Inmunología
Vol. 28 / Núm 1/ Enero-Marzo 2009: 32-45

The Th17 lineage: Answers to some immunological questions

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EL LINAJE TH17: RESPUESTAS A ALGUNAS CUESTIONES INMUNOLÓGICAS

Recibido: 1 Diciembre 2008
Aceptado: 26 Enero 2009

RESUMEN

En los últimos años se han estudiado exhaustivamente las funciones y las rutas de desarrollo del subtipo de células T helper especializado en la producción de IL-17 (Th17). Este linaje celular de células efectoras desempeña un papel decisivo tanto en la respuesta inmune a agentes infecciosos, como en inmunopatologías. Al igual que para los subtipos Th1 y Th2, la definición de Th17 está dirigida por citocinas y factores de transcripción específicos. La combinación de TGF-β e IL-6, y los factores de transcripción RORγt, RORα y Stat3 son esenciales para comprometer el subtipo Th17. IL-23 juega un papel clave en la estabilización del fenotipo y de la actividad patogénica de células productoras de IL-17. La citocina IL-21 producida por células Th17 participa en un mecanismo de retroalimentación para favorecer el desarrollo de células productoras de IL-17, mientras que las citocinas IL-27, IL-4, IFN-γ, IL-25 e IL-2 limitan el fenotipo Th17. Las células T reguladoras $CD4^+CD25^+Foxp3^+$ (Treg) siguen una ruta de desarrollo divergente al establecimiento de las células IL-17, aunque ambas alternativas son gobernadas por TGF-β, el cual dirige el destino de células $CD4^+$ naïve hacia uno u otro de estos subtipos celulares mutuamente excluyentes dependiendo de la presencia de IL-6. Además, datos recientes indican que células Treg ya establecidas pueden modificar su programa genético para convertirse en células Th17. En esta revisión se resumen y analizan los datos disponibles actualmente acerca de la biología de las células Th17.

PALABRAS CLAVE: Th17 / TGF-β / IL-6 / IL-23 / RORγt.

ABSTRACT

In recent years the function and developmental pathway for the T helper subset specialized in IL-17 production (Th17) have been exhaustively studied. This lineage of effector cells plays a decisive role in the immune response to infectious agents, as well as in immunopathologies. Similar to the Th1 and Th2 subsets, the Th17 definition is orchestrated by specific cytokines and transcription factors. A combination of TGF-β plus IL-6, and the transcription factors RORγt, RORα and Stat3 are essential for Th17 commitment. IL-23 plays a key role in the stabilization of the phenotype and in the promotion of the pathogenic activity of IL-17-producer cells. The IL-21 cytokine produced by Th17 cells participates in a feedback mechanism to favour this phenotype, while IL-27, IL-4, IFN-γ, IL-25 and IL-2 cytokines limit the Th17 response. $CD4^+CD25^+Foxp3^+$ regulator cells (Treg) follow a development pathway divergent to Th17 establishment, although both alternatives are governed by TGF-β that directs the fate of naïve $CD4^+$ cells to each of these mutually exclusive T cell subsets depending on the presence of IL-6. Furthermore, recent data indicate that preestablished Treg cells can switch its genetic program to become IL-17-producer cells. In this review we summarize and discuss the current available data about the biology of Th17 cells.

KEY WORDS: Th17 / TGF-β / IL-6 / IL-23 / RORγt.

THE IL-17 FAMILY

Since Mossman et al. proposed the model wherein CD4+ T helper cells were classified in Th1 and Th2 subtypes with different functions in immune responses according to the profile of cytokines produced[1], the Th1/Th2 paradigm provided a valuable tool to understand the interplay of innate and adaptive immunity and CD4+ T cell function. However, some discrepancies have arisen related to results that did not fit in the Th1/Th2 hypothesis. During the past years new studies have identified a distinct subset of CD4+ T cells that secrete IL-17 and the closely related cytokine IL-17F as well as other inflammatory cytokines such as IL-6 and IL-22[2,3].

Murine IL-17 was described as CTLA-8[4], and a 63% homologous human cytokine was soon found[5]. Currently, the IL-17 cytokines include a family of six members (IL-17A-F), with at least two of them having potent proinflammatory properties: IL-17A or CTLA-8 (the founder member of the family also named IL-17), and IL17-F. Both are produced by the recently described Th17 cell subset, are localized at the same chromosomal locus (1A4), share a 55% of homology at the protein level, and seem to have similar functions. IL-17A and IL-17F work mostly as homodimers, but IL-17A/F heterodimers have been recently described in several independent reports[6-8], suggesting a role in inflammatory response regulation for such IL-17 complexes. IL-17D and IL-17E (alternative names: IL-27 and IL-25) are the two members of the IL-17 family with lowest homology (16% at protein level) to IL-17A. None of them is produced by Th17 cells, and both of them, as discussed later, exert a negative control on the Th17 subset development.

IL-17 receptors are a family of five members of membrane proteins, IL-17RA-F[9]. Except for IL-17RA, each of these receptors has alternative splicing variants, and, for IL-17RB and IL-17RC, result in secreted soluble proteins which could serve to antagonize their ligands[10]. IL-17A and IL-17F bind to IL-17RA, although IL-17A binds with much higher affinity[11], which correlates with its greater potency in functions like induction of chemokine expression[8]. In addition to IL-17RA, IL-17F can also bind to IL-17RC. By signalling through IL-17RA, which is ubiquitously expressed, IL-17 can induce the production of different kinds of proteins, many of them related to inflammation, including chemokines (CXCL-1, CXCL-2, CXCL-8-10, CCL-2, CCL-20), cytokines (IL-6, TNFα, G-CSF, GM-CSF), proteins of the acute phase response, tissue remodelling factors (MMP1, MMP3, MMP9, MMP13, TIMP2), and anti-microbial products (β-defensins, mucins, calgranulins)[12]. The signal transduction of IL-17 is mainly mediated by NFκB and C/EBP transcription factors, with the involvement of MAP kinase pathways[13-16]. Moreover, TRAF6 was shown to be involved in the activation of NFκB by IL-17[17]. On the other hand, IL-17 can increase expression of some of its target genes through mRNA stabilization[18].

IL-17B and IL-17C are members of the family whose cellular sources are unknown yet, and whose biology seems unrelated to IL-17A. In this review, we will refer to IL-17A as IL-17.

CLUES TO THE IDENTIFICATION OF A NEW T HELPER CELL LINEAGE PRODUCING IL-17

The first suggestion of a new T helper subset, distinct from Th1 and Th2, was provided by the finding of T CD4+ cells producing high levels of IL-17 without expression of IFN-γ or IL-4, the respective prototype cytokines produced by Th1 and Th2[19]. Other important clues were supplied by studies on animal models of autoimmunity. Pathologies such as experimental autoimmune encephalomyelitis (EAE) and collagen-induced arthritis (CIA), or mouse models for human multiple sclerosis (MS) and rheumatoid arthritis (RA), have been traditionally considered as Th1-mediated diseases. However, over the years, a number of experimental results showed inconsistencies with the Th1/Th2 hypothesis. For example, depletion of the Th1 cytokine IFN-γ, its receptor IFN-γR, or the IL-12 receptor (IL-12Rβ2), the master inductor of the Th1 phenotype, increased the susceptibility to EAE[20-26].

With the discovery of IL-23 as an heterodimeric cytokine sharing its p40 subunit with IL-12[27], the relative contribution of IL-12 and IL-23 to chronic inflammation could be analyzed. The IL-12 heterodimer is composed by the p40 and p35 subunits, while IL-23 comprises p40 and a different p19 protein. By using mice lacking IL-23 *(p19-/-)*, IL-12 *(p35-/-)* or both cytokines *(p40-/-)*, it could be demonstrated that IL-23, and not IL-12, is the critical cytokine for autoimmune inflammation of the central nervous system (CNS) during EAE[28]. This study was followed by many other showing the role of IL-23 in inflammation[29-31]. In parallel with these findings, it was reported that IL-17-producer cells could be generated independently of the cytokines and transcription factors required for Th1 or Th2 differentiation. The development of Th17 from naïve cells was potently inhibited by IFN-γ and IL-4, whereas memory Th17 cells were resistant to suppression by Th1 or Th2 cytokines, indicating that these cells had a permanent commitment. All these data together indicated that a T helper lineage distinct from Th1 and Th2 can differentiate from naïve CD4+ cells to produce IL-17[2, 3].

In 2006, three independent reports provided the basis for understanding the process of Th17 cell promotion[32-34]. All of them found that transforming growth factor β (TGF-

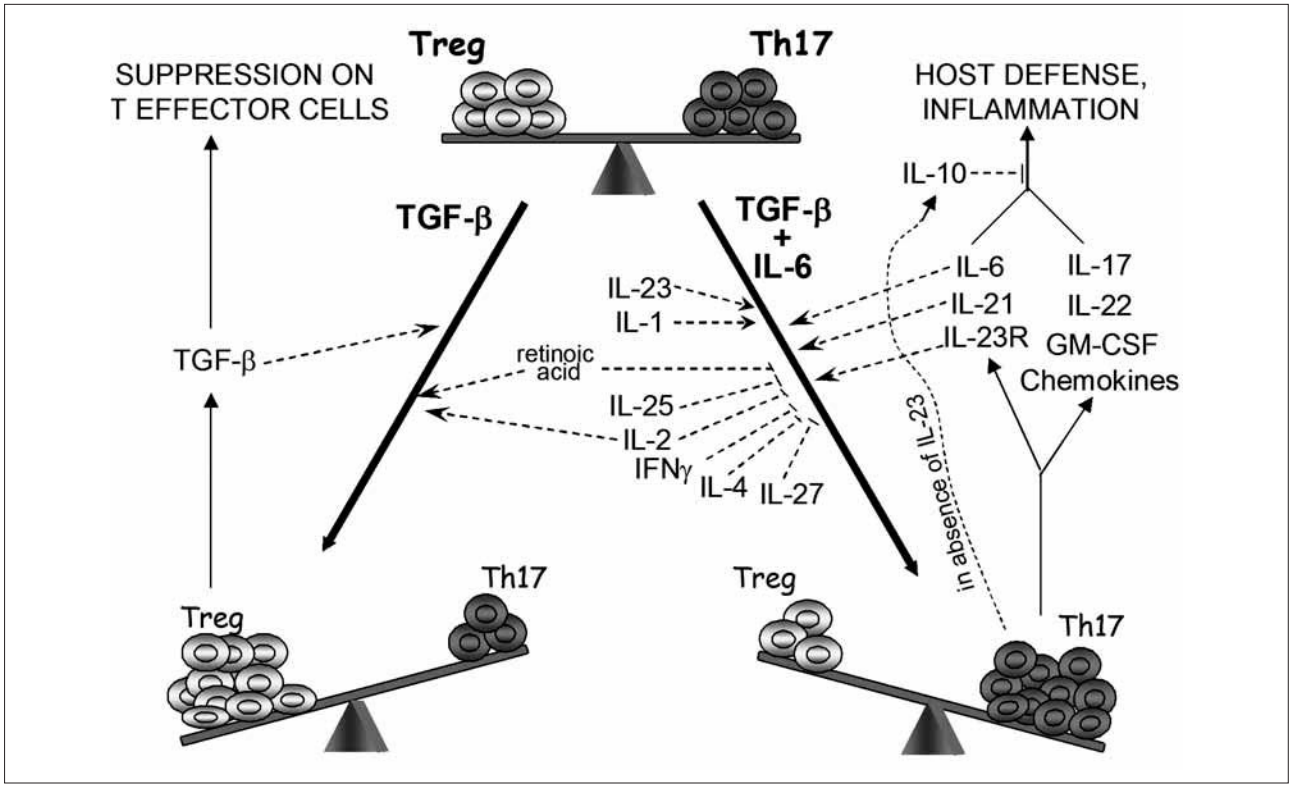

Figure 1. *The homeostasis of Treg and Th17 cells can be balanced to each phenotype by the cytokine environment. TGF-β is involved in both pathways, and IL-6 is the decisive cytokine directing the cell subset fate. In the absence of IL-6, TGF-β induces the establishment of Treg cells that in turn produce TGF-β and control inflammation and autoimmunity by suppression of T effector cells. This process is favoured by IL-2 and retinoids. The Th17 pathway is triggered by inflammatory cytokines like IL-6. Once Th17 cells are committed, the IL-23R is expressed, allowing responsiveness to IL-23, which stabilizes the phenotype of IL-17 producer cells. IL-6 and IL-21 are also produced, and both of them take part in an autocrine loop that collaborates in Th17 development. Negative regulators of Th17 differentiation are IL-27, IL-4, IFN-γ, IL-25, IL-2 and retinoids. In addition to IL-17, IL-21 and IL-6, Th17 cells secrete other products, like chemokines, GM-CSF and IL-22, all of them involved in host defence and inflammation. In the absence of IL-23, Th17 cells can also produce IL-10, which may control the pathogenic phenotype of IL-17 producer cells.*

β) is critical while IL-23 is not required for Th17 commitment. TGF-β is associated with immunosuppressive functions through the induction of Foxp3 expression and Treg cell activity with inhibitory properties on effector cells[35]. Nevertheless, it is now clear that TGF-β may also facilitate proinflammatory responses through Th17 development, although it needs to act in concert with inflammatory cytokines such as IL-6. Thus, TGF-β induction of Treg or Th17 subsets are mutually exclusive depending on the presence of IL-6[32, 34]. The finding of these reciprocal pathways promoted by TGF-β could explain the apparent discrepancy that TGF-β is involved in both anti- and pro-inflammatory events in the immune system.

Therefore, when the immune system is not activated, in the absence of inflammatory cytokines, TGF-β favours the generation of adaptive Treg cells, which prevent inflammation and autoimmunity. After infection, cytokines like IL-6 derived from the innate response can inhibit such process and collaborate with Treg-produced TGF-β to induce proinflammatory Th17 cells (Fig. 1).

TGF-β ACTION ON HUMAN TH17 ESTABLISHMENT

Despite the well-known role for TGF-β in murine Th17 differentiation, many doubts arose about such a function on human Th17. Several important reports in 2007 refuted the requirement of TGF-β in human IL-17 production[36-39]. While some of these studies considered that the central cytokines needed for human Th17 function were IL-23 and IL-1β, other found that IL-1, collaborates with IL-6; however, all of them emphasized that TGF-β was dispensable, suggesting important differences in the requirements for the differentiation of Th17 in humans and mice.

More recently, three simultaneously published reports assured that TGF-β was indeed required for the establishment of human Th17[40-42]. Several explanations for these divergent

conclusions are conceivable: i) the difficulty to obtain real naïve cells from human samples, a caution which has been ensured in the works of Manel et al.[41] and Volpe et al.[42]; ii) the importance of eliminating any residual contamination of platelets, a major store of TGF-β; iii) or the culture media used in which serum may contain endogenous TGF-β. These issues are discussed at length by O'Garra[43]. Thus, these new findings support that similar cytokine pathways are involved in Th17 development in mice and humans.

OTHER INFLUENTIAL CYTOKINES THAT FAVOUR TH17 ACTIVITY

As mentioned above, the early proposal that IL-23 directly drives differentiation to Th17 cells has been discontinued due to three main findings: i) IL-23-deficient mice produce Th17 cells which can be expanded *in vitro* by exogenous IL-23[33]; ii) although IL-23 can induce memory T cells to produce IL-17, naïve T cells do not express the receptor for IL-23[44]; and iii) it was clearly demonstrated that IL-23 is dispensable for initial IL-17 generation[2,3,29]. Nevertheless, there is no doubt about the essential role of IL-23 in the maintenance of Th17 cell activity. It has been proposed that IL-23 can have a function in promoting Th17 cell expansion or survival[45]. A recent report suggests that IL-23 maintains the Th17 phenotype without affecting proliferation or survival[46]. On the other hand, IL-23 has been demonstrated to maintain the pathogenic Th17 functions compared with culture under TGF-β and IL-6, depending on IL-10 production by Th17 cells[47].

Currently, IL-6 is the main partner of TGF-β in priming naïve T cells to IL-17 production[32,33,36,45,48,49]. It is able to inhibit the expression of Foxp3, which directs the differentiation of $CD4^+CD25^+$ Treg cells[50]. This may constitute the beginning of the Th17 commitment and the decline of Treg activity during immune responses. Moreover, IL-6 orchestrates a series of downstream cytokine-dependent signalling pathways to amplify Th17 cell differentiation. In fact, IL-6 is able to induce the expression of IL-23R in T cells making them responsive to the stabilizer phenotype of IL-23[48]. On the other hand, after induction of IL-17 expression, Th17 cells start to secrete IL-6 and IL-21, which in turn act as autocrine factors[49,51].

Although IL-21 does not look like an essential factor for Th17 lineage commitment, it is able to induce IL-17 expression in collaboration with TGF-β even in the absence of IL-6[48,51,52]. Furthermore, generation of Th17 cells is attenuated by blocking IL-21[53], and loss of its expression, or its receptor, results in defective Th17 differentiation. Similar to IL-6, IL-21 inhibits Foxp3 expression induced by TGF-β[51,52]. IL-21 is produced by Th17 cells under IL-6 induction and autocrinally induces its own synthesis and the expression of IL-23R to allow IL-23 responsiveness[48,51].

Another positive regulator of Th17 development is IL-1. It has been reported that IL-1, can increase the effect of IL-6 and TGF-β on Th17 definition[45]. Besides, the induction of antigen-specific Th17 cells is abrogated in IL-1R1-deficient mice, without effect on Th1 or Th2 cells[54]. However, the mechanism through which IL-1 influences Th17 differentiation is not determined yet.

The IL-18 signalling pathway might be involved in Th17 cell definition as well. Remarkably, the receptor of IL-18 (IL-18Rα), but not IL-18 itself, would be involved in this action. Gutcher et al.[55] showed that, while IL-18-deficient mice were susceptible to EAE, IL-18Rα-deficient animals were resistant to the disease showing a deficient Th17 response, and proposed that IL-18 signalling is involved in Th17-mediated immunopathology through binding of an unknown alternative ligand distinct from IL-18.

NEGATIVE INFLUENTIAL CYTOKINES FOR THE PROMOTION OF THE TH17 SUBSET

As Th1 and Th2 cells inhibit the polarization of one another through IFN-γ and IL-4, respectively[56], there is evidence that these cytokines antagonize initial Th17 development[2,3]. However, after Th17 cells become established effectors, IFN-γ and IL-4 cannot suppress or revert their phenotype.

One of the main negative regulators of Th17 development is IL-27[57,58], a cytokine structurally related to IL-6, but with many different actions. Definitive studies showing such damaging role of IL-27 on IL-17 producer cells were reported by Batten et al. and by Stumhofer et al.[59,60]. Both reports conclude that the absence of IL-27 signalling exacerbates chronic inflammation in correlation with increased number of Th17 cells. Moreover, IL-27 is able to promote IL-10 production[61], another negative player in the network of Th17 activity regulation[62].

IL-10 was first described as a product of Th2 cells, but it is now well-known that it is also produced by other cell types like Th1 cells or regulatory Tr1 cells, with an important role in limiting T-cell mediated immunopathology. The involvement of IL-10 in regulating the pathogenic function of Th17 cells has been definitively demonstrated by McGeachy et al.[47], who have described a non-pathogenic Th17 subtype expressing IL-10 generated by IL-6 and TGF-β in the absence of IL-23. Such type of cells are not only non-pathogenic in EAE, but they also prevent the induction of the disease in an IL-10-dependent way.

Another negative regulator of Th17 cells is IL-25 (IL-17E), identified by database searching for genes homologous to IL-17. Although IL-25 is included in the IL-17 family, it is not produced by Th17, but by Th2 and mast cells. This cytokine is involved in the expression of the Th2 products IL-5 and IL-13, and favours Th2 responses[63]. On the other hand, IL-25 deficiency is involved in pathologic inflammation, associated with increased expression of IL-17 and IL-23[64,65].

At least in mice, IL-2 also antagonizes Th17 activity. In spite of the essential function of IL-2 as growth factor of T effector cells, its deficiency leads to systemic autoimmune disease[66]. This is justified in part by its involvement in differentiation and survival of Treg cells[67]. Besides, a recent work has revealed that IL-2 constrains IL-17 production since IL-2 deficiency promotes differentiation of the Th17 cell subset in a Stat5-dependent mechanism[68].

ROLE OF TH17 CELLS IN HOST DEFENCE

The role of IL-17 in immune responses against infections has been widely demonstrated[69]. IL-17 is a potent activator of neutrophils, both through cell expansion and through regulation of chemokine expression for cell recruitment. Signalling mediated by IL-17 induce target cells to express CXCL-8 (IL-8) and G-CSF, which results in the generation and accumulation of neutrophils. In addition, IL-17 induces expression of various anti-microbial genes such as β-defensins. IL-17 deficiencies are associated with neutrophil defects leading to disease[70]. The lamina propria, constituent of mucosa located at the respiratory, gastrointestinal and urogenital tracts, is the only tissue where constitutive expression of IL-17 is detected. Due to the permanent interaction of this tissue with microbial flora, a defensive T cell population promptly responding to infection may be very useful[71].

Both IL-17- and IL-17RA-deficient mice show enhanced susceptibility to experimental *Klebsiella pneumoniae* pulmonary infection and reduced lung G-CSF and CXCL-1 in response to this infection[72,73]. In addition to *K. pneumoniae* IL-17F has also demonstrated to be involved in pulmonary recruitment of neutrophils[74]. Other bacteria preferentially inducing a Th17 response are *Borrelia burgdoferi*, *Bacteroides fragilis*, or *Mycobacterium tuberculosis*[19,75].

IL-17 seems to have also a role in protective immunity against fungal infections. Deficiency in IL-17R leads to decreased survival and increased damage in kidney after infection with *Candida albicans*, with delayed mobilization of peripheral neutrophils[76]. Another major human fungal pathogen which provokes a Th17 response is *Aspergillus fumigata*[77]. Other fungal infections activating the IL-23/IL-17 axis are *Cryptococcus neoformans* and *Pneumocystis carinii*[78,79].

IL-17 could be also defensive against some parasites, like infection with the protozoan *Toxoplasma gondii*[60,80]. However, and although an homologue of IL-17 is encoded by herpes virus Saimiri[81], the role of Th17 in viral infections is unclear. Several studies suggest a pathogenic activity of IL-17 rather than protective function[82,83]. A protective role of Th17 cells has only been clearly demonstrated against rotavirus infection[84].

ROLE OF TH17 CELLS IN IMMUNOPATHOLOGY

The Th17 subset has a key role in both induction and progress of immunopathologies. IL-17 and other cytokines related to the development and function of Th17 cells are closely associated with several immune disorders, not only in animal models, but also in human diseases. As we have mentioned before, the importance of the Th17 cell activity in autoimmunity was first demonstrated in mice deficient in the p19 chain of IL-23, which showed an important impairment of IL-17 production and were highly resistant to EAE[28] and CIA[31]. Using passive transfer studies in EAE, it was demonstrated that IL-17 producing cells are highly pathogenic and essential for the establishment of organ-specific inflammation[29]. On the other hand, neutralization of IL-23 can decrease IL-17 expression in the central nervous system and prevent EAE[30]. In addition, IL-17A deficient mice show reduced symptoms of EAE with delayed onset and early recovery[85], and are resistant to CIA[86]. In several independent assays it could be demonstrated that vaccination against IL-17 prevents EAE and CIA[87-89]. All these experiments highlighted the importance of the IL-23/IL-17 axis in the pathogenesis of several disorders that was previously thought to be mediated by the Th1 subset. Many previous data, inconsistent with the Th1 hypothesis, could be then understood[20-26]. The role of IL-17 in autoimmunity has been later underlined by the relationship between therapeutic treatments and IL-17 production impairment. Thus, IFN-β, currently the main therapy used for MS[90], inhibits IL-17 expression in peripheral and CNS infiltrates of T lymphocytes during EAE[91,92], and increases the expression of the IL-17 negative regulator IL-27[93]. On the other hand, after transfer of $ICOS^+$ cells to ICOS-deficient mice, that have enhanced susceptibility to EAE, lymph node cells showed decreased IL-17 production and could reduce the severity of the disease[94].

IL-17 seems to be also associated with the human diseases MS and RA. IL-17 and IL-23 are present in the sera, synovial fluids and synovial biopsies of RA patients[95-97]. In addition,

it is known that IL-17 mediates induction of IL-6 and IL-8 in RA synovial fibroblasts[98]. Before the explosion of the recent advances in the knowledge of the murine Th17 subset, high IL-17 mRNA expression had been observed in mononuclear cells of the blood and CSF in human MS[99], and a gene-microarray analysis of MS lesions had suggested that IL-17 and IL-6 could be possible targets for MS therapy[100]. Although human Th17 cells are less well characterized than the murine subset, some specific features in addition to IL-17 production have been proposed for them, mainly related with specific patterns of chemokine receptor expression, which suggest their involvement in migration of Th17 cells and recruitment of other inflammatory cells[101,102]. More recently, an elevated number of IL-23-expressing dendritic cells has been found in MS patients, concurrent with increased IL-17 production by T cells[103]. Moreover, evidence has been provided that IL-17 and IL-22 induce a breach in the blood-brain barrier and promote recruitment of additional $CD4^+$ lymphocytes[104]. Of particular interest has been the report of Tzartos et al., who found IL-17 mRNA and protein expression in active areas of MS lesions, where the cell sources of IL-17 were both infiltrating T cells and resident astrocytes and oligodendrocytes[105].

Teunissen et al.[106] described the upregulation of IL-17A in psoriasis, an autoimmune disorder affecting the skin. IL-17 cooperates with IL-22 in the induction of anti-microbial peptides as a function in host defence against pathogens. However, these defensive peptides can enhance the expression of factors related to psoriatic skin[107]. Moreover, expression of different products of Th17 cells has been found in psoriatic skin[39]. Another autoimmune disease that has been recently linked to inappropriate Th17 cell response is systemic lupus erythematosus (SLE). IL-17, IL-23 and the number of Th17 cells were higher in plasma from SLE patients than in control individuals, suggesting involvement of the IL-23/IL-17 axis in inflammatory immunity in SLE[108].

There are also data indicating that IL-17 and IL-23 are involved in animal models of induced colitis[109-111]. Patients with ulcerative colitis or Crohn's disease display an elevated expression of IL-17 in the intestinal mucosa, which is augmented during active exacerbations of inflammatory bowel disease[112,113]. All these data showing the contribution of IL-17 activity to autoimmune diseases justify clinical trials using a monoclonal antibody anti-IL-17 (AIN457) for Crohn's disease and psoriasis[114].

Allergic asthma is considered to be a Th2-dominant chronic inflammatory disease with a major involvement of eosinophil activity. However, some asthmatic processes seem to be mediated by neutrophil infiltration rather than by eosinophil cells, and have been described as "non-eosinophilic asthma"[115-119]. Some results in experimental models suggest that Th17 cells may be important for neutrophilic activity in acute airway inflammation[120-122]. These data lead some researchers to think that Th17 might be responsible for allergic processes mediated by neutrophil cells. However, the role of IL-17 in allergy is still largely unclear. Current results related to this issue have been discussed in length by Oboki et al.[123].

THE GENETIC PROGRAM GOVERNING TH17 COMMITMENT

In contrast to our wide understanding of the genetic and epigenetic control of Th1 and Th2 regulation, we are at the beginning of the discernment of the genetic mechanisms for the commitment of the Th17 subset. Th1 and Th2 development are initiated by TCR signalling in conjunction with master transcription factor regulators, T-bet for Th1 and GATA-3 for Th2, which trigger integrated signals with inheritable epigenetic changes that allow tissue-specific gene expression[124]. Each of these processes requires the participation of factors that are activated by specific cytokines. IL-12 signalling through Stat4 is associated with Th1 differentiation, and IL-4 through Stat6 governs Th2 cell fate[56]. The transcription factors RORγt and RORα seem to be hallmark regulators of Th17 development[71,125]. Both factors belong to the family of retinoic-acid-related orphan nuclear receptors, which in turn is included in the hormone nuclear receptor superfamily. The orphan label is due to the fact that the ligand for these receptors is unknown. While RORγ is broadly expressed, the isoform RORγt is exclusively found in cells of the immune system[126]. RORγt is coexpressed with IL-17 in the mucosa constituent lamina propria. Ivanov et al. found that RORγt is required for IL-17 expression in response to IL-6 and TGF-β, and RORγt-deficient mice have attenuated EAE and lack tissue-infiltrating Th17 cells[71]. Nevertheless, residual Th17 cells are still present in conditions of RORγ deficiency and EAE was not completely abolished. This can be justified by the results of Yang et al., showing that another related nuclear receptor, RORα, also induced by TGF-β and IL-6, can collaborate in Th17 promotion[125]. In fact, double deficiencies in RORα and RORγ impair Th17 generation and confer more resistance to inflammatory disease than RORγ deficiency alone. However, single RORα deficiency in T cells only resulted in modest decrease of IL-17 and IL-23R expression, and had a very moderate inhibition of EAE. Thus, these two ROR factors seem to have redundant functions acting in a synergistic way in promoting Th17 cells.

Upregulation of RORγt is Stat3-dependent[127]. This is not the only level at which Stat3 regulates Th17 development

since it can also induce the expression of IL-23R[128]. Moreover, the positive effect of IL-6, IL-21 and IL-23 on the Th17 subset is dependent on Stat3[48,51,129]. On the other hand, negative regulation of Th17 generation by Socs3 was found to act mainly on IL-23-mediated Stat3 phosphorylation[130]. A definitive result to ascribe a role for Stat3 in Th17 development was the finding that Stat3 deficiency resulted in defective Th17-cell differentiation *in vivo* and protection against EAE[128]. Collecting these data together, Stat3 seems to act at several levels during the process of Th17 phenotype definition, taking part in sequential and feedback loops of cytokine functions: first, IL-6 induces IL-21 production in a Stat3-dependent fashion. Once produced, IL-21 autocrinally stimulates its own synthesis and IL-23R expression through Stat3 activity. This allows IL-23 signalling leading to RORγt induction, which in turn up-regulates IL-23R expression in collaboration with Stat3. In addition, IL-21 cooperates with TGF-β to promote IL-17 expression with the involvement of Stat3 and RORγt (and/or RORα).

Interferon-regulatory factor 4 (IRF4), essential for the development of Th2 cells[131-133], is also critical for the generation of Th17 cells and for EAE induction[134]. IRF4-deficient mice did not develop EAE and showed a fault of IL-17 expression by T helper cells, while transfer of wild-type T helper cells allowed EAE susceptibility. Another feature of Irf4$^{-/-}$ T cells is the reduced expression of RORγt. However, overexpression of this factor in Irf4$^{-/-}$ T cells only restored Th17-cell differentiation partially. Thus, whether IRF4 acts upstream or downstream of RORγt is not clear.

Recently, it has been reported that Ets-1 deficiency is associated with increased expression of IL-17, IL-22 and IL-23R in response to IL-6 and TGF-β, indicating an enhanced efficiency to Th17 cell differentiation[134]. However, Ets-1 apparently does not affect directly the expression of these genes. Rather, Ets-1-deficient T cells produce less IL-2 and have impaired responsiveness in terms of IL-2-mediated inhibition of Th17 differentiation. The resistance to IL-2 suppression was caused by a defect downstream of Stat5 phosphorylation, but was not caused by a difference in the levels of RORγt.

Regarding direct regulation of IL-17 promoter, chromatin immunoprecipitation (Chip) assays determined that Stat3 directly binds to the murine IL-17A and IL-17F promoters[130]. Ichiyama et al. reported that RORγt directly binds to the IL-17 promoter, and found two potential ROR binding sites[135]. Besides, RORγt was sufficient for IL-17 promoter activation. Another recent work describes that the transcription factor Runx-1 collaborates with RORγt to activate this promoter, although in the absence of RORγt, Runx-1 was not able to induce IL-17 transcription[136]. Fewer information is available about the activity of the human IL-17 gene promoter, and it is unknown if RORγt is able to bind to it. Nevertheless, the minimal promoter has been defined in a region between 232 and 159 nucleotides upstream from the start transcription point. This region contains two NFAT recognition sites which seem to be functional after TCR stimulation at least in Jurkat cells[137]. However, no more data about the role of NFAT in the control of IL-17 expression have been reported up to now.

In spite of our poor understanding of the genetic programs directing Th17 cells development and activity, the current thought is that, as for Th1 and Th2 subsets, Th17 polarization obeys to an epigenetic control, most probably controlled by RORγt and RORα transcription factors, with contribution of other players like Stat3, IRF4, or other still unidentified factors. In mice, the genes for IL-17A and IL-17F are linked in chromosome 1, and their expression also appears to be linked, similarly to the IL-4, IL-5 and IL-13 locus in Th2 cells. The possibility of chromatin remodelling events allowing epigenetic control of Th17-cell development is supported by the results obtained by Akimzhamov et al.[138], who showed that Il17a and Il17f genes undergo H3 acetylation in response to TGF-β and IL-6, implying increased accessibility of the locus.

REGULATION OF THE TH17/Treg BALANCE

Current data indicate that the reciprocal developmental pathways for the generation of effector Th17 and Treg cells are controlled by the cytokine environment through an strict transcription program. It is established that CD4$^+$CD25$^+$Foxp3$^+$ Treg cells are generated in the thymus (natural Treg) or may be induced by TGF-β in the periphery from naïve CD4$^+$CD25$^-$ cells (induced or adaptive Treg)[67,139-142]. The dichotomy between the generation of Th17 and regulatory Foxp3$^+$ T cells was first demonstrated by Betelli et al. using IL-6, which completely inhibited the generation of Foxp3$^+$ Treg cells induced by TGF-β, directing the phenotype fate toward IL-17-producer cells[32].

TGF-β alone is able to induce the expression of both master transcription factors involved in each genetic program: RORγt for Th17 cells and Foxp3 for Treg cells (Fig. 2). In spite of this, TGF-β does not initiate Th17 differentiation unless pro-inflammatory factors, such as IL-6 or IL-21, are also present. In the absence of these cytokines, Foxp3 interacts with RORγt suppressing IL-17 transcription[135]. IL-6, IL-21, and IL-23 are able to relieve Foxp3 inhibition of RORγt, and to activate Stat3, allowing Th17 cell differentiation. Zhou et al. have proposed that this subtle decision can be influenced

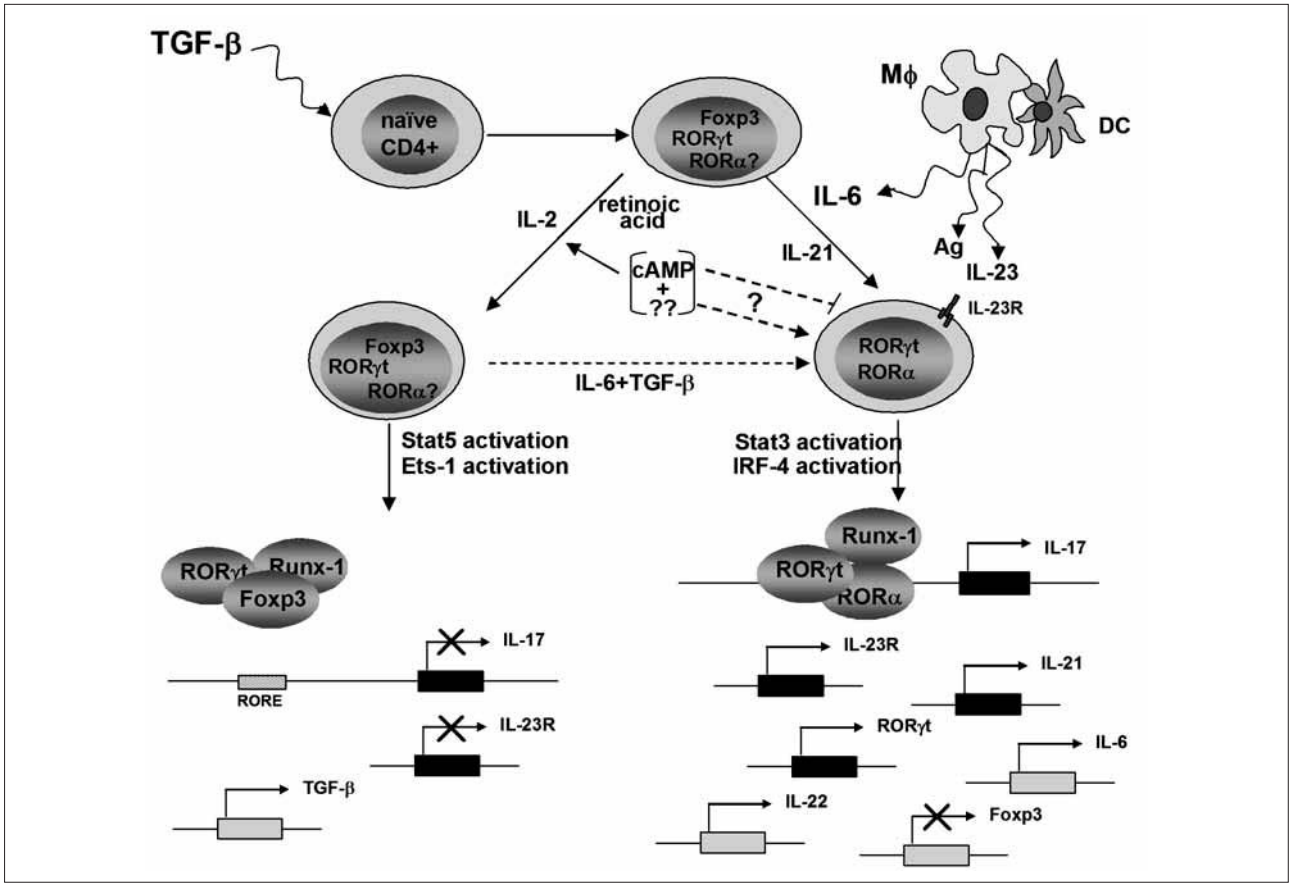

Figure 2. *Antagonic Treg and Th17 generations share an intrinsic genetic program. TGF-β activity on naïve CD4+ cells induces the expression of both Foxp3 and RORγt transcription factors. In the absence of IL-6, RORγt and Runx-1 are sequestered by Foxp3 binding; this interaction prevents contact with ROR elements (RORE) in the IL-17 promoter. Cascade signalling triggered by IL-6 leads in naïve, and probably in Treg defined cells, to Stat3 activation, RORγt expression, down-regulation of Foxp3 expression, and suppression of the binding of pre-existing Foxp3 to RORγt. In a Stat3-dependent mechanism, the activity of RORγt, RORα and Runx-1 allows IL-17 gene expression. The subsequent expression of IL-23R, IL-6 and IL-21 guide a feedback of signals to amplify and stabilize the Th17 phenotype. IRF4 activation by antigen (Ag) presentation also collaborates to Th17 cell differentiation, whether upstream or downstream of RORγt remaining to be determined. Dark boxes correspond to Stat3 regulated genes.*

by the environmental amount of TGF-β[143]. According to these authors, at low concentrations, TGF-β would synergize with interleukin IL-6 and IL-21, favouring Th17 cell differentiation, whereas high concentrations of TGF-β would repress IL-23R expression and favour Foxp3+ Treg cells. Runx1 has just been proposed as a new player in this network of transcription factors regulating Th17/Treg balance[136]. Results in this report show that Runx1, in addition to interact with RORγt on the IL-17 promoter, can be bound by Foxp3 and probably be neutralized in its ability to activate IL-17 transcription.

The function of IL-2, needed for Treg cell survival and negative regulator of Th17 cells, could constitute an additional control in the homeostasis of the Treg and Th17 subsets. On the other hand, retinoids appear to be other physiologic regulators of Th17/Treg differentiation[144-146]. Vitamin A derivatives are protective in animal models of autoimmune disease and current data suggest that retinoic acid produced by dendritic cells reciprocally regulates Th17 and Treg differentiation.

It is well-known that cAMP inhibits T cell proliferation[147,148]. This second messenger is involved in suppressor activity by Treg cells[149]. In effector cells, cAMP is strongly increased upon coactivation with Treg cells, and it was proposed that cAMP can be transferred from Treg to effector targets via cell contact-dependent gap junctions. Although the role of cAMP concerning to Th17 cell function is largely unclear, there are some indications of increased IL-17 production mediated by this second messenger. Chizzolini et al.[150], have demonstrated that prostaglandin E2, whose signalling leads to elevated cAMP, synergizes with IL-23 to favour Th17 expansion. Nevertheless,

the authors did not analyze if this effect is cAMP-dependent or not. Besides, Yadav et al.[151] found that the vasoactive intestinal peptide (VIP) induces Th17 differentiation, in a mechanism suppressed by inhibition of protein kinase A, a main target of cAMP signalling. On the other hand, our unpublished results suggest that some agents increasing cAMP favour *in vitro* Th17 development. Although more clarifying data about cAMP involvement in Th17 induction is needed, cAMP might be another mediator involved in the Th17/Treg balance, which, like intracellular signalling of TGF-β, could have a double function collaborating in Treg or Th17 differentiation depending on environmental signals.

A remarkable novel suggestion about the mechanisms of Th17/Treg balance has been made by Yang et al.[152], who suggest that proinflammatory cytokines produced in the inflamed tissue not only affect the generation of induced Treg cells, but also might inhibit the function of already existing Treg cells. In addition, the absence of Foxp3 expression in T cells leads to increased Th1 cell differentiation without enhancing Th17 development. These data could be interpreted as that the real opposite pathways are those followed by Th1 and Treg cells, while Th17 and Treg generation share intrinsic common programs which allow some plasticity to reprogramming the phenotype of Treg cells towards Th17 effectors. If confirmed, these data could have important implications in therapies against autoimmune diseases with Treg cells, and it should be required to complement them with suppression of IL-6 to avoid redifferentiation into pathogenic Th17 cells.

ACKNOWLEDGEMENTS

The authors thank M. J. Jerez for the careful grammar correction of this manuscript. This work was supported by grants from Instituto de Salud Carlos III (FIS-PI061012) and MM Foundation (MPY-1156/07).

DISCLOSURES

The author declares no financial conflicts of interest.

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