cells is that the former does not generate long-lived plasma cells [192]. The fact that iNKT cells can co-opt the follicular program but exhibit distinct features of the helper program suggest nonredundant functions executed by the iNKT and regular Tfh cells.

6.4.3 Follicular γδT Cells

γδ T cells develop largely in the thymus, generating their defining γδ TCR via recombination activating gene (RAG)-mediated V(D)J recombination [197]. Similar to NKT cells, γδ T cells express a semi-invariant TCR repertoire and recognize nonpeptidic phosphoantigens that are derived from microbial metabolites [197]. While conventional αβ T cells are mainly involved in adaptive antigen-specific immunity and give rise to long-term immune memory, γδ T cells respond rapidly with innate-like reactions. In mice and humans, a lack of conventional αβ T cells can still allow GC formation and antibody production under certain conditions [198]. This has led to the realization that γδ T cells can help B cells to initiate GCs formation. Indeed, a subset of human γδ T cells express CXCR5, located in the follicle, express ICOS and CD40L, secrete IL-2, IL-4, and IL-10, and stimulate B cells for Ab production [23, 24]. Interestingly, γδ T cells express IL-21R but do not produce IL-21, indicating that their development dependent on extrinsic sources of IL-21 [24]. Consistent with this notion, IL-21 treatment lead γδ T cells to upregulate Bcl6. Additional work is needed to elucidate how γδ T cells and conventional Tfh cells cooperate with each other to regulate the GC response.

6.4.4 Follicular CD8 Regulatory T Cells

The vast majority of studies on regulatory T cells are focused on FoxP3+ CD4 T cells. On the other hand, CD8 T cells can also give rise to a regulatory lineage. A CD8 subpopulation of T cells that can suppress T-cell help to B cells has been identified a decade ago [156]. It is shown that Qa-1-restricted CD8+ cells inhibit experimental autoimmune encephalomyelitis by targeting autoreactive CD4+ cells [34, 133]. This regulatory lineage of CD8 T cells in mice can directly suppress GC Tfh cells [107]. These CD8 regulatory T cells highly express CD44, ICOSL, and CXCR5. Unlike Tfr, they do not need Foxp3 or CTLA-4 but IL-15 and perforin to execute their suppressive functions. Disruption of regulatory interactions between

Qa-1-restricted CD8 regulatory T cells and Tfh cells leads to production of dsDNA-specific auto-antibodies and development of a lupus-like disease.

The participation of conventional CD4 T cells, γδ T cells, and NKT cells in positive promotion of the GC response and humoral immunity and the involvement of CD4 Tfr cells and CD8 regulatory T cells to regulate these processes highlight the evolutionary need to generate useful antibody responses while maintaining self tolerance in the GC.

6.5 Tfh Cells and Diseases

6.5.1 Tfh and Primary Immuno-deficiencies

Abnormalities in Tfh functions cause human diseases [42, 136]. Mutations in SH2D1A, the gene encoding the SAP adaptor protein, cause XLP disease. XLP patients are highly susceptible to viral infections, particularly infection by Epstein-Barr virus (EBV) [29, 192]. On the one hand, this is likely because SAP-deficient T cells cannot help B cells to generate normal GCs and a proper antibody response [28, 44, 171]. On the other hand, CD8 T cells deficient in SAP are very inefficient in killing B-cell targets due to a reduced ability to adhere to antigen-presenting B cells [221].

CD40 is essential for B cell proliferation, differentiation and survival during a T-dependent B-cell response, and CD40L is highly expressed by activated CD4 T cells. When CD40L-CD40 interactions are blocked, the GC development is abrogated [57, 75]. As a result, patients with mutations that affect the CD40L or CD40 gene suffer from hyper-IgM syndrome, which is characterized by an elevated serum IgM but severely reduced serum IgG, IgA and IgE concentration [41]. Furthermore, CD40L-defiecient patients have abnormal formation of memory B cells and aberrant somatic hypermutation, consistent with CD40L-CD40 signals being required for T-dependent humoral responses [1].

With its high expression in Tfh cells, ICOS is essential for GC formation. Similarly, ICOSL expressed by B cells is necessary for a proper GC response. ICOS-ICOSL engagement generates signals to CD4 T cells for the maintenance and differentiation of Tfh cells. At the same time, ICOS controls the follicular recruitment of activated CD4 T cells in a costimulation-independent mode that requires ICOSL expression by follicular bystander B cells [215]. A deficiency in ICOS can cause common variable immunodeficiency (CVID), which is characterized by defective immunoglobulin production, particularly that of IgG and IgA [70, 209].

STAT3 deficiency cause impaired antibody responses in a form of hyper-IgE syndrome [135, 149]. As discussed above, STAT3 signaling is essential for B cells to generate memory B cells and long-lived plasma cells and for T cells to differentiate into Tfh cells. As a result, patients suffering from hyper-IgE syndrome with STAT3 mutations exhibit impaired memory B cells and a reduced number of circulating CD4+CXCR5+ T cells.

6.5.2 *Tfh in Acquired Immuno-deficiencies and Autoimmunity*

CD4+ T cells are targets for acute HIV infection, which is accompanied by vigorous humoral immune responses, characterized by polyclonal B-cell activation, GC and plasmablast formation, hypergammaglobulinaemia and virus-specific antibodies. On the other hand, HIV-infected people do not typically produce neutralizing antibodies. Increased Tfh frequencies in lymph nodes and enrichment of HIV-infected T cells in the Tfh compartment have been observed in HIV-infected individuals. Although the frequency of Tfh cells positively correlates with GC numbers and amounts of virus-specific IgG, and anti-retroviral therapy would reduce the numbers of Tfh cells [168], an increasing number of Tfh cells would not necessarily lead to neutralizing antibodies. It has been suggested that Tfh cells from HIV-infected individuals cannot promote B-cell differentiation in vitro, because increased PDL1 expression on GC B cells inhibits Tfh cell function [45]. These results suggest PD-1-PDL1 blockade can be a useful strategy in developing HIV vaccines. Human circulating PD-1+CXCR3-CXCR5+ memory Tfh Cells are functional and are highly correlated with a neutralizing HIV antibody response [131].

CXCR5+ T cells are detected in target organs of autoimmune disorders, suggesting that Tfh cells may directly participate in autoimmune inflammation. Quantitation of circulating Tfh-like cell frequencies may serve as a biomarker of disease activities [80]. In lupus patients, an increased number of circulating CD4+CXCR5+PD1hi cells, CXCR5+ICOShi cells or ICOShi T cells along with increased serum levels of IL-21 and CXCL13 are typically associated with more severe end-organ damage [89, 184, 185]. In Sjögren's syndrome, increased frequencies of circulating CD4+PD1hi T cells, CD4+CXCR5+ICOShi T cells or CD4+CXCR5+CCR6+ (TH17-type) T cells are more frequently found in the patients with higher serum levels of autoantibodies [120, 184]. In Juvenile dermatomyositis, higher frequencies of TH2- and TH17-like subsets among circulating CD4+CD45RO+CXCR5+ T cells are associated with more severe diseases [151]. In autoimmune thyroiditis, increased frequencies of circulating CD4+CXCR5+PD1hi T cells or CD4+CXCR5+ICOShi T cells are associated with increased titers of serum autoantibodies against thyroid stimulating hormone receptor and thyroglobulin [222]. In myasthenia gravis, increased frequencies of circulating CD4+CXCR5+ T cells or CXCR5+CD57+ T cells are also found in the patients with increased disease severity [177, 190]. Clearly, increased circulation of CXCR5+ T cells is indicative of disease activities in multiple autoimmune conditions and may be exploited for clinical disease monitoring. On the other hand, whether such close association reflects in any way causal relationship between Tfh cells and the disease remains to be further investigated.

6.5.3 Tfh Cells and Tumors

Peripheral T-cell lymphomas (PTCLs) are rare hematological malignancies that include follicular T-cell lymphoma (FTCL), angioimmunoblastic T-cell lymphoma (AITL), and PTCL-not otherwise specified (PTCL-NOS) [47, 86]. FTCL and AITL are characterized by increased numbers of CXCR5+PD1hiICOShiOX40+SAP-hiBCL-6+MAF+ malignant cells and their heightened expression of CXCL13 and IL-21. In addition, these characteristics are often accompanied by abnormal humoral features such as follicular hyperplasia, hypergammaglobulinaemia and autoantibody production in PTCLs. This latter feature might reflect abnormal functions of malignant Tfh cells. Clinical evidence indicates Tfh-associated molecules such as PD-1 and CXCL13 might be targets for treating AITL and FTCL by targeting Tfh cells. Some genetic abnormalities are found to associate with PTCLs. For example, mutations in isocitrate dehydrogenase 2 (IDH2) and TET2 have been found in AITL and PTCL-NOS. On the other hand, IDH2 mutations do not seem to predict clinical outcomes for the AITL [26], whereas patients with TET2 mutations often have more severe clinical phenotypes than those without TET2 mutations [119].

6.6 Tfh Cells Studied by Live Imaging

6.6.1 Technical Aspects

Many aspects of the development and function of Tfh cells have been directly revealed by 2-photon (2P) tissue imaging in animal models, from the mode of T–B interactions to the characteristics of Tfh cellular dynamic in GCs.

One of the most distinct characteristics of immune cells is their highly dynamic nature. Many immune cell types, including lymphocytes, macrophage, DCs, and natural killer (NK) cells are continuously circulating throughout the body, migrating in different tissues [68] and interacting with each other. Starting with a series of three papers [20, 148, 187] in 2002, immunologists have begun to study by a dynamic live imaging approach how dynamic immune cell behaviors contribute to effective immune responses in vivo. Compared with conventional single-photon confocal microscopy, 2P microscopy offers two main advantages [25, 49, 62, 208]. In single-photon microscopy, upon excitation, fluorophore molecules absorb energy from one photon and then emit photon to release energy. On the contrary, in 2P microscopy, fluorophore molecules absorb two photons simultaneously. Such events happen so rarely that 2P microscopy can confine the excitation area to focal point of an objective lens effectively [93]. The spatial restricted excitation provides several crucial advantages over confocal microscopy for intravital imaging. First, higher resolution and brighter images can be obtained for regions deep inside tissues. Because compared with visible or UV light used for confocal microscopy,

near-infrared lasers for 2P microscopy can penetrate deeper with less decline and scattering. Based on this principle, immunologists can visualize objects at a depth of 100–1,000 μm with 2P microscopy. Second, near-infrared lasers can minimize photobleaching, the damage of fluorophores and tissues, which is important for live imaging over an extended period time. The other main advantage of 2P microscopy is the nonlinear optical effects such as the second-harmonic generation [27, 93, 208]. When the intense lasers pass through a highly polarized material, second-harmonic emission at half the wavelength of the original light is generated. Using this effect, many biological structures, including collagen fiber, bone, and muscle can be visualized without fluorophore probes.

6.6.2 Dynamic Views of Tfh Cells

Lymph nodes are a major site where antigen is collected, detected, and responded to by the adaptive immune system. T cells migrate in an apparently random walk-like manner to scan antigen at a speed of about 10–12 μm/min [148, 187]. The migration of lymphocytes is partially driven by chemotaxis, particularly CCL21, CXCL13 and their respective receptor CCR7 and CXCR5. The main sources of those chemokines are stromal cells such as FRCs and FDCs.

When T cells encounter antigen-presenting DCs, they arrest and interact for several hours [21, 148, 187]. This T-DC interaction process can be divided into three phases [145]. First, once finding an antigen-bearing DC, T cell stops intermittently and then migrates again to contact with several DCs. In the same time, calcium flux is induced by TCR signaling [210]. Then the calcium signaling acutely reduces cell mobility, resulting in gene transcription, cytokine secretion, and cell proliferation. In the third phase, after usually more than 10 h interaction, T cells begin to end contact with DC and resume their rapid migration [32]. Subsequently, activated CD4 T cells can start to interact with cognate B cells near the follicle.

It has been demonstrated that activated B cells search T cell help by moving toward the T-B border [61, 173]. By 2P microscopy, this dynamic process has been visualized. Initially activated T cells move to T-B border by down-regulating CCR7 and up-regulating CXCR5, while activated B cells migrate toward the T-cell zone by up-regulating CCR7. Then at the T-B border, antigen-specific T and B cells meet up and form long-lasting mobile conjugate pairs with B cell leading the way [165]. The conjugate formation critically depends on T-cell antigen receptor signaling and inside-out activation of integrin-mediated adhesion. Maintenance of T-B conjugate in vivo depends on SH2 domain-containing adaptor protein SAP [171]. Without SAP, T-cell activation by DCs and migration to the T-B border are not affected, but the duration of cogante T-B contacts is severely reduced [171]. 2P imaging analysis also reveals the importance of noncognate interactions between activated T cells and the ensemble of bystander B cells for Tfh recruitment into the follicle [215]. Moreover, Tfh cells can migrate between different GCs that contain cognate B cells [183].

The GC is histologically separated into the light zone (LZ) and the dark zone (DZ), while the mechanism involved has not been fully understood [199]. In a mature GC, the LZ is sustained by the FDC networks which secret CXCL13, while the DZ is supported by CRC networks which produce CXCL12 [12]. The LZ contains the majority of Tfh cells and GC B cells so-called centrocytes [180], while the DZ includes centroblast type GC B cells [3, 78, 180]. Unexpectedly, while longlasting T–B contact is frequently observed at follicle edge, they much rarely happen in the follicle [4, 105, 171]. What is more, most of T-B contacts in GC are even less than 5 min [4], while some of noncognate T-B contacts usually can last about 5 min at T–B boarder [171]. It is not clear whether the reduction in contact duration results from T- and B-cell intrinsic changes or external changes in follicle and GC environment. One possible explanation is that shorter contacts may facilitate competition among GC B cells for Tfh help in order to select for high-affinity antibodies. Indeed, GC B cells can continuously cycle between the LZ and DZ, competing for contacts with Tfh cells [200].

Novel imaging tools have begun to aid our studies of Tfh cell biology. For example, photo-activated genetic probes have been introduced for observing GC B-cell migration [200]. Genetically encoded calcium indicators allow long-term observation of intracellular calcium flux of activated T cells in vivo [153]. Future technical development will improve further our ability to visualize Tfh cell-related dynamics and multiple functional parameters simultaneously in even deeper regions of lymphoid organs with even better time resolution.

6.7 Future Directions

As a newly defined Th subset, Tfh cells have been intensely studied over the last few years. Unresolved issues abound. First and foremost, while we know the key factors necessary for the Tfh development (e.g., Bcl6), we cannot yet pinpoint the biological processes that is being regulated by such factors. How Bcl6 and other transcription factors control Tfh differentiation and functional specialization remains a centerpiece of future studies in the field. Second, while epigenetic studies of other Th subsets have generated a wealth of information on how effector T-cell lineages are maintained, much remains to be explored for Tfh cells in these regards. In this context, investigation of how Tfh features are maintained in the memory phase of an immune response could turn out to be very informative. In addition, potential roles of noncoding RNA species in regulating Tfh functions have just begun to be appreciated, and studies of these aspects are clearly needed. Third, while we appreciate the existence of Tfr cells in the follicle, we are yet to fully grasp the essence of Tfr functions. Future studies have to reveal their regulatory targets, GC B cells or Tfh cells or both, and cellular mechanisms by which they function. A related issue is the conceptual integration of Tfh, Tfr, and other follicular T cells such as CD8 T cells, NKT cells, and γδ T cells. Fourth, it is amply clear that Tfh cells are involved in disease processes, either when pathological

conditions result from their functional abnormalities or when they can be informative diagnostic and prognostic markers for underlying pathological conditions. Therefore, studies of human Tfh cells in pre-clinic and clinical settings hold the promise of ultimate translation of our understanding of Tfh biology into strategies of improving human health.

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Chapter 7
Differentiation, Regulation and Function of Th9 Cells

Lei Jia and Changyou Wu

Abstract Naïve $CD4^+$ T cells are activated and differentiate to distinct lineages of T helper (Th) cells, which are involved in physiological and pathological processes by obtaining the potential to produce different lineage-specific cytokines that mediate adaptive immunity. In the past decade, our knowledge of Th cells has been significantly expanded with the findings of new lineages. Interleukin (IL)-9 producing T cells are recently identified. In consideration of the ability to preferentially secret IL-9, these cells are termed Th9 cells. Given the multiple function of IL-9, Th9 cells participate in the lesion of many diseases, such as allergic inflammation, tumor, and parasitosis. In this chapter, we will focus on the cytokines, co-stimulatory factors, and transcriptional signaling pathways, which regulate Th9 cells development as well as stability, plasticity, and the multiple roles of Th9 cells in vivo.

Keywords Th9 cell · Differentiation · Transcription factor · Human diseases

Abbreviations

APC	Antigen presenting cell
IL	Interleukin
CNS	Conserved non-coding sequences
TSS	Transcription starting site
Th	T helper
Treg	Regulatory T cell
iTreg	Inducible regulatory T cell
IFN-γ	Interferon γ
ILC	Innate lymphoid cell
NKT	Natural killer T cell
TGF-β	Transforming growth factor-β

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B. Sun (ed.), *T Helper Cell Differentiation and Their Function*, Advances in Experimental Medicine and Biology 841,
DOI 10.1007/978-94-017-9487-9_7

TCR	T cell receptor
PBMC	Peripheral blood mononuclear cell
EAE	Experimental autoimmune encephalomyelitis
AHR	Airway Hyper-responsiveness
Der f	Dermatophagoides farina
RA	Rheumatoid Arthritis
MPE	Malignant pleural effusion
AD	Atopic Dermatitis
NF-κB	Nuclear factor κB
PDK1	Pyruvate dehydrogenase kinase, isoenzyme 1
TNFRF	Tumor necrosis factor receptor superfamily
CGRP	Calcitonin gene-related peptide
PD-L2	Programmed cell death ligand 2
HEL	Hen egg lysozyme
OVA	Ovalbumin
JAK	Janus kinase
STAT	Signal transducer and activator of transcription
GATA	GATA binding protein
IRF	Interferon response factor
CIS	Cytokine-induced SH-2 protein
SOCS	Suppressor of cytokine signaling
Foxp3	Forkhead box protein 3
NICD	Notch intracellular domain
Sfpi	Spleen focus forming virus (SFFV) proviral integration oncogene
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
NFAT	Nuclear factor of activated T cells
PI3K	Phosphoinositide 3-kinase
MAPK	Mitogen-activated protein kinase
BCL6	B cell lymphoma6
MHC II	Major histocompatibility complex class II

7.1 Introduction

Adaptive immune system is orchestrated by various T helper (Th) cells, which generate from naïve $CD4^+$ T cells after stimulation of different antigens and produce subset-specific cytokines that regulate immune responses. The initial defined Th cells are Th1 and Th2 cells based on their production of IFN-γ and IL-4, respectively [50]. IL-9, identified by Van Snick and colleagues more than two decades ago and initially described as T cell growth factor III, mainly comes from T lymphocytes, including long-term T cell lines, antigen-specific T cells and naïve

T cells [80]. Many preliminary functions of IL-9 were studies in the Th2 cell-mediated immunity; moreover, the regulatory expression of IL-9 protein was tested in BALB/c mice with *Leishmania* infection, which generated Th2-biased immunity. Hence, IL-9 was defined as Th2 cell cytokine [18].

Besides T cells, bone marrow-derived mucosal mast cells (BMMC) and eosinophils were also identified as sources of IL-9, and in addition to IL-1, LPS, and IL-10, kit-ligand could synergistically enhance the production of IL-9 [22,70]. As to naïve $CD4^+$ T cells, stimulation with IL-4 and TGF-β milieu could synergistically enhance IL-2-dependent IL-9 production, and this effect was inhibited by INF-γ [67]. Yet it was not clear whether IL-9 was secreted by IL-4-producing Th2 cells or by a unique T cell lineage. Until 2008, two groups demonstrated that a combination of IL-4 and TGF-β promotes the development of naïve $CD4^+$ T cell to a brand-new IL-9-producing subset, termed Th9 cell, and FACS staining with IL-4- and IL-9-specific mAbs demonstrated IL-4/IL-9 double-positive Th cells could not be found [82, 13]. Besides of IL-9, Th9 cells also produce IL-10 and IL-21. However, their functions in Th9 cells are still unclear [21].

Th9 cell development requires a cocktail of cytokines. In addition to IL-4 and TGF-β, IL-2, IL-1, IL-33, and IL-25 can promote IL-9 production from T cells [2, 5, 61], while IFN-γ, IL-27 suppress the expression of IL-9 [67, 68, 85]. Transcription factors STAT6, IRF4, GATA3, PU.1, and/or STAT5 are required for Th9 cell development [13, 16, 20, 71, 82, 88, 90], indicating a complex signaling transduction network in the differentiation of Th9 cells (Fig. 7.1). These pros and cons factors that regulate Th9 cell generation will be discussed in the following part of the chapter. Given the pleiotropic functions of Th9 cells are based on their production, it is of priority to figure out the basic information of lineage-specific cytokine, IL-9.

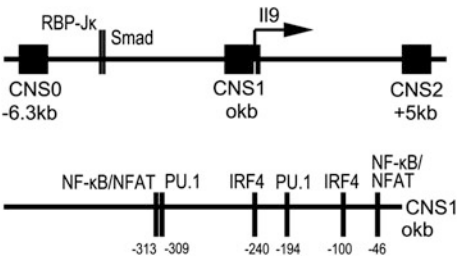

Fig. 7.1 Transcription factors and *Il9* gene. The ideograph of Il9 gene comprises three conserved non-coding sequences (CNS), CNS0, CNS1, CNS2, and Th9-development-involved transcription factor binding sites. CNS0 is −6.3 kb from the transcription starting site (TSS), CNS1 is the promoter of *Il9* gene expression, and CNS2 is +5 kb from TSS. PU.1 has two binding sites which are −194b and −309b from TSS. IRF4-binding sites (−100b, 1240b) and NF-κB/NFAT sites (−46b, −313b) are determined within 1 kb from TSS. In contrast, Smad and RBP-Jκ (−4065b and −4270b, respectively) are about 2 kb from CNS0

7.2 The Biology of IL-9

IL-9, first identified in the late 1980s, was characterized as a member of T cell and mast cell growth factors termed p40 and proved to have pleiotropic functions in immune system [80]. The evidence from cloning and complete amino acid sequencing of p40 indicated that it is structurally different from other T cell growth factors [69, 87], and then was renamed as IL-9 based on its biological functions in inflammatory immunity. The diverse functions of IL-9 based on the binding to IL-9 receptors, which express on multiple kinds of cells, will be discussed later in this part.

7.2.1 Il9 Gene

The *Il9* locus, linked to Th2 cytokine loci, is about 3.2 Mb telomeric from the *Il5/Il13/Il4* loci. The mouse *Il9* gene is located on chromosome 13, whereas humane *Il9* gene is on chromosome 5. However, a similar structure with five exons and four introns stretching about 4kd is shared by human and murine *Il9* gene [49]. Three conserved noncoding sequences (CNS) have been determined in upstream and downstream of *Il9* locus (Fig. 7.1 upper part). CNS1, which locates in the upstream of *Il9*locus, is the promoter of *Il9* expression, and stimulatory transcriptional factors binding to this area have been identified [9, 57] (Fig. 7.1 lower part). CNS2, located at +5kb from the transcription starting site (TSS), is conserved between murine and human genomes [9]. However, it is not conserved between murine and canine genomes [57]. Compared with CNS2, CNS0, located at −6.3 kb from TSS, is conserved among multiple species. CNS1, the promoter of *Il9* gene, responses to multiple transcription factors including PU.1, IRF4, NF-κBand Smad/Notch complexes [9, 16, 25, 71]. Meanwhile, the sequence of the promoter in Th9 cell is mostly acetylated and suppressive modification of the chromatin, H3K27 trimethylation, can be hardly detected compared with other lineages of $CD4^+$ T helper cells [9]. These findings indicate that CNS1 do possess regulatory functions to IL-9 production in Th9 cells.

7.2.2 IL-9 and IL-9R

IL-9, a 14kd glycoprotein, consists of 144 amino acids along with a leader sequence of 18 amino acids [62]. Compared with 55 % at protein level between human and murine, homology level is up to 69 % at nucleotide level [62]. Although human cells response to murine IL-9, human IL-9 has no effect on murine cells [63].

IL-9 receptor (IL-9R) consists of two chains, which are the α chain (IL-9Rα) and the common γ chain shared by other cytokines including IL-2, IL-4 and IL-17 [65, 64].

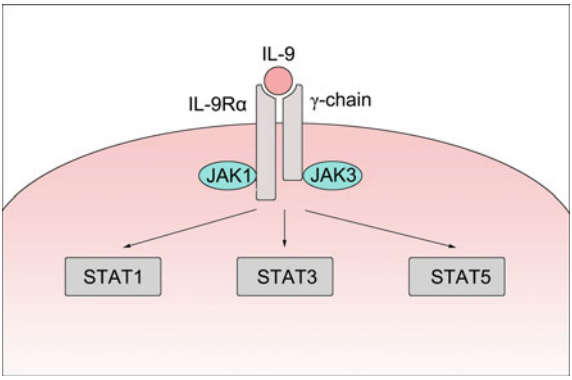

Fig. 7.2 The IL-9 receptor signaling complex. IL-9 receptor consists of the α-chain (IL-9Rα) and the γ-chain which can be activated by IL-9 and then promotes the phosphorylation of Janus kinase 1 (JAK1) and JAK3 leading to the activation of signal transducer and activator of transcription 1 (STAT1), STAT3 and STAT5

Murine IL-9R gene composes of nine exons and eight introns encoding a protein containing 468 amino acids and two hydrophobic domains spanning residues 15–37 and 271–291. IL-9R is proved to be a member of hematopoietin receptor superfamily based on the presence of the WSWS motif in the extracellular domain composed of 233 amino acids containing two potential N-linked glycosylation sites and six cysteines [23]. Human IL-9R gene encodes a sequence with 522 amino acids, which is 53 % homology with murine IL-9R.

Interleukin-9 (IL-9) activates a heterodimeric receptor that consists of the IL-9 receptor α-chain (IL-9Rα) and the γ-chain and promotes the cross-phosphorylation of Janus kinase 1 (JAK1) and JAK3. This leads to the activation of signal transducer and activator of transcription 1 (STAT1), STAT3, and STAT5, and the upregulation of IL-9-inducible gene transcription (Fig. 7.2).

7.2.3 IL-9 Production by Multiple Cells

Data from early studies suggested that IL-9 is a Th2 cytokine [14, 18]. Recent studies by two distinct groups reveal that IL-9 is produced by a new lineage of Th cell, termed Th9 cells [82]. Besides the production of IL-9 from Th9 cells, multiple cells possess the ability to produce IL-9.

IL-9 is also found to be secreted by Th17 cells [52, 72], Treg cells [15, 43] and NKT cells [27, 35]. In a papain-provoked model of airway inflammation, innate lymphoid cells (ILCs), but not Th9 cells, represent the main source of IL-9 [83] (Fig. 7.3).

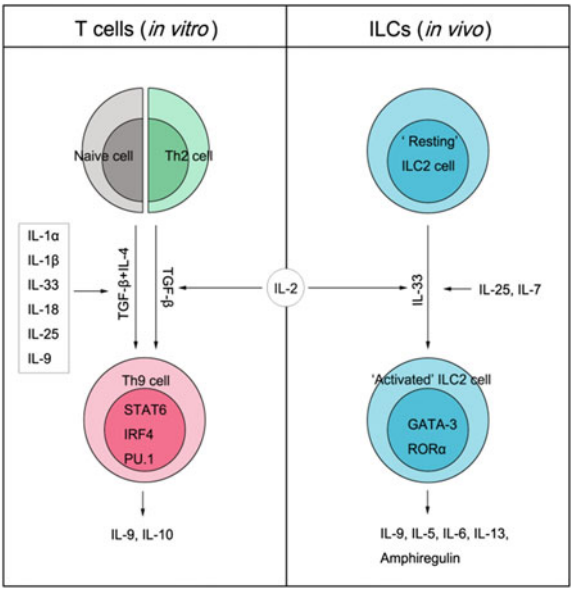

Fig. 7.3 IL-9 is produced by multiple cells. Although in vitro stimulation of T cells with TGF-β and IL-4 results in Th9 differentiation, the main detectable IL-9-producing cell type in vivo is the ILC2 cells. These cells are activated in vitro and in vivo by the epithelial cell–derived cytokine IL-33 and produce IL-9 after exposure to IL-2, a cytokine that also positively affects IL-9 production in T cells. A variety of other cytokines have been described as enhancing IL-9 production in T cells in vitro, including members of the IL-1 family and IL-25, and some also have a stimulatory effect on IL-9 production in ILC2 cells. Th9 cells differentiated in vitro produce IL-9 and IL-10 but not IL-4, IL-5 or IL-13, whereas IL-9-producing ILC2 cells secrete large amounts of IL-5 and IL-13. The transcription factors STAT6, IRF4 and PU.1 contribute to Th9 differentiation, whereas the transcription factors GATA-3 and RORα seem to be involved in ILC2 development

IL-9 plays critical roles in the induction and progress of different pathological mechanisms by binding the IL-9 receptor, which has a common γ chain along with specific α chain that delivers its signals to the target cells.

7.2.4 Immunological Function of IL-9

A wide spectrum of functions in both hematopoietic and nonhematopoietic cells has been attributed to IL-9. In addition to its effects on the survival and proliferation of T cells and mast cells, its postulated activities include the modulation of B cell responses, as well as antiapoptotic effects on neurons and the induction of chemokines in epithelial and muscle cells. IL-9 serves its diverse biologic functions via IL-9R, a cytokine receptor that consists of a ligand-specific α-subunit and a common γ-chain that is shared with the IL-2, IL-4, IL-7, IL-15, and IL-21 receptor complexes. The binding of IL-9 to IL-9R increases heterodimerization of the

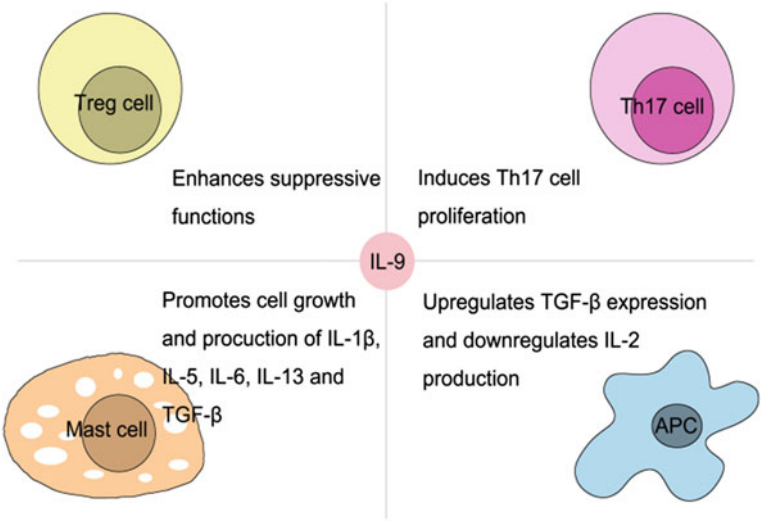

Fig. 7.4 Targets of IL-9 function. Interleukin-9 (IL-9) has been shown to have various effects on different cell types. These effects include activating mast cells to secrete several products, including IL-13, which exerts its effects on the epithelial cells of the lung and gut. In addition, IL-9 seems to have a direct effect on regulatory T (T_{reg}) cells, T helper 17 (Th17) cells, and antigen-presenting cells (APCs). TGF-β, transforming growth factor-β

IL-9R α-subunit with the common γ-chain and induces activation of the kinases JAK1 and JAK3, which results in phosphorylation of IL-9R on a single tyrosine residue; that residue then acts as a docking site for transcription factors STAT1, STAT3, and STAT5, which are then activated by the Jak kinases associated with the receptor. Studies of a set of mutations in the gene encoding IL-9R have elucidated the contributions of those STAT factors to various in vitro IL-9 effects, such as cell proliferation and prevention of apoptosis [84] (Fig. 7.4).

7.3 The Differentiation of Th9 Cells

7.3.1 Th9 Cell Development

7.3.1.1 Cytokines

The differentiation of Th9 cells relies on a cytokine pool in the culture conditions. Foremost among the cytokines are IL-4 and TGF-β. Two decades ago, it is proved that naïve $CD4^+$ T cells primed with the combination of IL-4 and TGF-β can acquire the potential to secret IL-9 cytokine. Compared with stimulation with anti-CD3 and anti-CD28 antibodies, the production of IL-9 from murine $CD4^+$ T cells

was significantly increased in the situation with additional TGF-β and further enhanced by the addition of IL-4, which, by itself, has little influence [67, 68]. Data from IL-2 knockout mice indicates that IL-2 is essential for the production of IL-9 by T cells. However, the IL-2-plus-TGF-β-mediated IL-9 production from IL-4 knockout $CD4^+$ T cells demonstrates an IL-4-independent way [67, 68]. Transcription factors downstream of IL-4 including STAT6, IRF4, GATA3 are required for Th9 cell development [13, 20, 71, 82] (Fig. 7.5). TGF-β signaling in Th9 cells depends on the activation of Smad and the expression of PU.1 [16, 20]. Meanwhile, Th2 cells cultured with TGF-β produce high level of IL-9 and convert to Th9 cells [82]. Significantly, the development of Th9 cells requires combined signals from IL-4 and TGF-β that would otherwise differentiate to distinct Th subsets because IL-4 alone leads to Th2 cell polarization and single TGF-β signal results in inducible regulatory T cell (iTreg) differentiation [28, 56].

The development of Th9 subset is promoted by IL-2 and its downstream transcription factor STAT5, while interferon γ (IFN-γ) suppresses the production of IL-9 from T helper cells by neutralizing the function of IL-4 [67, 68]. A recent report indicates that naïve $CD4^+$ T cells cultured with a combination of IL-4, TGF-β, and IFN-γ tend to acquire the phenotype of Th1 cells other than Th9 cells. The co-expression IFN-γ and surface marker CD103 depend on T-bet and STAT6 signaling induced Eomesodermin [77].

IL-1, as well as IL-33, can induce IL-9 production from T cells [5, 61]. IL-33 can initiate IL-9 production from human $CD4^+$ T cells and basophils isolated from peripheral blood. IL-1 family members contribute to the development of Th9 cells by the activation of nuclear factor κB (NF-κB), which, as discussed later in the chapter, is an important activator of IL9 gene.

Compared with other lineage $CD4^+$ T cells, differentiated Th9 cells express high level mRNA for IL-17 receptor B (IL-17RB), the receptor of IL-25. Transgenic IL-25 promotes IL-9 production in vivo. Moreover, transgenic and retroviral overexpression of IL-17RB in T cells results in IL-25-induced IL-9 production in an IL-4-independent manner. Blocking IL-25 in IL-25 transgenic mice reduces the development of IL-9-dependent inflammation. This evidence supports the critical role of IL-25 in IL-9-dependent immunity [2].

The observation of the increased IL-9 level from *Il23r*$^{-/-}$ T cell in experimental autoimmune encephalomyelitis (EAE) model indicates that IL-23 can inhibit IL-9 production [24]. Given the ability of Th17 cells to produce a certain amount of IL-9, as well as the ability of IL-23 to enhance and/or maintain Th17 development, IL-23 may contribute to the specific functions of Th17 cells in inflammations [36, 74].

IL-21 possesses the potential to promote IL-9 production, and blocking IL-21 leads to the reduction of IL-9 secreting, whereas IL-27 could suppress IL-9 production in a dose dependent manner [85].

7.3.1.2 Co-stimulatory Factors

There are emerging findings to support the crucial roles that co-stimulatory signals play in the development of Th9 cells and the production of IL-9. In addition to the signals from the combination of T cell receptor (TCR) and presented antigens, Kaplan and colleagues have observed that TCR transgenic T cells develop poorly to Th9 cells in the absence of anti-CD28 [29]. Meanwhile, in the study of apoptosis inducing factors they find that plate-coated anti-CD28 leads to more IL-9 production than soluble anti-CD28 [75]. Along with TCR induced signaling, co-stimulation via anti-CD28 depend on PDK1 (pyruvate dehydrogenase kinase, isoenzyme 1)-mediated activation of NF-κB [55]. These evidences indicate a strong potential of anti-CD28 in the activation and induction of IL-9 producing cells (Fig. 7.5).

In the study of airway inflammation models, Xiao and colleagues demonstrated a strong potential of tumor necrosis factor receptor superfamily (TNFRF) member OX40 to induce IL-9 secreting from T cells [86]. The combination of OX40 with OX40L overexpressed on the surfaces of antigen presenting cells results in a striking increase of IL-9 production in vitro (Fig. 7.5). This IL-9-inducing potential is specific as they did not observe the same increase of Treg and Th17 boom even under the respective culture conditions. The OX40-induce IL-9 effect was proved in vivo. When treated with antibody to OX40, mouse models developed allergic airway inflammation characterized with eosinophil inflammation and goblet cell metaplasia [86].

The translocation of NATFc2 induced by calcitonin gene-related peptide (CGRP) also contributes to the production of IL-9 from T cells [48]. CGRP promotes IL-9 secreting in a cAMP/PKA-dependent manner and results in enhanced expression of Sfpi1 (PU.1) and GATA3.

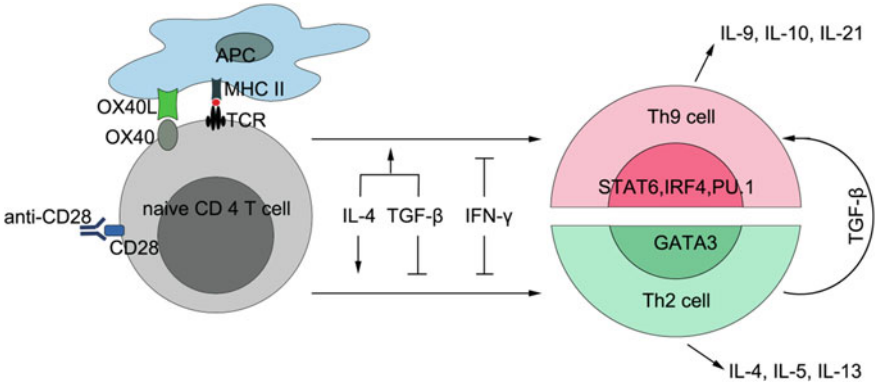

Fig. 7.5 Differentiation of Th9 Cells. Antigen presenting cells (APCs) present treated antigens to naïve CD4 T cells. Naïve cells are activated and differentiate to different Th cell subsets. In the presence of IL-4, naïve $CD4^+$ T cells differentiate into Th2 cells. However, in the preset of TGF-β, naïve cells differentiate to Th9 cells. In addition, TGF-β seems to convert Th2 cells into Th9 cells

A recent study by Kerzerho and colleagues indicated that programmed cell death ligand 2 (PD-L2) downregulates the differentiation of Th9 cells in an Aspergillus fumigatus-induced model of chronic airway hyper-responsiveness (AHR). Blocking PD-L2 results in an increase of IL-9 production albeit no effect on IL-4 produced by Th2 cells [32].

7.3.2 Antigen-Specific Th9 Cell

There are sufficient evidences to prove that there do exist Th9 cells in vivo. $CD4^+IL\text{-}9^+IL\text{-}13^-$ IFN-γ^- T cells can be found in the peripheral blood of allergic patients, compared with the rare detection level of IL-9-production T cells from nonallergic donors. Mice models challenged with house dust might suffer allergic airway disease and an elevated IL-9 production and Th9 cell differentiation can be detected in the target models [26]. In the study of melanoma, Kupper and colleagues have found a considerable high level of $CD4^+$ $IL\text{-}9^+IL\text{-}4^-IL\text{-}17^-IFN\text{-}\gamma^-$ T cells in mice models [60]. Meanwhile, adoptive transfer of tumor-antigen-specific Th9 cells into both wild type and transgenic mice suppress melanoma growth indicating the significant role of antigen-specific Th9 cells in anti-tumor immunity [60]. Th9 cells can also be found in both normal and inflamed skin [12, 60].

In the majority of reported studies, the in vitro differentiation of Th9 cells usually required coated anti-CD3 antibody in combination of anti-CD28 antibody in the culture plates. Meanwhile, the polarization of Th9 cells is induced by cytokines cocktail. However, natural models of Th9 cell generation depends on the activation mediated by APC presented specific antigens. Several groups have yielded data concerning antigen-specific Th9 cells generated in natural models of Th9 cell development.

In murine systems, Van Snick and colleagues immunize mice models and antigen-specific Th9 lines generate by challenging the pre-sensitized CD4 T cells with antigen in vitro [79]. Gery and colleagues study TCR transgenic CD4 T cells with TCR specific against hen egg lysozyme (HEL), and these transgenic CD4 T cells are strongly activated in cultures with HEL presented by APCs and develop into IL-9 producing T cells [76]. As discussed above, $CD4^+$ $IL\text{-}9^+IL\text{-}4^-IL\text{-}17^-IFN\text{-}\gamma^-$ T cells can be identified during the progress of house dust mite-induced lung inflammation [60]. However, the identification of antigen–specific IL-9-producing CD4 T cells has been more challenging depending on the different mice models people used. Using IL-9 fate reporter mice, Th9 cells were detected using an ovalbumin (OVA) model of airway inflammation, but not in a papain-induced lung inflammation. This may due to the preferential activation of innate versus adaptive immune cells by each stimuli [83].

Antigen-specific Th9 cells also can be found in human diseases. High level of IL-9 production can be detected from the presensitized cells isolated from the

peripheral blood of atopic infants when challenged with house dust mite or cat allergen in vitro [89]. Similar response can be detected in peripheral blood mononuclear cells (PBMCs), which are obtained from adult atopic asthmatics and incubated with Dermatophagoides farina (Der f) extract [78].

Both in vitro generated and in vivo existing Th9 cells do possess the potential to produce IL-9. However, it is of great importance that Th9 cells generated from the activation of either antibodies or specific antigens presented by APCs express remarkable phenotypes [76, 78].

7.4 The Regulation of Th9 Cells

Th9 cells develop in a cocktail of different cytokines. These cytokines and their downstream signaling pathways do not function alone but in a complex network. Here, we will focus on several transcriptional signal pathways that regulate Il9 expression and Th9 cell polarization (Fig. 7.6).

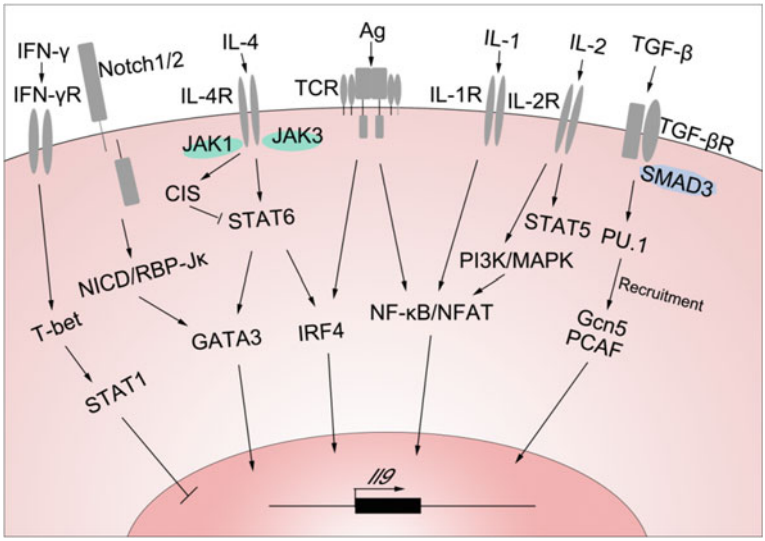

Fig. 7.6 Transcriptional network involved in *Il9* expression. Transcription factors including PU.1, downstream of TGF-β signals, and IL-4-activated STAT6 that promotes expression of IRF4, as well as IL-1 family members activated NF-κB directly bind to *Il9* promoter, and upregulate the expression of the *Il9* gene in Th9 cells. Meanwhile, transcription factors T-bet and Foxp3, which contribute to the development of Th1 and Treg cells, respectively, down-regulate IL-9 synthesis. Abbreviations: IRF4, Interferon Response Factor 4; GATA-3, GATA-binding protein 3; NICD, Notch intracellular domain, RBP-Jk, recombination signal binding protein for immunglobulin kJ region; NFAT, nuclear factor of activated T cells

7.4.1 Stimulatory Signal Transduction Network

7.4.1.1 IL-4/STAT6/IRF4-and/or-GATA3 Pathway

Triggering of the IL-4Ra with IL-4 leads to the activation of Janus kinase JAK1 and JAK3, recruitment and phosphorylation of signal transducer and activator of transcription (STAT)6 and the subsequent expression of genes that regulate Th2 cell development [31]. Notably, given the importance of IL-4 in the development of Th9 cells, phosphorylated STAT6 is indispensable in IL-9 production and Stat6-deficient $CD4^+$ T cells are limited to acquire the potential of IL-9 production under Th9 polarization culture [20, 82]. Compared with STAT6, STAT3, which is required for Th2 cells differentiation and is activated in the progress of Th9 development, is not essential for Th9 cell differentiation [20, 73].

Upon activation, phosphorylated STAT6 facilitates the transcription of *Gata3* and *Irf4*, which are both indispensable in the generation of Th2 and Th9 cells [71, 93]. *Gata3* mRNA rises throughout the development of Th2 cells and is in a STAT6-dependent manner. Although the expression of GATA3 is slight higher in Th9 cells than Th2 cells, GATA3 cannot be induced with anti-CD3 alone in Th9 cells as is inducible in Th2 cells, indicating a crucial regulation of *Gata3* expression in the two subsets [20]. In addition, several groups have demonstrated that *Gata3* transcription can occur in a STAT6-dependent as well as STAT6-independent fashion. Although GATA3 could induce Th2 cytokine in *Stat6*-deficient Th2 culture, ectopic GATA3 did not rescue IL-9 production in Stat6-deficient Th9 polarization condition [20, 53], indicating the involvement of additional factors dependent on STAT6 activity in the regulation of *Il9* expression. This STAT6-deficient Th9 polarization is Notch-dependent, which will be discussed in the following part of the chapter.

Another downstream factor of STAT6 is interferon response factor (IRF)4. *Irf4*-deficient T cells fail to differentiate into Th9 cells, thus diminish the development of allergic inflammation in *Irf4*-deficient mice models [6, 41, 71], although this inflammation depends on a complex network of Th2, Th17, and Th9 cells. Compared with an increase production of IL-9 after the transduction of *Irf4* into wildtype Th9 culture, the transduction of *Irf4* into *Stat6*-deficient culture did not rescue IL-9 expression, demonstrating STAT6-dependent manner of IRF4 in the development of Th9 cells [20].

A recent work from Dong and colleagues demonstrates that there exists a negative feedback regulation of IL-4-induced-STAT6 signaling pathway in the development of Th2 and Th9 cells [88]. Stimulation of IL-4R results in the expression of the cytokine-induced SH-2 protein (CIS), a member of the suppressor of cytokine signaling (SOCS) family, and leads to the inhibition of STAT5 and STAT6 phosphorylation. The interference of Cis gene brings about an increase expression of IL-9 and IRF4 and greater susceptibility to development of experimental allergic asthma.

7.4.1.2 TGF-β/PU.1 Signaling

TGF-β boosts the expression of forkhead box (Fox)p3 in naïve $CD4^+$ T cells and induces the development of iTreg [46]. Binding of TGF-β to TGF-βR leads to the phosphorylation of the SMAD family member, and results in the translocation of SMAD protein to the nucleus as well as the transcription of SMAD-dependent genes [47]. TGF-β-activated SMAD3 binding to *Il9* locus cooperates with Notch intracellular domain (NICD) and recombination signal binding protein for immunoglobulin kappa J region (RBP-Jκ) [16]. TGF-β-induced expression of PU.1 is required for the production of IL-9 [9].

Murine PU.1 is encoded within the Sfpi1 [spleen focus forming virus (SFFV) proviral integration oncogene] locus. The Sfpi1-deficient CD4 T cells fail to differentiate to Th9 cells with the stimulation of IL-4 and TGF-β. Meanwhile, the Sfpi1-deficient transgenic mice reduce the severity of allergic airway inflammation [10]. Transduction of PU.1 into Th2 and Th9 culture leads to an increased IL-9 production and a decreased Th2 cytokines secretion. On the contrary, the reducing of PU.1 expression leads to increased Th2 cytokines production [8, 10]. Based on the ability to suppress Th2 phenotype and enhance Th9 phenotype, the downstream factor of TGF-β, PU.1 functions as the bridge that Th2 cells converts to Th9 phenotype.

In Th2 cells, transduced PU.1 interferes the binding of GATA3 and IRF4 to gene locus and influences the transcription of cytokine genes [1, 8]. In PU.1-deficient Th2 cells, Kaplan and colleagues observed an increasing binding of IRF4 to *Il10* locus and GATA3 to *Il4* locus, indicating the potential of PU.1 to modify the functions of Th2 cytokine related transcriptional factors [21].

In Th9 cells, PU.1 functions by directly binding to *Il9* locus and recruiting the histone acetyltransferase (HAT) proteins Gcn5 [Kat2a, K(lysine) acetyltransferase 2A] and PCAF [Kat2b, K(lysine) acetyltransferase 2B][9, 21], resulting in an open chromatin formation at the *Il9* locus, thereby facilitating the binding of transcription factors like IRF4 and enabling expression of the *Il9* gene. PU.1-deficient Th9 cultures have decreased total histone H3 acetylation and decreased acetylation of several specific histone residues including H3K9/18, H3K14, H4K5, H4K8and H4K16 at the *Il9* locus. Decreased histone acetylation at the *Il9* locus is coincident with decreased association of the HAT proteins Gcn5 and PCAF, and increased association of histone deacetylase (HDAC) proteins HDAC1 and HDAC2 observed in the absence of PU.1 [21]. PU.1 cooperates directly with Gcn5 and interfering with siRNA for Gcn5 leads to a decreased production of IL-9. However, other cytokines, IL-10 and IL-21, produced by Th9 cells are not affected [21]. Thus, this finding indicates that PU.1/Gcn5 complex is crucial in Th9 cell development.

Human PU.1 can similarly promote IL-9 production and is required for the development of Th9 cells. Decreasing the expression of PU.1 results in the reduction of IL-9 level [9]. PU.1 expression can be observed in many human diseases, which will be discussed in detail in Th9 cell function part. For example,

$CD4^+$ $IL^-9^+PU.1^+$ T cells can be found in inflamed skin [12]. Along with the increased expression of Spfi1, Th9 cultures from atopic infants indicate an increased production of IL-9 [89]. High level of IL-9 can be detected in the peripheral blood of patients suffering atopic asthma [26]. These evidences demonstrate a significant promotion of PU.1 in the production of IL-9 in human cells.

7.4.1.3 NF-κB Mediated Transduction Pathways

The mammalian Rel/NF-κb transcription factors family consist of RelA, c-Rel, RelB, NF-κB1 (p50 and its precursor p105)and NF-κB2 (p52 and its precursor p100), which play critical roles in the immune system by regulating several processes ranging from the development and survival of lymphocytes and lymphoid organs to the adjustment of immune responses and malignant transformation. NF-κB signaling consists of both the classical (p50-RelA) and the alternative (p52-RelB) pathways [81]. NF-κB mediated transduction pathways are involved in the induction of IL-9.

A recent study by Li and colleagues identifies a previously unknown mechanism of Th9 induction in mice. The targeted costimulatory factor is OX40, which induces both NF-κB signaling pathways. However, the classical pathway is transient, and the alternative way sustains for a long time, leading to Th9 development [86]. In the classical pathway, the activation of p50-RelA heterodimers is not required for OX40-induced IL-9 expression. Meanwhile, in the alternative pathway, OX40 activated the ubiquitin ligase TRAF6, which triggered the induction of NF-κB-inducing kinase (NIK) in $CD4^+$ T cells, subsequently resulting in IL-9 production. Ectopic expression of p52-RelB leads to elevated production of IL-9, and OX40-induced IL-9 disappears in the both p52-deficient and TRAF6-deficient Th9 polarization culture [86].

NF-κB, in cooperation with NFAT1 (nuclear factor of activated T cells), is required for TCR-induced IL-9 production from Th9 cells [25]. In vivo binding of NFAT1 and NF-κB (p65) to the IL-9 promoter was observed. NFAT1 binding induced a transcriptionally active chromatin configuration at the IL-9 promoter locus, whereas NF-κB (p65) binding transactivated the IL-9 promoter. NFAT1-deficient mice show a significant reduction of IL-9 expression, resulting from altering histone modifications and chromatin structure, at least partly through recruitment of the HAT p300 to the *Il9* promoter. In parallel, knockdown of NF-κB (p65) also diminishes IL-9 expression [25].

IL-2 activates the phosphoinositide 3-kinase (PI3K) and p38 mitogen-activated protein kinase (MAPK) pathways, leading to the triggering of NF-κB pathway and the activation of downstream Il9 gene expression [19]. In addition to IL-2, IL-1, IL-33 and IL-25 have been shown to signal through NF-κB pathway to enhance the production of IL-9 [2, 3, 5].

7.4.1.4 IL-2/STAT5 Derived Pathway

In parallel to NF-κB pathway, IL-2 induces STAT5, which directly binds to *Il9* locus and promotes the expression of IL-9 [88, 90]. IL-2 binds to the specific IL-2 receptor consisting of three subunits, IL-2α (CD25), IL-2Rβ (CD122)and the common γ-chain (CD132) leading to the proliferation and differentiation of T cells [45]. IL-2 is indispensable for IL-9 production by Th9 cells as it has been identified that IL-2-deficient $CD4^+$ T cells cannot produce IL-9 in the absence of exogenous IL-2 [67, 68]. IL-2 signaling via STAT5 constrains Th17 cell generation [34]. Hence, it is reasonable that IL-2-STAT5 signaling promotes IL-9 production from Th9 cells by inhibiting Th17 cell development at the early stage of Th9 cell differentiation.

A recent study confirms that IL-2-JAK3-STAT5 signaling is required for Th9 differentiation. The IL-2-induced IL-9 production also relies on the inhibited B cell lymphoma6(BCL6) expression, as overexpression of BCL6 impairs Th9 cell development [39].

7.4.1.5 Notch, Smad and RBP-Jκ Co-operation Signaling

Elyaman et al. recently demonstrates that Notch pathway cooperated with TGF-β signaling to induce IL-9 production. In experimental autoimmune encephalomyelitis (EAE) model, they reports that Notch1- and Notch2-deficient Th9 cells show decreased IL-9 production and that Jagged2 but not Delta-like 1 is able to induce IL-9 production in the presence of TGF-β alone [16]. These findings indicate that Notch pathway and Smad3 signaling cooperate in the induction of Th9 cells (Fig. 7.6).

The NICD, acting as a co-factor with RBP-Jκin binding to *Il9* promoters, is released when triggering Notch signaling pathway. The activation of Jagged2 leads to the increase of Smad3 protein and phospho-Smad3 activated by TGF-β. Meanwhile, NICD interacts with Smad3, which together with RBP-Jκ bind directly to the *Il9* promoter and enhance IL-9 production in Th9 cultures [16].

7.4.2 Transcriptional-Factors-Mediated Opposing Signaling

Transcription factors in favor of the development of one lineage may suppress the generation of another T cell subset. The Th1-associated transcription factors T-bet and Runx3 decrease the expression of *Il9* gene and the production of IL-9 in Th9 cells. Likewise, the Treg cell lineage factor Foxp3 also impairs IL-9 production in Th9 cells. On the contrary, the expression of both T-bet and Foxp3 are suppressed by IL-4/STAT6 signaling in the development of Th9 cells [20]. As discussed above, overexpression of BCL6 impairs Th9 cell development [39]. Retroviral transduction of any of these factors into developing Th9 cells impairs the production of IL-9 [20].

7.5 The Plasticity and Stability of Th9 Cells

Emerging data indicate that $CD4^+$ T cells exhibit various degree of plasticity, the potential to change their phenotypes to another lineage when exposed to specific cytokines environment [54, 91]. Tan et al. demonstrates that when stimulation with cytokine milieu specific for Th1 or Th17, Th9 cells shows low level of phenotype change, in contrast with a high level phenotype switching from Th9 to Th2 phenotype [76, 78]. It is reported that TGF-β reprograms Th2 cells to lose their characteristic profile and switch to IL-9-secreting cells [82]. Meanwhile, it is of great note that, unlike the observation in vitro, a considerable portion of Th9 cells acquire Th1 cell phenotype and produce Th1 type cytokine, IFN-γ in vivo [76, 78]. This phenomenon may be due to multiple environmental signals including the local cytokine milieu in disease models and organs, shaping Th9 cell phenotype to improve and facilitate local adaptive immunity.

The stability of Th9 cell lineage in vivo is still under debate. Data that support relatively high plasticity in Th9 cells are mainly based on adoptive transfer of in vitro generated Th9 cells into mice models. Adoptive transfer of MOG35-55-specific Th9 cells induces EAE symptoms concomitant with the potential to produce IFN-γ and IL-17 [24]. On the contrary, several works reveal that neutralization of IL-9 diminishes the effects evoked by adoptively transferred Th9 cells, indicating the stability of the IL-9-producing ability in distinct disease models and organs [60, 71]. Nevertheless, from the studies we discussed above, blocking IL-9 in recipients of transferred Th9 cells is able to neutralize the function of transferred $IL\text{-}9^+$ T cells. This evidence indicate that in an appropriate environment of local environment of cytokine cocktail, Th9 cells may maintain the potential of IL-9 production, so as to mediates local immunity in vivo.

By studying the Th9 cell paradigm, stability and plasticity, we may reveal what Th9 cells do in other Th cell-mediated adaptive immunity, as well as how Th9 cells influence the progress of diseases.

7.6 Th9 Cells and Diseases

As $CD4^+$ T cells regulate immunity and inflammation by the differentiation to specific lineages and acquisition the potential to produce lineage-specific cytokines, which are secreted into local environment and play the crucial roles of Th cells. Likewise, Th9 cells produce IL-9, IL-10, and IL-21, even though the roles of IL-10 and IL-21 in Th9 cells function is not clear [13, 30, 76, 78]. Hence, in the following review of Th9 functions, we will focus on the biological functions of IL-9 in immune system as well as the physiological and pathophysiological functions of Th9 cells (Fig. 7.7).

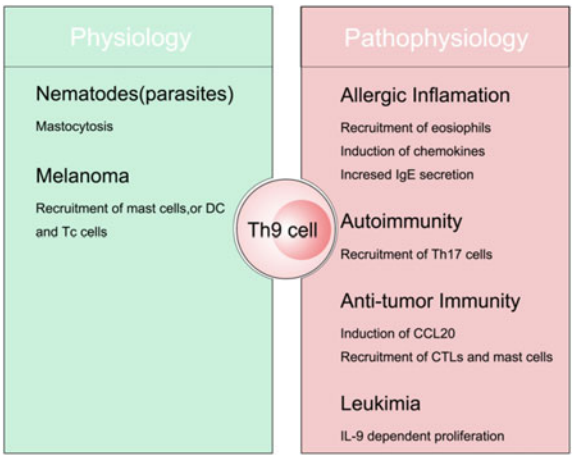

Fig. 7.7 The functions of Th9 cells

7.7 Airway Inflammation

7.7.1 Asthmaand Airway Hyper-Responsiveness (AHR)

Distinct reports have demonstrated the important role of transferred allergic pathologies in lung. The restimulation with ovalbumin (OVA) leads to allergic airway disease in transferred DO11.10 Th9 cells into recipients [172], whereas the treatment of recipient mice by neutralization of IL-9 is able to block many features of the inflammation including airway hyperresponsiveness, eosinophil recruitment and goblet cell metaplasia. A similar finding is observed in PU.1-dificient mice. PU.1 is required for Th9 cell development and IL-9 production, therefore, indicating the key role of Th9 cell-derived IL-9 in the induction of airway inflammation and expansion of inflammatory cells [9]. Exposure of mice to house dust mite results in the rapid accumulation Th9 cells that produce IL-9 in the absence of IL-13, IL-10 and IFN-γ, which directly correlated with the severity of AHR and anti-IL-9 treatment decreased airway inflammation [26]. A recent study of chronic AHR induced by intranasal Aspergillus fumigatus lysates reveals that Th9 cells did not coexpress IL-4, IL-10, and IL-13 [32]

7.7.2 Tuberculosis

The frequency of Th9 cells detected in tuberculous pleural effusion increases during the progress of Mycobacterium tuberculosis infection [90]. Tuberculous pleural effusion and supernatants of cultured pleural mesothelial cells were chemotactic for Th9 cells, whereas IL-9 promotes the pleural mesothelial cell repairing. Moreover, pleural mesothelial cells, acting as antigen presenting cells, promote Th9 cell differentiation by presenting antigen [90].

7.8 Autoimmune Diseases

7.8.1 Experimental Autoimmune Encephalomyelitis (EAE)

Jager et al. generate Experimental Autoimmune Encephalomyelitis (EAE) models by the Transfer of MOG35-55-specific Th9 cells differentiated in vitro into recipients, indicating that Th9 cells also induce central nervous system inflammation. Along with the recovery of the lesions of EAE, the transferred cells obtain the potential to produce IL-17 and IFN-γ, in addition to IL-9, IL-10, and IL-4 [24]. IL-9 is identified to induce the expression of chemokine CC ligand (CCL)20 in astrocytes, thereby attracting Th17 cells into the central nervous system (CNS) of EAE models [92]. Furthermore, MOG35-55-induced EAE in IFN-γ-deficient mice was severely exacerbated and could be ameliorated by the neutralization of IL-9 [51].

However, there exists conflicting reports regarding to the function of IL-9 in EAE models. On one hand, several studies support the role of Th9 cells in the lesion of EAE, and blocking IL-9 results in a diminished pathology [37, 38]. On the other hand, IL-9 may also promote Treg function and inhibit EAE development [17]. A recent report by Elyaman et al. demonstrates that Notch receptors and Smad3 signaling cooperate in the induction of Th9 cells and signaling through Jagged2 expanded Treg cells and suppressed EAE before antigen immunization, but worsened EAE concurrently with immunization by favoring Th17 cell development [16], suggesting that context-dependent effects of IL-9 in the progress of CNS disease.

7.8.2 Rheumatoid Arthritis (RA)

In the study of RA by Khan et al. multiplex kits were tested on serial plasma samples obtained from six RA patients at baseline and multiple time points (3, 6, and 9 months) post-treatment with rituximab. The profile of significantly elevated immunomodulators in the plasma of RA patients treated with rituximab lead to an increased IL-9 production [33].

7.8.3 Transplantation

IL-9 is critically involved in heart transplant eosinophilia in conjunction with IL-4 and IL-5. The Th2 alloimmune response in CD8-deficient mice was associated with the accumulation of IL-9 mRNA in the rejected graft. In IL-9-deficient recipients depleted of CD8 T cells, eosinophil infiltration of heart allografts did not develop, but

rejection still occurred. In the major histocompatibility complex class II (MHC II) disparate model, heart allografts from IL-9 transgenic donors were acutely rejected, whereas grafts from wild-type donors did not develop rejection [59]. Despite the fact that IL-9 promotes IL-5 production from alloreactive T cells, IL-9-deficient recipients of skin allografts still developed eosinophilic graft infiltrates and neither IL-9 nor IL-9R deficiency modified Th2-type allograft rejection [58]. Mast cells are crucial for allograft tolerance, through the inability to induce tolerance in mast-cell-deficient mice. Activated Treg cells produce high levels of IL-9, which seems of great importance in mast cell recruitment to, and activation in, tolerant tissue. Neutralization of IL-9 greatly accelerates allograft rejection in tolerant mice [43].

7.9 Anti-tumor Immunity

7.9.1 Melanoma

In the study of tumor immunity in mice models by Purwar et al. *Il9r*-dificient mice showed accelerated tumor growth, and administration of recombinant IL-9 (rIL-9) to tumor-bearing wild type and Rag1-dificient mice inhibited melanoma as well as lung carcinoma growth. Adoptive transfer of tumor-antigen-specific Th9 cells into both WT and Rag1-deficient mice suppressed melanoma growth, which was abrogated by treatment with neutralizing antibodies to IL-9. Exogenous rIL-9 inhibited tumor growth in Rag1-dificient mice, but not in mast-cell-deficient mice, indicating a mast cell mediated antitumor immunity [60]. Another study by Lu et al. demonstrates the suppression of adoptive transferred OVA-specific Th9 cells in the growth of pulmonary melanoma, indicating that IL-9 may induce CCL20 production and subsequently recruit dendritic cells (DCs) as well as cytotoxic CD8 T cells (cytotoxic T lymphocyte, CTL) to eliminate tumors [42].

7.9.2 Malignant Pleural Effusion (MPE)

The work of Bu et al. demonstrates that Th9 cells in both MPE and peripheral blood expressed a high level of CCR6, enabling both MPE and supernatants of cultured pleural mesothelial cells inducing the migration of Th9 cells. The induction of CCL20 leads to significantly increased Th9 cells chemotaxis induced by MPE or supernatants. Their data revealed that recruitment of Th9 cells into MPE could be induced by pleural CCL20 and that the majority of Th9 cells in MPE displayed the effector memory cells phenotype, $CD45RO^{+}CD45RA^{-}CD62L^{-}$ [7].

7.10 Skin Diseases

Th9 cells may play a crucial role in inflamed skin, because $CD4^+$ $PU.1^+$ cells can be detected within the cellular infiltrate of some inflammatory cutaneous diseases, i.e., atopic dermatitis (AD), chronic plaque psoriasis, and chronic lesions of allergic contact dermatitis (ACD) [12].

7.10.1 Atopic Dermatitis (AD)

Three groups have reported that IL-9 expression increases in AD patients compared with control group [4, 11, 44], demonstrating the essential role of IL-9 in AD, an inflammatory disorder of the skin characterized by an impaired immune response. Data from Ma et al. show no significant difference of IL-9 production between simple AD patients and AD patients with allergic rhinitis or asthma, and IL-9 can promote the secretion of VEGF by keratinocytes in a time- and dose-dependent manner, indicating that the pathogenesis of AD may be mediated by the increased release of VEGF by keratinocyes after IL-9 stimulation [44].

7.10.2 Psoriasis

Besides the predominant role of Th1-Th17 balance in the pathogenesis of psoriasis, studies of K5.hTGF-beta1 transgenic mice, exhibiting a psoriasis-like phenotype, by Wolf and colleagues reveal the link between Th9 and Th17 cells in psoriasis [68]. They have found that increased IL-9R and IL-9 expression in the skin and intradermal IL-9 injection induced Th17-related inflammation. IL-9 also promoted angiogenesis and VEGF and CD31 overexpression in mice in vivo and increased tube formation of human endothelial cells in vitro. Moreover, the application of anti-psoriatic acting anti-IL-17 antibody reduces skin IL-9 mRNA and serum IL-9 levels in targeted models, indicating that IL-9 may play a role in the development of psoriatic lesions through Th17-associated inflammation and angiogenesis [68]. Schlapbach et al. also reveals an increase of IL-9 producing T cells in the skin lesions of psoriasis, further confirming that Th9 cells may contribute to human inflammatory skin disease [66].

7.10.3 Parasitosis

7.10.3.1 Gastrointestinal Worm Infection

Flawell and colleagues use IL-9-deficient and IL-9-fluorescent reporter mice to study host-protective immunity, and reveal that Th9 cells and type 2 innate lymphoid cells (ILC2s) were major sources of infection-induced IL-9 production, the adoptive transfer of Th9 cells, but not Th2 cells, caused rapid worm expulsion, marked basophilia, and increased mast cell numbers in Rag2-deficient hosts, indicating the essential role of IL-9 for the rapid expulsion of the parasitic worm Nippostrongylus brasiliensis [40].

7.10.3.2 Lymphatic Filariasis

Study of lymphatic filariasis by Anuradhar et al. demonstrates that filarialantigen-specificTh9 cells, but notIL9$^+$ Th2 cells, expand in a chronic helminth infection [3]. Moreover, the per cell production of IL-9 is significantly higher in Th9 cells compared with IL9$^+$ Th2 cells, indicating that the Th9 cells are the predominant CD4$^+$ T cell subset producing IL-9 in the context of human infection.

7.11 Conclusions and Prospective Future

The development of Th9 cells from naïve CD4$^+$ T cells is triggered by antigen or antibodies in response to a cocktail of cytokines including IL-4, TGF-β, IL-2, and enhanced by IL-1 family and IL-25, and inhibited by IFN-γ and IL-27. Unlike other lineages of Th cells have the specific transcription factors, i.e., T-bet for Th1 cells, GATA3 for Th2 cells, Foxp3 for Treg cells, and RORγt for Th17 cells, many transcription factors, including IRF4, PU.1, GATA3 et al., are involved in the generation of Th9 cells, indicating a complex transduction network in the regulation of Th9 cell differentiation. Triggering of the IL-4R leads to the activation of JAK1 and JAK3, the recruitment and phosphorylation of STAT6, IRF4, and GATA3 to the promoter of *Il9* gene. TGF-β-induced expression of PU.1 is required for the production of IL-9. Meanwhile, TGF-β-activated Smad3 binding to Il9 locus cooperates with NICD and RBP-Jκ, resulting in IL-9 expression. NF-κBmediated TCR signal and IL-2 signal are of significant importance for the prime of Th9 differentiation. T-bet, Foxp3 and BCL6 impair Th9 cell development. Nevertheless, we are far from the comprehensive inspection of the transcriptional regulation of Th9 cells generation. Th9 cells play a notable role in immune responses by the acquisition of IL-9 production. Th9 cells are responsible for the lesions of allergic airway inflammation, autoimmune diseases such as EAE and RA. However, Th9

cell-derived IL-9 suppresses melanoma growth and eliminates the intestinal nematodes infection, demonstrating a protective role in health.

After the study of IL-9 producing cells for more than two decades, we are still far from the truce of Th9 cells. Although recent reports provide clues for the regulation of *Il9* expression, it is still unclear that whether there are transcription factors and genes, which are indispensable for Th9 cell function, aside from the expression of IL-9 protein. Since TGF-β can "transprogram" Th2 cells to IL-9 producing Th9 cells, whether other effector Th cells obtain the potential of changing to Th9 cells and vice versa. We know both of Th17 and Treg can be the source of IL-9, and we do not know the exact relationships among the three subsets in the pathophysiological progress. Meanwhile, there remain plenty of questions regarding to the cooperation of Th9 cells and other effector Th cells, the specific immune response strictly dependent on Th9 cells and the therapeutic illumination of diseases that Th9 cells involve in.

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Chapter 8
The Biology and Functions of Th22 Cells

Lei Jia and Changyou Wu

Abstract T helper (Th) cells develop from naïve $CD4^+$ T cells under lineage-specific culture conditions and are nominated by their lineage-specific cytokines. Th22 cells, new players in adoptive immune responses, are identified by the production of interleukin (IL)-22. Plenty of observations are obtained over the past few years indicating that IL-22 is produced by activated T cells including Th22 cells, Th17 cells, Th1 cells, innate lymphoid cells and some nonlymphocytes. IL-22 functions synergistically with IL-17 or tumor necrosis factor (TNF), however, it plays different roles by IL-22/IL-22 receptor signal transductions in pathologic processes, including inflammations, autoimmunity, tumor, and digestive organs damages. In this chapter, we focus on the biology of IL-22, the generation and regulation of Th22 cells, the possible signal pathways that involved in the functions of Th22 cells, as well as the relationship between Th22 cells and various diseases.

Keywords IL-22 STAT · Th22 cell · Disease

Abbreviations

TNF	Tumor necrosis factor
IL-TIF	IL-10-related-T-cell-derived inducible factor
DC	Dendritic cell
ILC	Innate lymphoid cell
LTi cell	Lymphoid tissue inducer cell
NCR	Natural cytotoxicity triggering receptor
IL-22 BP	IL-22 binding protein
AHR	Aryl hydrocarbon receptor
CCR	Chemokine receptor

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B. Sun (ed.), *T Helper Cell Differentiation and Their Function*, Advances in Experimental Medicine and Biology 841,
DOI 10.1007/978-94-017-9487-9_8

CCL	Chemokine ligand
SIRT	Sirtuin
ROR	Retinoic acid receptor-related orphan receptor

8.1 Introduction

Interleukin (IL)-22 was first described as IL-10-related-T-cell-derived inducible factor (IL-TIF) by studying murine IL-9-stimulated BW5147 T lymphoma cells in 2000 [23] and then followed by the identification of human IL-22 in two studies by Renauld and colleagues [23, 24]. IL-22, along with IL-10, IL-19, IL-20, IL-24, and IL-26, belongs to IL-10 family [120]. Like all members of IL-10 family, IL-22 acts via a transmembrane receptor complex composed of IL-22R1 chain and IL-10R2 chain. And IL-22R1 chain is also used as a subunit of IL-20 and IL-24 receptor [90]. A soluble single chain termed IL-22-binding protein (IL-22BP) could bind to IL-22 and thus prevent the binding of IL-22 to IL-22R resulting in inhibition of IL-22 functions [18, 121]. Rather than immune cells, IL-22 targets cells at barrier surfaces such as skin and tissues of digestive and respiratory system as well as kidney and joints [102, 116]. As long as IL-22 functions on target cells through signal pathways transduced by IL-22-IL-22R complex, studying the regulation of this signal may offer therapeutic opportunities for these organs/tissues damages [91].

Th22 cells were identified by the production of IL-22 without IFN-γ, IL-4 and IL-17 from Th Cells [20, 106], Like other lineages of Th cells, the function of Th22 cells are most mediated by its lineage-specific cytokine, IL-22. The generation of Th22 cells depends on the transcription factor aryl hydrocarbon receptor (AHR) [86, 106] and the engagement of AHR leads to the increase of IL-22 production and a decrease of IL-17 expression. Th22 cells express the chemokine receptor CCR6 and the skin homing receptor CCR4 and CCR10 indicating their crucial roles in skin diseases [20, 106]. Given the pleiotropic functions of IL-22 on different cells, in addition to skin diseases, Th22 cells are closely associated with various diseases, such as infections, autoimmune diseases, hepatitis, pancreatitis, rheumatoid arthritis (RA), and tumors, which we will discuss in detail in the following part of the chapter.

8.2 The Biology of IL-22

8.2.1 IL-22

As a member of IL-10 family, IL-22 possesses a similar structure with IL-10 for which IL-22 was originally termed 'IL-10-related-T-cell-derived inducible factor (IL-TIF)'. Human *Il22* gene is located on chromosome 12q15, while other IL-10

family cytokines, *Il19*, *Il20*, and *Il24* are located on chromosome 1q32, and they share a similar intron-exon structure containing five (IL-10, IL-19, IL-20, IL-22, IL-26) or six (IL-24) protein coding exon encoding 138–195 amino acids. Both murine and human Il9 gene are composed of 179 amino acids with 22 % (murine) and 25 % (human) overall sequence identity with *Il10* gene [25].

The structure of IL-22 expressed in Drosophila melanogaster S2 cells [IL-22 (Dm)] and Escherichia coli [IL-22(Ec)] has been studied by crystallization and X-ray diffraction, indicating that IL-22 has a bundle-like structure composed of α-helices (helices pre-A and helices A-F) [74, 124]. Although IL-22 has three potential N-linked glycosylation sites and two of which were glycosylated in insect cells, these N-linked glycosylation causes only minor structural changes to the cytokine. However, 1–4 A main-chain differences are observed between the six IL-22(Dm) monomers at regions corresponding to the IL-22R1 and IL-10R2 binding sites [124]. Compared to IL-10, the hIL-22 dimer does not present an interpenetration of the secondary-structure elements but indicates interface interactions between monomers. Structural differences demonstrate that, while a homodimer of IL-10 is required for signaling, hIL-22 most probably binds to its receptor as a monomer [74].

8.2.2 IL-22 Receptor

IL-22 receptor (IL-22R) is a heterodimeric complex composed of two subunits, IL-22R1 and IL-10R2, both of which contains three parts including extracellular part, transmembrane part, and intracellular part [55, 122]. The intracellular part of IL-22R1 contains four Tyr-X-X-Gln motifs which represent putative STAT (signal transducer and activator of transcription) recruitment sites [55], indicating IL-22 signal transduces via the combination of IL-22 and IL-22R1. The extracellular parts of IL-22R1 and IL-10R2 consist of the amino-terminal D1 domain and the close-to-membrane D2 domain, and there are three and four putative N-linked glycosylation sites in IL-22R1 and IL-10R2, respectively [5, 47, 55, 128].

IL-22 has a high affinity for IL-22R1 subunit but no affinity for IL-10R2 [47, 63, 64]. However, IL-10R2 has a measurable affinity for IL-22-IL22R1 complex as well as peptides derived from IL-22 amino acid sequence [47, 118, 128]. These studies reveal a multistep progress of the binding of IL-22 to IL-22 receptor, which are also observed by Fouser et al. [121]. The binding of IL-22 to IL-22R1 results in conformational change of IL-22, which in turn favors the secondary binding of IL-22-IL22R1 complex to IL-10R2. The contact of IL-22 and IL-22R1 involves the L2-4 loop in IL-22R1 D1 domain and the AB loop and helix F of IL-22, in which Thr70, Arg73 and Lys162 are crucial for the binding. Data also demonstrate that the IL-22R and IL-10R2 binding sites are juxtaposed on adjacent IL-22 surfaces contributed mostly by helices A, D, and F and loop AB [5, 121].

IL-10R2 is also a part of receptor complexes of IL-10, IL-26, IL-28 and IL-29 [90]. Meanwhile, IL-22R1 is also used by IL-20 and IL-24 in a complex of IL-22R1

and IL-20R2 [21, 110]. Despite of the difference of R2 subunit, IL-22R1-IL-20R2 complex seems to mediate similar signaling transduction as IL-22R complex. However, it is of note that IL-20 and IL-24 can act via a second receptor complex, the IL-20R1-IL-20R2 complex, which may lead to different effects [22, 110].

IL-22R1 are expressed only by nonhematopoietic cell lineages, such as cells from liver, pancreas, kidney and barrier surfaces like skin, intestine, and lung, but not found in tissues or cells containing high proportion of immune cells, such as bone marrow, spleen, thymus, and peripheral blood mononuclear cells [102, 116].

8.2.3 IL-22 Binding Protein (IL-22BP)

IL-22 BP, which is encoded by IL-22R1-independent gene, is a soluble single chain with 210 amino acid [21, 55, 112, 113, 125]. IL-22 has higher affinity for IL-22BP than IL-22R1 [64, 117]. Structural analysis demonstrates that the binding of IL-22 to IL-22BP overlap the binding surface of IL-22 which is also involved in the binding of IL-22R1, indicating the inhibitory function of IL-22BP [18, 121].

IL-22BP can be detected in normal tissues from lymphatic organs (thymus, spleen, and lymph nodes), gastrointestinal system, lung, skin, and female reproductive system (placenta and breast) [22, 55, 113]. In these tissues, IL-22BP is expressed on immature dendritic cells (DCs) and decreased on mature DCs [12, 43, 68], which is consistent with the acts of IL-22BP in the pathological process in these organs. The level of IL-22 increases during acute inflammation, while IL-22BP is down-regulated [43, 101, 117]. In tissues suffering acute inflammation, immature DCs are activated and mature and leave the undisturbed tissues that they reside in. And during chronic inflammation, the increased level of dysregulated DCs was observed [31].

8.2.4 Multiple Sources of IL-22

IL-22 is produced by various cells and the foremost source is $CD4^+$ T cells. IL-22 was originally thought to be a Th1-associated cytokine. As new lineages of Th cells are identified, Th22 and Th17 cells are revealed to be crucial IL-22 producers [20, 61, 106]. Though Th17 cells can produce IL-22, Th17-cell polarization culture, TGF-β plus IL-6, is not the optimal cytokine milieu for the production of IL-22, because of the inhibitory role of TGF-β in the expression of IL-22 [35]. Furthermore, the production of IL-17A from Th17 cells depend on the transcription fact or retinoic acid receptor-related orphan receptor (ROR) γt and RORα, whereas IL-22 expression requires aryl hydrocarbon receptor (AHR) [108, 127]. Spits and colleagues describe a previously uncharacterized IL-22-producing human helper T-cell population, Th22 cells, distinct from both Th17 cells and Th1 cells, which coexpressed the chemokine receptor CCR6 and the skin-homing receptors CCR4 and

CCR10 with the preferential production of IL-22 [106]. And even cultured in Th1-, Th2-, Th17- or Treg-polarization conditions, Th22 cells still continue to produce IL-22 [26].

Besides Th cells, $CD8^+$ T cells also acquire the potential to produce IL-22, which is observed in patients with atopic dermatitis (AD) [39]. Additionally, γδ T cells have been found to co-express IL-22 and IL-17A [67, 97].

Besides T cells, innate immune cells, blood-derived NK cells also express IL-22 [127]. In addition, in response to IL-23, mucosa-associated lymphoid tissues-residing NK cells (termed NK22) can produce IL-22 and also express NKp44, CCR6 and transcription factors RORγt, RORα, AHR, and IRF4, which offer protection and regulation of mucosal homeostasis rather than the classical role of NK cells [11, 65].

IL-22 also can be produced by several subsets of innate lymphoid cells (ILCs), which can co-express NK cells and myeloid cell markers [93]. $IL\text{-}22^+$ ILCs, including lymphoid tissue inducer (LTi) cells and natural cytotoxicity triggering receptor (NCR)- positive ILCs as well as $NKp46^+$ ILCs can express IL-22 in response to the stimulation of IL-23 alone [57, 87, 93]. Additionally, a recent study by Powrie and colleagues indicates that $IL\text{-}22^+$ ILCs drive IL-23-dependent innate intestinal pathology [9] (Fig. 8.1).

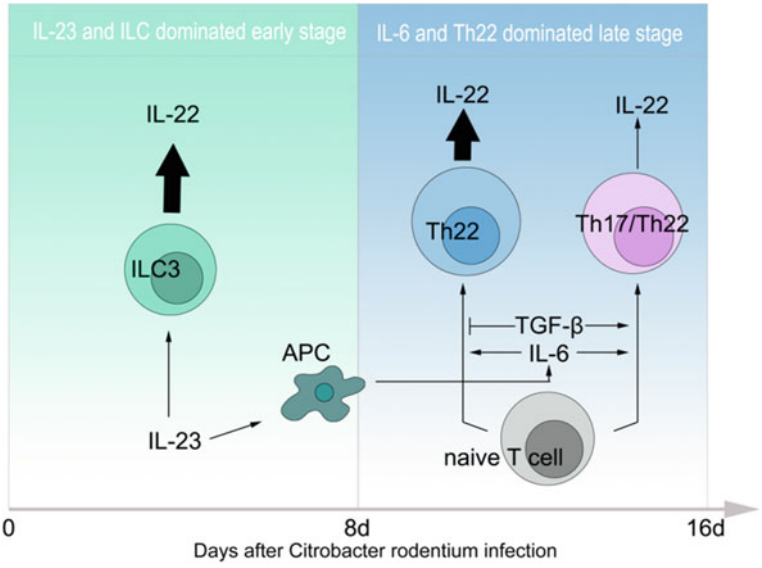

Fig. 8.1 Th22 cells play a critical role in the late-stage of intestinal pathogen immunity. IL-23-mediated IL-22 production by group 3 innate lymphoid cells (ILC3 s) plays a critical role in the early-stage of *Citrobacter rodentium* infection. However, IL-6-mediated differentiation and/or accumulation of Th22 cells and their production of IL-22 dominate the elimination of the pathogen in the later stage. IL-23 can induce IL-6 production by antigen-presenting cells, which then further contributes to the induction of Th22 cells. TGF-β can induce the development of Th17 cells and suppress the generation of Th22 cells

8.2.5 IL-22 Acts via IL-22-IL-22R1 Pathway

IL-22 binding to IL-22R1-IL10R2 complex leads to a cascade of downstream signal pathways. IL-22 primarily signals through Janus kinases, associated with IL-22R subunits and STAT molecules [59] (Fig. 8.2). IL-22 signaling utilizes Jenus kinase (JAK) 1 and tyrosine kinase (TYK) 2 to propagate the downstream phosphorylation signals including mitogen-activated protein kinase (MAPK) pathways (ERK1/2, MEK1/2, JNK, and p38 kinase), the phosphoinositide 3-kinase (PI3K)-AKT-mammalian target of rapamycin (mTOR) pathway and STATs [59, 72, 98]. Although STAT1 and/or STAT5 are weakly activated, phosphorylation of STAT3 at Tyr705 residue is the main event revealed in IL-22-exposed primary cells [116]. However, the phosphorylation of Lys686 accounts for the phosphorylation of Tyr705 residue and deacetylase sirtuin (SIRT) 1 inhibits IL-22-induced STAT3 activation [76, 96]. And these modifications of STATs regulate the expression of their target *Il22* gene. Furthermore, in vivo studies demonstrate that STAT3 phosphorylation is indispensable for the function of IL-22 on epithelial cells. Becker and colleagues found that intestinal epithelial STAT3 activation regulates immune homeostasis in the gut by promoting IL-22-dependent mucosal wound healing [84]. Meanwhile, Artis and colleagues revealed that IL-22 is critical in the maintenance of normal barrier homeostasis. Consistent with that, murine studies indicates signaling by IL-22-IL-22R is of great importance in the promotion of antimicrobial immunity, inflammation, and tissue repair at barrier surfaces [99] (Fig. 8.2).

8.3 Th22 Cell Differentiation and Regulation

Navie $CD4^+$ T cells can acquire Th22 cell phenotype in the presence of IL-6 and TNF-α and further promoted by IL-1β [20]. Duhen et al. [20] demonstrated that Th22 cells produce IL-22 but neither IL-17 nor IFN-γ as well as a high level of aryl hydrocarbon receptor (AHR) but low or undetectable level of the Th17 and Th1 transcription factors RORγt and T-bet. However, the production of IL-22 can be inhibited by a higher dose (5 ng/ml) of TGF-β. These findings indicate an IL-6- and TNF-dependent way in Th22 cell differentiation.

However, Fujita et al. [32] have identified a largely but not completely IL-6- and TNF-dependent way in Th22 cell generation. They isolated Langerhans cells (LCs; $HLA\text{-}DR^+$ $CD207^+$ cells) and dermal DCs ($HLA\text{-}DR^{hi}CD11c^+$ $BDCA\text{-}1^+$ cells) from normal human epidermis and dermis, respectively. LCs were more powerful in the induction of Th22 cells from peripheral blood T cells and naive $CD4^+$ T cells than dermal DCs. Furthermore, they revealed that in vitro-generated LC-type DCs induced Th22 cells more efficiently than monocyte-derived DCs and the induced IL-22 expression was more correlated with IFN-γ than IL-17.

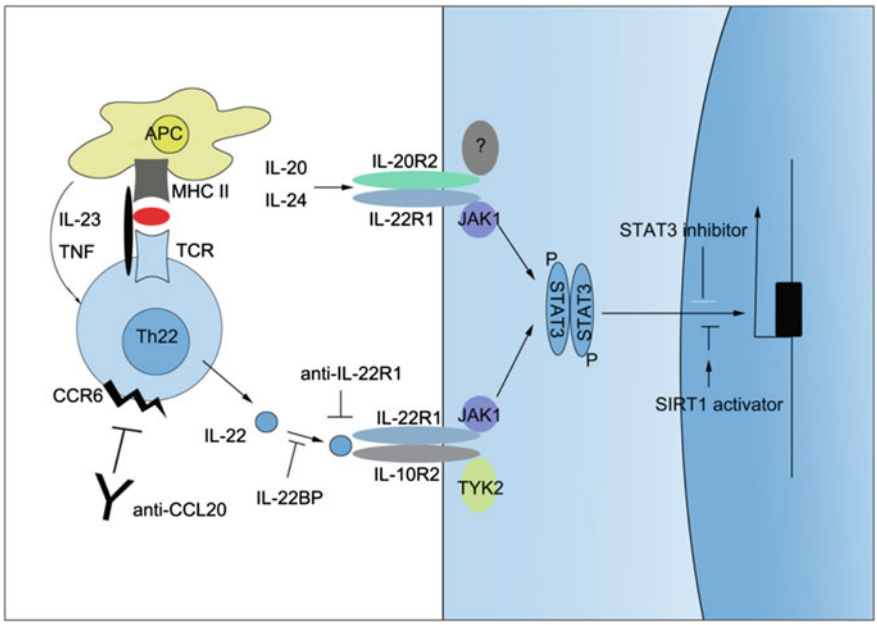

Fig. 8.2 IL-22-IL-22R1 signaling pathway. The binding of IL-22 to IL-22R1-IL10R2 complex utilizes Jenus kinase (JAK) 1 and tyrosine kinase (TYK) 2 to propagate the downstream phosphorylation of STAT3, which in turn enhance the expression of relative genes. The production of IL-22 from Th22 cells can be increased by IL-23 and TNF produced by dendritic cells. Furthermore, the binding of IL-22 to IL-22R can be inhibited by IL-22BP and neutralization of IL-22R1. STATs inhibitor and deacetylase sirtuin (SIRT) 1 can decrease STAT3 acetylation resulting in decreasing genes expression. IL-22R1 is also used by IL-20 and IL-24 in a complex of IL-22R1 and IL-20R2, which seems to mediate similar signaling transduction as IL-22R complex

8.3.1 The Functions of IL-22 and Th22 Cells

8.3.1.1 The Effects of IL-22 on Diverse Cells

IL-22 targets on epithelial cells, hepatocytes, pancreatic cells, and special types of fibroblasts, and mediates host defense in vivo.

Epithelial Cells

IL-22 regulates different innate mechanisms to eliminate invasive pathogens that encounter epithelial cells of the skin, gut and respiratory tract [3, 119, 132]. This prevention mechanism of IL-22 mainly depends on the following functions of this cytokine, the enhancement of innate defense, the regulation of cell development, the production of specific chemokines that mediate the mobility of effector cells and the synergistic effects with other molecules.

The anti-bacterial proteins induced by IL-22 are different in distinct areas of the body according to the complexity of pathogens that enter bacteria barrier [3, 7, 95, 116, 119, 132]. In keratinocyte, IL-22 can induce the production of β-defensin 2, β-defensin 3, S100A7, S100A8, S100A9, and lipocalin 2. With the stimulation of IL-22, intestinal epithelial cells can produce β-defensin 2, S100A8, S100A9 and regenerating islet-derived protein (REG) family members including REG3β, REG3γ, and REG1α. As to bronchial epithelial cells, IL-22 can induce the expression of β-defensin 2, S100A7, S100A12, and lipocalin 2. Moreover, IL-22 also induces mucus-associated proteins, i.e., mucin 1 (MUC1) in colonic and tracheal epithelial cells as well as MUC3, MUC10, and MUC13 in colonic epithelial cells [3, 101].

IL-22 can dramatically cut down the production of proteins, such as keratin (KAT) 1, KRT 10, profilaggrin, involucrin, loricrin, kallikrein 7, desmocollion 1 and late cornified envelop protein 1B, that are critical for keratinocytes development in the recovery and renewing of epidemis.

Hepatocytes

The effects of IL-22 on hepatocytes lie in two aspects, the induction of acute-phrase proteins and the protection against damages. On one hand, the expression of acute-phrase proteins including serum amyloid A, α-antichymotripsin, haptoglobin, and lipopolysaccharide (LPS)-binding protein is elevated by IL-22 [25, 60, 116, 117, 130]. Furthermore, IL-22 can enhance the production of acute-phrase proteins induced by LI-6, TNF, or LPS (Dumoutier et al. [25, 60, 116, 117, 131]. On the other hand, anti-apoptotic proteins, B cell lymphoma (BCL)-2, BCL-XL and myeloid cell leukaemia sequence 1 (MCL1) and mitogenic proteins, cyclin D1, p21 and cyclin-dependent kinase (CDK) 4, which are induced by IL-22, can promote cellular proliferation in vitro [83, 85] but not in vivo [109, 117]. IL-22 also acts on liver stem/progenitor cells which enable liver reconstruction after severe injury. In murine and human livers with chronic HBV infection, IL-22 promotes cellular proliferation via STAT3 [28, 29].

Pancreatic Cells

Though high level of IL-22R1 has been observed in pancreas, only a few effects of IL-22 have been identified and mainly relate to acinar cells, which produce antimicrobial proteins REG3β, REG3γ, and osteopontin [1, 126]. IL-22 enhances the expression of B cell lymphoma (BCL)-2, BCL-XL in acinar cells and also upregulate the production of REG1 and REG2 in pancreatic islet cells [29, 42].

Fibroblasts

Several observations demonstrate that IL-22 also acts on fibroblasts and their derivatives from skin, gut and joint. High level of IL-22 leads to the proliferation of synovial fibroblasts from patients with RA as well as an increase of monocytes-attracting chemokine CCL2 [44]. And IL-22-induced NF-κB ligand (RANKL, also known as CD245) binds to RANK on monocytes resulting in the development to bone-degrading osteoclasts [52]. However, these effects of IL-22 cannot be observed in human colonic myofibroblasts and murine lung fibroblasts [2, 62]. Instead, IL-22 can enhance the production of anti-inflammatory factors (IL-11) as well as inflammatory mediators (IL-6, CXCLs) in human colonic myofibroblasts [2, 62]. Additionally, IL-22 is found to increase the expression of ECM protein in murine dermal fibroblasts but not in human colonic myofibroblasts [2, 62, 71].

8.3.1.2 Th22/IL-22 and Diseases

Skin Inflammation

Psoriasis

Psoriasis is a typical Th1-mediated chronic skin disease with characteristic skin changes including acanthosis, hypogranulosis, parakeratosis. After the identification of Th17 cell, the overloading IL-23/Th17 pathway has been considered to be crucial for the lesion of this skin disorder. As the first organ-specific disease model in which the roles of IL-22 have been studied, psoriasis is now considered as a Th1/Th17/Th22-mediated inflammatory disease, as an increased circulating Th17, Th22, and Th1 cells has been identified in psoriasis [48].

IL-22 expression in both psoriatic skin and peripheral blood from patients are higher than these from healthy controls [6, 119]. Furthermore, a significant decrease of IL-22 has been observed after anti-psoriatic treatment [10, 119]. IL-22-producing cells enrich in psoriatic skin, and moreover, isolated T cells from psoriatic lesion produce high level of IL-22 and most of $CD4^+$ T cells are Th22 and Th17 cells with about 10 % of Th1 cells [6, 26, 78]. Meanwhile, over-representation of IL-17A and IL-22 producing $CD8^+$ T cells was observed in lesional skin, suggesting their involvement in the pathogenesis of psoriasis [88]. Streptococcal extracts induced upregulation of mRNA expression for IFN-γ, IL-17, IL-22, IP-10, as well as epidermal cell mediators (CXCL8, CXCL9, CXCL10, and CXCL11) in psoriatic patients [30].

During the lesion of psoriasis, the high level of IL-22 enhances the expression of anti-microbial peptides including β-defensins, S100A7, S100A8, S100A9, and lipocalin 2 [37, 119]. Neutrophil-attracting chemokines such as CXCL1, CXCL5, and CXCL8 are over-expressed in patients' epidermis [38]. In addition, IL-22 can induce CCL20 which recruits CCR6-bearing cells such as Th22, Th17, and dendritic cells [37, 40, 77, 115]. TNF-α contributes to the enhancement of IL-22

function on keratinocytes via increasing expression of IL-22R and STAT3 during the lesion of psoriasis [115]. Additionally, IL-6 promotes the differentiation of Th22 cells, and an increased level of IL-6 is also observed in psoriasis [20] (Fig. 8.3).

Atopic Dermatitis

The expression of IL-22 is elevated in the inflamed skin of patients with AD [116]. Moreover, the increased level of IL-22 derived from Th22 cells and Tc22 cells ($CD8^+$ $IL\text{-}22^+$ T cells), most of which also produce IL-13 [78, 105]. IL-22 can enhance hBD-2 production via STAT3 in keratinocytes of AD patients [50]. Furthermore, serum level of IL-22 significantly correlates with CCL17, which specifically binds to CCR4 and induces chemotaxis of T cells in AD patients [41]. To date, the detail function of Th22 cells in AD progress has not be revealed, however, considering the effects of IL-22 in keratinocytes, it may contribute to epithelial acanthosis which is observed in chronic AD.

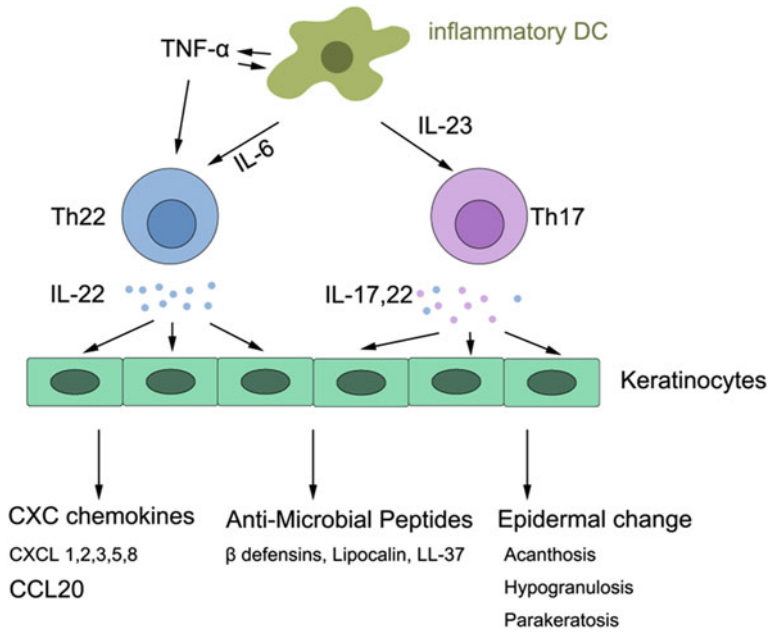

Fig. 8.3 Potential role of IL-22 in the pathophysiology of psoriasis. Differentiation of Th17 cells is induced by IL-6, IL-23, and TGF-β while Th22 cells are induced by TNF-α and IL-6. IL-22 produced by Th17 and Th22 is able to induce neutrophil-recruiting chemokines such as CXCL1, CXCL5, and CXCL8 in keratinocytes. In addition, IL-22 can induce CCL20 which recruits CCR6-bearing cells such as Th22, Th17, and dendritic cells. IL-17 cooperates with IL-22 to induce the production antibacterial peptides such as β-defensins, lipocalin, and LL-37. IL-17 and IL-22 are also thought to contribute to skin changes including acanthosis, hypogranulosis, parakeratosis in psoriasis

Allergic Contact Dermatitis

Recent studies demonstrate that IL-22-producing cells are enriched in thelesional skin of allergic contact dermatitis (ACD), especially in Nikel-allergic ACD [26, 56]. Meanwhile, a high level of IL-22 can be detected in serum of patients suffering ACD [26, 89]. Although ACD is a common skin disease, the exact role of Th22 cells and IL-22 still remained uncovered.

Infection

IL-22 plays a critical role in host defense against extra pathogens by promoting the expression of β-defensin 2, β-defensin 3, S100A7, S100A8, S100A9, and lipocalin 2 [3, 7, 95, 116, 119, 132] indicating the importance of IL-22 in innate immune response. Here we discuss the present findings of IL-22 in immune response to bacteria, virus, and fungi.

IL-22 is indispensable for the elimination of intestinal Gram-negative bacteria *Citrobacter rodentium* by inducing Reg family proteins and Th22 cells dominate the late phrase of this pathogen infection (Fig. 8.1) [132]. IL-22 is negligible for the host defense to Klebsiella pneumonia in pulmonary infection [3]. However, murine experiments demonstrate that IL-22 is also important for the host defense to intracellular bacteria such as M. avium, Listeria monocytogenes, and Salmonella enterica [36, 94, 114].

Th22 cells and other IL-22-producing cells decrease dramatically during HIV infection and an increase of IL-22 can be detected after antivirus therapy [53]. As discussed above, the function of IL-22 is notable in hepatitis B virus infection in both murine and human [29].

IL-22 limits the Asperigillus fumogatus burden during murine lung infection [34]. And IL-22 is required for the protection of kidney and stomach but not skin from the intragastrical infection of Candida albicans [17, 49].

Autoimmune Diseases

Inflammatory Bowel Disease

IBD is a chronic bowel disease with disorders of intestinal immune responses and consists of Crohn's disease (CD) and ulcerative colitis (UC). High level of IL-22 can be detected in inflamed intestines of IBD patients, furthermore, Th22 cells are observed to locate throughout the intestinal wall in CD patients and settle in the lamina propria in CD patients [2]. In addition to local expression, IL-22 production in CD patients' blood is significantly increased and is consistent with the severity of CD [117]. Moreover, the number of memory Th22 cells is increased in CD and correlates inversely with extant of mucosal inflammation [19].

Based on the present understandings of the functions in IBD, IL-22 possesses both protective and pathogenic role in IBD. On one hand, IL-22 can promote

intestinal epithelial cell migration and proliferation [7]. Hence, the increased expression of IL-22 may help to heal the wound intestines. On the other hand, Brand et al. [7] observed increased expression of IL-22 as well as IL-22R in CD. IL-22-induced proinflammatory cytokines and β-defensin 2 can be detected in colonic lesion indicating the inflammatory function of IL-22 [111]. However, these findings on IL-22 function may offer therapeutic opportunities for IBD, even though the exact role of Th22 cells in IBD still need to be further investigated.

Allergic Asthma

It has been well recognized that Th2 cells dominate the pathogenesis of asthma and Th17 cells aggravate the severity of the disease. Recent studies have found that the expression of IL-22 is increased in both asthma patients and murine asthma models [4, 27]. In murine ovalbumin-induced asthma models, the application of IL-22 can reduce pulmonary eosinophil infiltration, chemokines expression and airway hypertension [4, 104]. Meanwhile, neutralization of IL-22 leads to the aggravation of pulmonary inflammation and the enhancement of goblet cell hyperplasia [4, 103]. The protective role of IL-22 in asthma has not been fully revealed. Based on the present information, IL-22 may inhibit CCL17 and IL-25 production of lung epithelial cells [103, 104] and decrease the damage of lung epithelia [79], and it seems to be an IL-10-dependent mechanism [75]. However, IL-22 can synergize with IL-17 to promote lung inflammation and fibrosis [100].

Systemic Sclerosis

Systemic sclerosis (SS) is a Th2/Th17-cell-mediated autoimmune disease and characterized with high-level autoantibodies, vascular abnormalities, and fibrosis of skin and organs. Along with Th2 and Th17 cells, the percentage of Th22 cells increase in patients of SS [107]. In correlation with the increased Th22 cells, serum IL-22, but not IL-17 is dramatically enhanced in SS [70]. As skin infiltrating Th22 cells express high levels of fibroblast growth factors and fibrosis-associated chemokine CCL7 [26], Th22 cells may be responsible for fibrosis of SS patients' skin and internal organs. Moreover, pulmonary fibrosis is closely associated with the increasing level of Th22 cells in SS patients suffering interstitial lung disease [107]. Pretreatment with collagen V can inhibits fibrosis and reduces the expression of IL-22, IL-6 and IL-17 in murine models of bleomycin-induced pulmonary fibrosis [8]. Taking together, Th22 subset plays an important role in the fibrosis of SS.

Systemic Lupus Erythematosus

Systemic lupus erythematosus (SLE) is a systemic disease with autoantibodies boost and immune-disorder-mediated tissue and organ lesions. Pan et al. [82] observed a dramatically decrease of IL-22 expression in serum of SLE patients compare with healthy controls. However, no significant difference was revealed between less active SLE and more active SLE [82] indicating that there is no

correlation between IL-22 level and the activity of SLE. However, the subsequent study by Cheng et al. [13] indicated that the decreased IL-22 expression in patients' plasma positively correlated with the disease activity. The conflicting result may due to the small sample size of the previous research. Therefore, the decreased IL-22 production may indicate the protective role of IL-22 in SLE.

Rheumatoid Arthritis

A high-level *Il22* expression can be detected in synovial tissues in patients suffering RA and IL-22 induces the proliferation and chemokine CCL2 production of synovial fibroblasts [44, 83]. Moreover, Th22 cells increase in the peripheral blood of RA patients and correlate with the elevated level of plasma IL-22, which positively correlates with rheumatoid factor and disease severity [15, 58, 131].

IL-22 was shown to promote osteoclastogenesis, which may be responsible for the reduced severe arthritis in IL-22-deficient mice [33]. Furthermore, Kim et al. [53] demonstrate that IL-22 promotes osteoclastogenesis in RA through the induction of receptor activator of nuclear kappa-B ligand (RANKL) as well as the p38 MAPK/NF-κB and JAK2/STAT3 signaling pathways in human synovial fibroblasts. Neutralization of IL-22 leads to significant reduction of inflammation and bone erosion in IL-1R antagonist (IL-Ra)-deficient mice [66]. However, recent studies reveal an inhibitory role of IL-22 in collagen-induced arthritis in association with increased levels of IL-10 [92]. These protective and pathogenic roles of IL-22 may depend on the different phrases of arthritis.

Hepatitis and Pancreatitis

The level of IL-22 expression and IL-22-producing cells are enhanced during the lesion of chronic hepatitis B virus and hepatitis C virus infection in human beings [16, 83]. Several in vitro and in vivo studies with distinct murine models indicate a protective role in liver damage. The pretreatment with IL-22 can ease concanavalin A-induced liver injury, while neutralization of IL-22 aggravates damage [85]. Hydrodynamic gene delivery of IL-22 cDNA results in IL-22 protein expression, STAT3 activation, and expression of several anti-apoptotic proteins, and leads to the protection from CCL4- and FasL-induced liver injury [81]. Additionally, IL-22 shows protective effect in IL-22-deficient and IL-22-overexpression murine models [83, 129]. Furthermore, IL-22 can help to heal liver injury from acute and chronic alcohol-induced damage [51, 80, 123]. The protective effects of IL-22 also can be observed in ischaemia-reperfusion as well as bacterial and parasitic infection murine models [14, 69, 94]. These protection manners of IL-22 mainly depend on STAT3-meidated production of anti-apoptic, mitogenic and antitoxidant materials during liver injury. IL-22 also enhances the proliferation of liver stem/progenitor cells in mice indicating an important role in organ regeneration [29].

IL-22 influences pancreatitis process by enhancing the expression of REG3β, REG3γ, and osteopontin [1, 126] as well as B cell lymphoma (BCL)-2, BCL-XL, which inhibit autophagosome formation by binding to beclin 1 in acinar cells [29, 42]. IL-22 also contributes to the proliferation of pancreatic islet cells by upregulating REG1 and REG2 [29, 42].

Tumor

In murine colon carcinoma models, though the increased expression of IL-22 does not inhibit the growth and metastasis of syngeneic Colon 26/IL-22 tumor, it does prolong the survival rate compared with the mice inoculated with parent tumors [73]. Furthermore, IL-22 can accelerate the development and progression of hepatocellular carcinoma by studying IL-2-transgenic mice. An enhanced tumor formation can be observed in IL-22-overexpression mice and a decreased tumor cell proliferation can be revealed in IL-22-deficient mice [45, 83]. IL-22 itself does not appear to promote oncogenesis but through its downstream STAT3, which mediates signal transduction to regulate relative gene expression and promote cell proliferation [45, 83].

Th22 cells and IL-22 are associated with various tumors inducing colon cancer, gastric cancer, hepatocellular carcinoma as well as small- and large-cell lung cancer [45, 46, 54, 133]. Recent studies demonstrates that a high level of Th22 cells and IL-22 can be detected in primary tumor tissue, malignant pleural effusion (MPE) and serum of patients with nonsmall cell lung cancer indicating the crucial role of Th22 cells in the genesis of tumor. Hence, targeting Th22 cells and IL-22 may provide a new thinking for tumor therapy.

8.4 Conclusion and Prospective Future

Th22 cells are identified as a new lineage of Th cell family by their specific production of IL-22, which act on epithelial cells and keratinocytes and has been linked to skin homeostasis and inflammation. However, the function of IL-22 cannot represent the role of Th22 cells in immune response for IL-22 can be produce by other activated T cells such as Th17, $CD8^+$ T cells as well as innate immune cells like NK cells. Th22 cells express the chemokine receptor CCR6 and the skin-homing receptors CCR4 and CCR10 as well as a high level of aryl hydrocarbon receptor (AHR) but low or under-detectable level of T-bet and RORγt. The development of Th22 cells from naïve $CD4^+$ T cells can be promoted by IL-6 and TNF-α and suppressed by TGF-β.

However, a variety of issues on Th22 cells still remain unclear. First, the detail information about Th22 cell differentiation including transduction pathways and the regulatory factors involved in the development progress. Second, as other T cells and innate immune cells can produce IL-22, it is of interest to figure out the relationships among these cells in local immune response. Last but not least,

although the functions of Th22 cells and IL-22 are observed in multiple diseases, the effects of treatments related to Th22 cells in clinical affairs still cannot be evaluated with the present observations of Th22 cells and related cytokines.

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Erratum to: Th1/Th2 Cell Differentiation and Molecular Signals

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Erratum to:
Chapter 2 in: B. Sun (ed.), *T Helper Cell Differentiation and Their Function*, DOI 10.1007/978-94-017-9487-9_2

Figure 2.1 in the original chapter unfortunately was wrong. The correct figure is given below:

The online version of the original chapter can be found under DOI 10.1007/978-94-017-9487-9_2

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B. Sun (ed.), *T Helper Cell Differentiation and Their Function*, Advances in Experimental Medicine and Biology 841, DOI 10.1007/978-94-017-9487-9_9

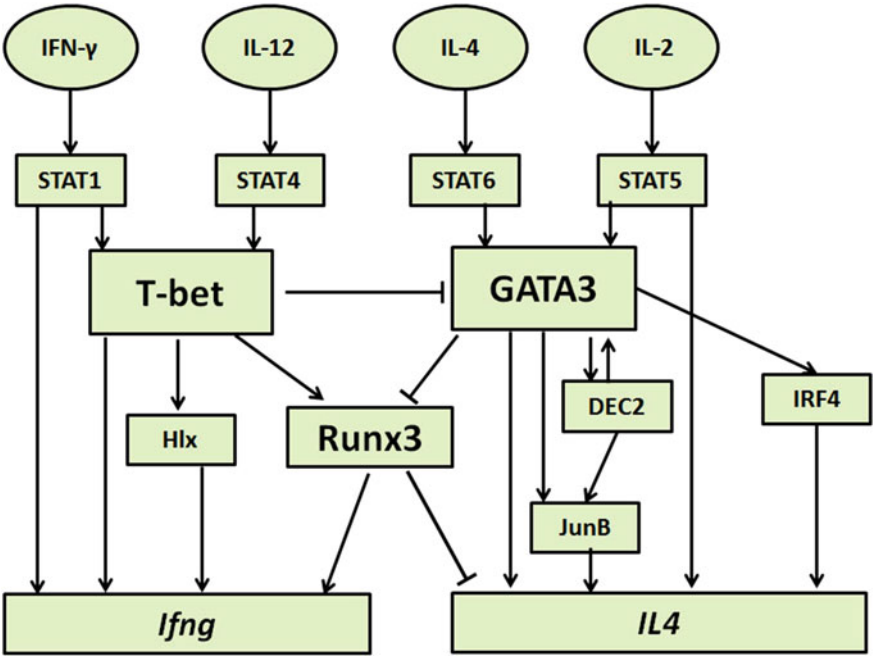

Fig. 2.1 The network of transcription factors in Th1/2 cells. The activation of signal transducer and activator of transcription (STAT) proteins by different cytokines plays a critical role in inducing the expression of the lineage-specific master regulators T-bet (Th1), GATA3 (Th2). The STAT proteins also collaborate with the master regulators and some secondary transcription factors, whose expression is controlled by the master regulators, for the induction of cytokine genes. Positive or negative regulation among these transcription factors occurs at the gene expression level and/or at the protein level through protein–protein interaction, forming a sophisticated transcriptional regulatory network during Th1/2 cell differentiation [191]