Review

Heterogeneity of human effector CD4⁺ T cells
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Abstract

For many years the heterogeneity of CD4⁺ T-helper (Th) cells has been limited to Th1 and Th2 cells, which have been considered not only to be responsible for different types of protective responses, but also for the pathogenesis of many disorders. Th1 cells are indeed protective against intracellular microbes and they are thought to play a pathogenic role in organ-specific autoimmune and other chronic inflammatory disorders. Th2 cells provide protection against helminths, but are also responsible for the pathogenesis of allergic diseases. The identification and cloning of new cytokines has allowed one to enlarge the series of functional subsets of CD4⁺ Th effector cells. In particular, CD4⁺ Th cells producing IL-17 and IL-22, named Th17, have been initially implicated in the pathogenesis of many chronic inflammatory disorders instead of Th1 cells. However, the more recent studies in both humans and mice suggest that Th17 cells exhibit a high plasticity toward Th1 cells and that both Th17 and Th1 cells may be pathogenic. More recently, another two subsets of effector CD4⁺ Th cells, named Th9 and Th22 cells, have been described, even if their pathophysiological meaning is still unclear. Despite the heterogeneity of CD4⁺ effector Th cells being higher than previously thought and some of their subsets exhibiting high plasticity, the Th1/Th2 paradigm still maintains a strong validity.

Introduction

CD4⁺ T-helper (Th) lymphocytes represent a heterogeneous population of cells that play an essential role in adaptive immunity. These cells include effector cells, which are devoted to protection against pathogens, and regulatory T cells (Tregs), which protect against effector responses to autoantigens and also against responses to exogenous antigens when they may become dangerous for the host. The term Th derived from the observation that these cells were critical for helping B cells to produce antibodies in the primary response (humoral immunity). On the other hand, CD4⁺ T cells were also found to be responsible for the so-called cell-mediated immunity, or delayed-type hypersensitivity, which was characterized by the ability of these cells to induce inflammatory reactions mainly characterized by the activation of macrophages. The prototypic cell-mediated immune response was considered to be the skin papular reaction induced by intradermal injection of tuberculin or purified protein derivative (PPD) in animals infected with tubercular bacilli or in humans naturally infected by Mycobacterium tuberculosis or vaccinated with Bacillus Calmette-Guérin (BCG).

The first demonstration of the existence of at least two different populations of CD4⁺ effector T cells was given in 1972 by Parish and Liew [1]. Injection of multiple doses of flagellin in Wistar rats allowed them to demonstrate that suppression of delayed-type hypersensitivity was observed when enhancement of antibody response occurred, suggesting an inverse relationship between humoral and cell-mediated immune response. In 1986 Mosmann and his coworkers showed that the functional heterogeneity of murine CD4⁺ T cells was due to their different profile of cytokine production [2], a finding that was also confirmed in humans [3,4]. Murine and human CD4⁺ T cells were categorized into two main subsets, which were defined as Th type 1 (Th1) or Th type 2 (Th2) [2-4].

The T-helper type 1/T-helper type 2 paradigm

The reason for the heterogeneity of effector CD4⁺ Th cells is mainly related to their protective function, because it enables the best type of response according to the nature of the invading microorganism. Th1 cells produce high levels of IFNγ and are responsible for both phagocyte activation and the production of opsonizing and complement-fixing antibodies, thus playing an important role in protection against intracellular pathogens. Th2 cells produce IL-4, IL-5, IL-9 and IL-13, thus being mainly involved in the protection against parasitic helminths [5]. IL-4 and IL-13 are the major mediator of IgE class switching in B cells [6]. IgE binds to FcεRI on...
basophils and mast cells, and their interaction with a multivalent ligand induces cross-linking of FcεRI – which leads to the secretion of active mediators such as histamine and serotonin, and to the production of several cytokines and chemokines, including IL-4, IL-13, TNFα and eotaxins. IL-5 positively regulates a large number of eosinophil functions, including eosinophilopoiesis, bone marrow release, activation and survival [7]. IL-9 production following infection by helminths contributes to the general mast cell and IgE response characteristic of these infections [8]. In addition to its effect on mast cells and lymphocytes, IL-9 induces mucin production in epithelial cells [9]. To say that Th1 cells are responsible for cell-mediated immunity and Th2 cells are responsible for humoral immunity, however, is not correct. Indeed, Th1 cells allow the production of IgG2a antibodies in mice and of IgM, IgA, IgG1, IgG2 and IgG3 antibodies in humans, whereas Th2 cells induce IgG4, and IgE antibodies in mice and IgM, IgG4 and IgE in humans.

The mechanisms responsible for Th1 or Th2 polarization were also discovered. Based on the observation that IL-12 and IFNγ, two cytokines produced by dendritic cells, acted as powerful inducers of human Th1 polarization [10-12], I hypothesized that the type of innate immunity response was the main conditioning mechanism for the type of subsequent adaptive immunity [13]. This hypothesis was found to be true when the existence in dendritic cells of the so-called Toll-like receptors was observed [14]. The Toll-like receptors are able to interact with a group of highly conserved structures of many bacteria and viruses, and this interaction usually results in the production by dendritic cells of high amounts of IL-12 and/or IFNγ, thus explaining the Th1 polarization usually induced by microbial infections. Indeed, IFNγ produced by dendritic cells and/or IFNγ produced by natural killer cells upon stimulation by IL-12 activates the signal transducer and activator of transcription (STAT)-1 in the naïve CD4 T cells. Activated STAT-1 upregulates T-box expressed in T cells (T-bet) expression, which in turn induces early T-cell IFNγ production and upregulates IL-12Rβ2 expression. The IL-12Rβ2-expressing T cells can then directly respond to IL-12 that, through activation of STAT-4, induces high IFNγ production and sustains the expression of IL-12Rβ2 [15].

Collaboration between interferons and IL-12 therefore induces full Th1 differentiation [15-18]. At later stages of Th1 differentiation, IL-18Rα is also upregulated. IL-18Rα upregulation requires IL-12/STAT-4 signalling and is further increased by IFNγ. IL-12 and IL-18 jointly induce IFNγ production by Th1 cells in the absence of T-cell receptor stimulation. Such antigen-independent cytokine production is probably important for amplifying Th1 responses by recruiting other pre-existing Th1 cells [19,20].

At that time it was also found that Th2 polarization was mainly due to the early production of IL-4 during the primary response [12]. The cell and the mechanisms responsible for this early IL-4 production, however, remained unclear for a long time. Only recently was it found that IL-4 could be produced by the naïve Th cell itself, upon Notch triggering, as a consequence of the expression by the dendritic cells of its ligand Jagged-1 in both mice and humans [21-23]. Another possibility is the production by other cell types, such as mast cells and macrophages present in the gut of worm-infested animals or lung epithelial cells, of a more recently discovered cytokine, named IL-25. IL-25 can induce the early production of IL-4 by a non-T, a non-B, c-kit+, a FcεRI+ cell or by the Th naïve cell itself, thus allowing its Th2 polarization [24,25].

The interaction of the endogenous and/or exogenous IL-4 with its receptor results in the activation of STAT-6, which in turn upregulates GATA-binding protein-3 (GATA-3) and c-maf expression [26-30]. GATA-3 has been reported to induce its own expression [31], probably when it reaches a threshold level. GATA-3 binds to regions of the Il4/Il13 loci, including DNaseI hypersensitive site Va and conserved noncoding sequence-1 sites. GATA-3 alone, however, is not sufficient to induce IL-4 production. IL-2-mediated activation of STAT-5 plays a critical role in inducing/maintaining accessibility at the second intron HSII and HSIII DNase I hypersensitive sites of the Il4 locus [32]. Indeed, STAT-5 is bound to these two sites in Th2 cells, but not in Th1 cells. The collaboration of STAT-5 and GATA-3 accounts for full Th2 differentiation in vitro [33]. Of note, there is a mutual regulation of Th1 and Th2 polarization induced not only by IL-4 and IFNγ, respectively, but also by the Th1-specific and Th2-specific transcription factors. Accordingly, GATA-3 has been reported to downregulate STAT-4 [34]. Strong STAT-5 activation inhibits T-bet expression [32]. On the other hand, T-bet can suppress GATA-3 expression [35].

In addition to Th1 and Th2 cells, another series of CD4+ T cells were identified as able to produce at the same Th1-related and Th2-related cytokines, which were named Th type 0 (Th0) cells [36]. Because of their functional differences, Th1 and Th2 cells – in addition to having different protective functions against invading pathogens – also contribute to the development of different human disorders: Th1 cells have been thought to be involved in the pathogenesis of organ-specific autoimmune diseases, as well as other chronic inflammatory disorders such as Crohn’s disease, sarcoidosis and atherosclerosis [37]; and Th2 cells certainly play a central role in the development of allergic disorders [38].

**Beyond the T-helper type 1/T-helper type 2 paradigm**

**The discovery of T-helper type 17 cells**

The Th1/Th2 paradigm was maintained until some years ago when a third subset of CD4+ effector Th cells, named Th type 17 (Th17) cells, was identified [39,40]. Although the existence of IL-17 as a product of activated CD4+ T cells has been known for more than 10 years, only recently was the existence of Th17 cells as a distinct subset recognized
The breakthrough leading to the discovery of the Th17 lineage came from murine models of autoimmunity. Experimental autoimmune encephalomyelitis (EAE), collagen-induced arthritis and inflammatory bowel disorders have historically been associated with unchecked Th1 responses, largely based on studies in which disease development was ablated by neutralizing the IL-12p40 chain or by targeting the p40 or IL-12Rβ1 genes [41].

This initial concept of Th1 association with autoimmune disorders, however, required an adjustment with the unexpected discovery that mice deficient in IFNγ or IFNγ receptor were not resistant to EAE but were actually more susceptible to central nervous system autoimmunity [42-44]. Moreover, the link with IL-12 in these diseases was called into question by the discovery that a new IL-12 family member, IL-23, shares with IL-12 the p40 subunit – the heterodimer of IL-12 being composed of p40 and p35, and that of IL-23 being composed of p40 and p19 [39]. IL-23 also shares with IL-12 a chain of its receptor – the IL-12 receptor being composed of IL-12Rβ1 and IL-12Rβ2 chains, and that of IL-23 being composed of IL-12Rβ1 and IL-23R chains. After this discovery, it was found that EAE and collagen-induced arthritis did not develop in mice deficient in the IL-23p19 subunit or the IL-23R chain, whereas the diseases could develop in those deficient in the IL-12p35 subunit or the IL-12Rβ2 chain, suggesting that at least in these models IL-23 but not IL-12 is critically linked to autoimmunity [40,45,46].

Based on these and other findings, a new role for Th17 cells in immunopathology and the distinct origin of Th1 cells and Th17 cells under differential IL-12 or IL-23 conditioning was proposed [40,45]. More recently, however, a completely different pathway of murine Th17 origin has been described [47-49]. Although IL-23 appeared to be required for Th17-induced immunopathology, different groups independently demonstrated that transforming growth factor beta (TGFβ) was required for initiation and that IL-6 was a critical co-factor for Th17 differentiation [47-49]. Of note, the Th17-polarizing cytokine TGFβ was already known for its ability to promote the development of Foxp3+ Tregs, but only in the absence of IL-6 [49]. Murine Th17 cells express a master transcription factor different from Th1 and Th2 cells, an orphan receptor known as retinoic acid-related orphan receptor (ROR)γt [50]. A second orphan receptor, named RORα, has also been found to contribute to the development of murine Th17 cells [51]. The STAT-3 transcription factor is also essential for the murine Th17 development, although whether it acts directly or through the activation of RORγt is still unclear [52].

The distinctive cytokine of murine Th17 cells, IL-17A, is involved in the recruitment, activation and migration of neutrophil granulocytes by inducing the production of colony-stimulatory factors and CXCL8 [53] by both macrophages and tissue resident cells. The other cytokines produced by murine Th17 cells, such as IL-17F, IL-21 and IL-22, can also contribute to the activation of mononuclear and/or resident cells and therefore may induce and/or maintain a chronic inflammatory process. Because of their unique ability to recruit neutrophils, however, the main protective function of Th17 cells appears to be the clearance of extracellular pathogens, including fungi [54].

Nevertheless, the major emphasis on the pathophysiology of murine Th17 cells was placed on their determinant or even exclusive pathogenic role on models of autoimmunity. This concept was immediately extrapolated to human disorders that are considered equivalent to the above-mentioned murine models, such as multiple sclerosis, rheumatoid arthritis and inflammatory bowel disorders [55], but also to psoriasis, contact dermatitis and atopic dermatitis [56]. Th17 cells were therefore thought to be the pathogenic cells in virtually all chronic inflammatory disorders, where the effect of Th1 cells – which had been shown to be important in hundreds of previous studies – was underscored or even seen as protective against the Th17-mediated inflammation [57].

**Human T-helper type 17 cells may be different from murine T-helper type 17 cells**

The studies in humans showed that Th17 cells express RORC, CCR6, CCR4 and the IL-23R, but also CD161 [58] the equivalent of murine NK1.1 [59], and they produce IL-17A, IL-17F, IL-22, IL-26 and the chemokine CCL20 [60-63]. Of note, a substantial proportion of human Th17 cells produce IFNγ in addition to IL-17A, and these cells were named Th17/Th1 [61]. Both Th17 cells and Th17/Th1 cells also expressed the IL-12Rβ2 chain and the Th1-related transcription factor T-bet [61]. Finally, stimulation of human Th17 cells in the presence of IL-12 downregulated RORC and upregulated T-bet, and enabled these cells to produce IFNγ in addition to IL-17A [61].

Another difference between murine and human Th17 cells was found with regard to their origin. While murine Th17 cells originate from a naïve Th cell in the presence of TGFβ and IL-6 [47-49], human Th17 cells exclusively originate in the presence of IL-1β and IL-23 from a small subset of naïve CD4+ Th cells that express CD161, which are present in the umbilical cord blood and newborn thymus [58]. The role of TGFβ in the differentiation of human Th17 cells is controversial, with some studies indicating that the addition in the culture of exogenous TGFβ is critical for the induction of RORC expression [64-66], while other studies do not [58,62,63,67]. Our studies demonstrate that the CD161+ precursors of human Th17 cells present in the umbilical cord blood and thymus already express RORC and IL-23R *ex vivo* [58], and that exogenous TGFβ may only have an indirect role in the development of Th17 cells, mainly due to its strong suppressive activity on the proliferation of Th1 cells [68]. The expression of CD161 by human Th17 cells has been recently confirmed in both the circulation and the gut of subjects with

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Crohn’s disease [69]. Circulating CD161+ T cells migrate to gut lymphoid tissue, where in normal subjects they require the presence of IL-1β and IL-23 to become fully mature Th17 cells, whereas in the gut of subjects with Crohn’s disease the presence of IL-23 is sufficient for this maturation [69].

Boniface and colleagues have recently shown that prostaglandin E2 can act directly on both human and murine T cells to enhance Th17 development [70]. In fact, prostaglandin E2 – acting via the prostaglandin receptor EP2-mediated and EP4-mediated signalling and cAMP pathways – induces upregulation of IL-23 and IL-1 receptor expression in human T cells. Furthermore, prostaglandin E2 synergizes with IL-1β and IL-23 to drive RORC, IL-17, IL-17F, CCL20, and CCR6 expression. Similar results have been obtained by Napolitani and colleagues, who showed that prostaglandin E2 triggering of the EP2 and EP4 receptors expressed on T cells led to a rapid increase of RORC and a decrease of T-bet mRNA [71]. In addition, prostaglandin E2 could favour the enrichment of IL-17-producing cells at inflammatory sites by preferentially inhibiting proliferation of CCR6- T cells, which include Th1 cells, but not proliferation of CCR6+ Th17 cells. Finally, prostaglandin E2 can directly act on T cells and promote Th17 responses independently of the presence of IL-23 while it synergized with IL-1 and IL-6 to favour IL-17 release.

The critical role for IL-1 signalling in the differentiation of Th17 cells has recently been demonstrated even in mice, and it has been shown that in presence of IL-1 the addition of exogenous TGFβ is not essential [72].

Another interesting point is the possible relationship between human Th17 cells and Tregs. A transdifferentiation of Tregs into Th17 cells has been described recently [73,74], as well as the existence of cells coexpressing IL-17 and Foxp3 [75,76]. Our findings showing that Th17 cells derive from CD161+ T-cell precursors and maintain CD161 expression even at the stage of memory T cells, whereas Tregs were never been shown to express this marker, apparently argue against such a possibility.

**Role of T-helper type 1 and T-helper type 17 cells in pathogenicity**

The demonstration of a potential plasticity of human Th17 cells to Th1 cells [61] was of great importance with regard to the controversial issue of the respective roles of the two effector cell types in the pathogenesis of murine and human autoimmune disorders, as well as of other chronic inflammatory disorders. An important clarification on the pathogenicity of murine Th17 cells recently came from four independent studies [77-80].

First, not only did the propagation of committed Th17 precursors in the presence of IL-23 without TGFβ result in a progressive extinction of IL-17A and IL-17F and promote the emergence of IFNγ-producing cells that lacked IL-17 expression, but their stimulation with IL-12 induced a rapid, STAT4-dependent and T-bet-dependent transition marked by an extinction of RORγt, RORα, IL-17A and IL-17F and an induction of a Th1-like expression signature [77]. Moreover, the potential plasticity of Th17 to Th1 cells, but not of Th1 to Th17 cells, has been reported [78]. These findings support the substantial developmental plasticity of the Th17 lineage already observed in humans [61], which identified a mechanism for latent Th1-like responsiveness of Th17 cells and provided the basis for understanding the relationship between Th17-mediated and Th1-mediated pathophysiology.

Indeed, it has recently been shown that Th17 cells can promote pancreatic inflammation, but can only induce type 1 insulin-dependent diabetes mellitus efficiently in lymphopenic mice after conversion into Th1 cells [79]. Accordingly, highly purified Th17 cells from BDC2.5NOD mice shift into Th1-like cells in NOD/SCID recipient mice. The transferred Th17 cells, completely devoid of IFNγ at the time of transfer, rapidly converted to secrete IFNγ in the NOD/SCID recipients. More importantly, the development of insulin-dependent diabetes mellitus was prevented by the treatment with anti-IFNγ-specific antibody but not with anti-IL17A-specific antibody [80]. The plasticity of Th17 cells into Th1 cells initially observed in humans [61] is therefore now confirmed in mice and provides the basis for supporting again the major pathogenic role of Th17-derived Th1 cells in murine autoimmune or other chronic inflammatory disorders. The relationship between the Th17-derived Th1 cells and the classic Th1 cells obtained in response to IL-12-mediated polarization, however, is not yet clear.

**T-helper type 9 cells**

An additional subset of CD4+ effector T cells able to produce IL-9 has been recently described [81,82]. IL-9 was previously known as a Th2-derived cytokine [83], and was found to be important in inducing the mucus hypersecretion in asthmatic subjects [84] and to contribute to the development of tuberculosis by reducing IFNγ production in peripheral blood mononuclear cells stimulated with *M. tuberculosis* antigens [85]. When murine Th2 cells were cultured in the presence of IL-4 and TGFβ, they lost the capacity to produce IL-4, IL-5 and IL-13, but they maintained the ability to produce IL-9 in addition to IL-10 [81,82]. The IL-9+IL-10+ T cells demonstrated no regulatory properties despite producing abundant IL-10. By contrast, their adoptive transfer into recombination-activating gene 1-deficient mice induced colitis and peripheral neuritis, the severity of which aggravated whether these cells were transferred with CD45RBhigh effector T cells. This novel Th subset therefore lacks suppressive function and constitutes a distinct population of effector T cells that promote tissue inflammation [81].

More recently, it was found that IL-9 is produced in high amounts not only by Th2 and Th9 cells but also by Th17 cells...
More importantly, IL-9 appeared to be a key molecule that affects both differentiation of Th17 cells and Treg function. IL-9 synergized with TGFβ1 to differentiate naïve CD4+ T cells into Th17 cells, while IL-9 secretion by Th17 cells was regulated by IL-23. Interestingly, IL-9 enhanced the suppressive function of Foxp3+CD4+ Tregs in vitro and the absence of IL-9 signalling weakened the suppressive activity of Tregs in vivo, leading to an increase in effector cells and worsening of EAE. Accordingly, it has been recently shown that both IL-9 neutralization and IL-9 receptor deficiency attenuate EAE, and this effect correlates with decreases of Th17 cells and IL-6-producing macrophages in the central nervous system, as well as of mast cell numbers in the regional lymph nodes [86]. These findings suggest a novel role of IL-9 as a regulator of pathogenic versus protective mechanisms of immune responses [86,87].
T-helper type 22 cells

IL-22 was originally described in mice and humans as a cytokine characteristic of fully differentiated Th17 cells [60]. Recently, however, a distinct subset of human skin-homing memory T cells has been shown to produce IL-22, but neither IL-17 nor IFNγ [88,89]. Differentiation of IL-22 producing T cells, now named Th22 cells, could be promoted by stimulation of naive T cells in the presence of IL-6 and TNF or by the presence of plasmacytoid dendritic cells, and appears to be independent of RORC but dependent upon the aryl hydrocarbon receptor [88,89]. The human Th22 cell population coexpresses the chemokine receptor CCR6 and the skin-homing receptors CCR4 and CCR10, which led to hypotheses that these cells may be important in skin homeostasis and pathology [88,89].

Concluding remarks

Very recent studies demonstrate that CD4+ Th effector cells represent a population much more heterogeneous than previously suggested. Beyond Th1 cells and Th2 cells, Th17 cells, Th9 cells and Th11 cells have now been recognized. These main populations of CD4+ effector T cells are depicted in Figure 1. Moreover, CD4+ T cells seem to exhibit a great plasticity not only in the context of effector responses, but also of regulatory responses. Th2 cells can shift to Th1 cells or to Th9 cells, whereas Th17 cells can shift to Th1 cells. Among different CD4+ effector T cells, the Th1 cells appear to be the more stable. Although this complex situation makes it more difficult to extrapolate the role of different subsets of CD4+ effector cells, the validity of the Th1/Th2 paradigm seems to maintain its validity. Indeed, there is no doubt regarding the main and essential role of Th2 responses against allergens in accounting for the great majority of pathophysiological manifestations in allergic subjects. On the other hand, even if Th17 cells certainly play a pathogenic role in autoimmune disorders and other chronic inflammatory disorders, classic Th1 cells or Th17-derived Th1 cells are also co-pathogenic or even truly responsible for the inflammatory processes that underlie these diseases.

Competing interests

The authors declare that they have no competing interests.

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