humane weight-loss limit more rapidly (80% of original mass, which required that mice be killed), in some cases as many as 2 weeks earlier than controls (Fig. 1 and data not shown). Indeed, by 6 weeks after transfer, the 80% mass-loss target had been reached by over 40% of *Rag1*$^{-/-}$ recipients of *Il17a*$^{-/-}$ CD45RB^{hi} cells (Fig. 1a,b and data not shown). Disease incidence was 94.3% and 92.1% for recipients of wild-type cells and *Il17a*$^{-/-}$ cells, respectively. Thus, CD45RB^{hi} cells unable to produce IL-17A induced an aggressive wasting disease *in vivo*, which demonstrates IL-17A is not critical in the initiation of T cell–induced colitis in this model. Moreover, it seems that in this model system, IL-17A delayed the kinetics of disease onset, which emphasizes a previously unappreciated protective function for IL-17A in intestinal inflammation. Notably, the cotransfer of IL-17A-deficient regulatory T cells completely inhibited colitis in this model (Supplementary Fig. 1 online), which suggests that although IL-17A may be protective, regulatory T cell–mediated suppression is IL-17A independent.

More T_H1-associated inflammation in recipients of *Il17a*$^{-/-}$ T cells

To gain insight into the mechanisms driving the accelerated colitis in the absence of IL-17A, we examined recipients of *Il17a*$^{-/-}$ CD45RB^{hi} T cells at 2 weeks and 4 weeks after adoptive transfer. Overall body mass was maintained or slightly higher in the first 2 weeks after transfer in all groups (Fig. 1b and data not shown). However, greater organ thickness, cellular infiltration and edema and disrupted tissue architecture were already observable in ascending and descending colon tissue from mice at autopsy 2 weeks after transfer (Fig. 1c,d). In the colons of recipients of wild-type cells, we found normal mucosa, minimal submucosa and unremarkable muscularis externa (Fig. 1d, i). By day 14, colons from mice given *Il17a*$^{-/-}$ cells had an overall thinner wall, with mild to moderate submucosal edema (Fig. 1d, ii, and e), in contrast to mice given wild-type cells, which had a much thicker colon (Fig. 1d, iii, and e), mainly due to moderately to considerably greater thickness at the level of the submucosa and muscularis (Supplementary Fig. 2 online). The overall severity of cellular infiltration, generally assigned a score as 'inflammation', was equivalent in the groups (Fig. 1e); however, several recipients of *Il17a*$^{-/-}$ cells had severe ulceration with considerable loss of mucosal epithelial cells (Fig. 1d, iv, v, and e). The transition to ulcerated epithelium was notable (Fig. 1d, v). By day 28, colons from mice that received wild-type cells showed similar inflammation without the severe mucosal epithelial ulceration (Fig. 1d, vi,vii). We assigned scores to histopathological criteria semiquantitatively (Fig. 1e). In general, the severity of ulceration, inflammation, edema and observable tissue damage was greater in individual mice with the greatest weight loss in the group, concomitant with meaningful differences in body mass at day 28. We concluded that the greater severity of wasting disease mediated by *Il17a*$^{-/-}$ CD45RB^{hi} T cells was probably not due to differences in cellular infiltration of the colon (Fig. 1e) but may instead have been due to relative changes in effector T cell function.

Next we assessed the expression of T_H1 cell–associated cytokines traditionally associated with T cell–mediated IBD. Quantitative RT-PCR showed that the expression of mRNA transcripts encoding T_H1-associated factors was higher in colon tissue from recipients of *Il17a*$^{-/-}$ cells than in that of recipients of wild-type cells (Fig. 1f). Notably, *Ifng* expression was threefold higher in recipients of *Il17a*$^{-/-}$ T cells. *Tnf* expression was not similarly higher (Fig. 1f). In addition, expression of *Spp1* mRNA, which encodes osteopontin, a cytokine that amplifies T_H1-type responses through the induction of IL-12 and other mechanisms[23,24], was ninefold higher in recipients of *Il17a*$^{-/-}$ cells (Fig. 1f). *Il6* expression trended upward in the group of recipients of *Il17a*$^{-/-}$ cells, but this result was not statistically significant. We found no difference in *Tnf* expression in recipients of *Il17a*$^{-/-}$ cells or wild-type cells (Fig. 1f). We detected these differences in cytokine mRNA expression at 28 d after cell transfer but not at 14 d after cell transfer or in samples obtained at the disease endpoint (typically 8–10 weeks), when epithelial damage and cellular infiltration was extensive (data not shown). Additionally, expression of *Il17a* and *Il17f* mRNA was nearly undetectable in recipient colon tissue obtained at 28 days after transfer (Supplementary Fig. 3 online), which suggests that neither IL-17A nor IL-17F contributes much to the disease in this model.

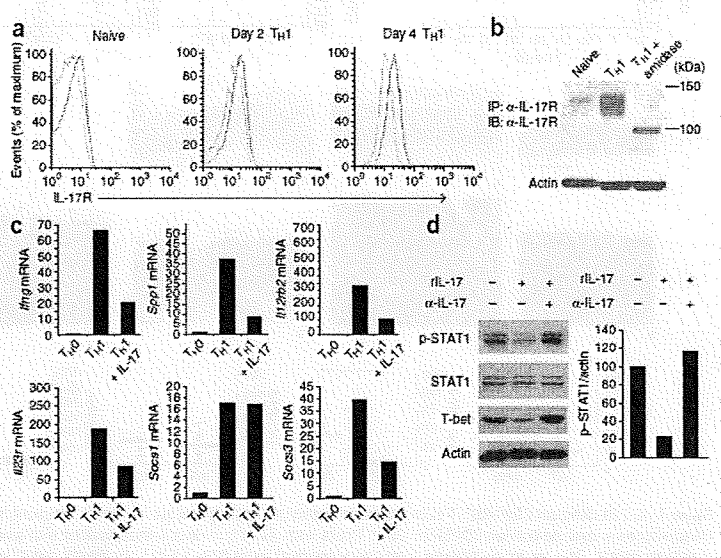

Figure 2 IL-17 modulates T_H1 differentiation. (**a**) Flow cytometry analysis of IL-17R on the cell surface of naive CD4$^+$ T cells assessed directly after isolation (Naive) or after 2 d or 4 d of culture in T_H1-polarizing conditions. Light lines, isotype-matched control antibody; dark lines, antibody to IL-17R. Data are representative of two experiments. (**b**) Immunoblot analysis (IB) of the immunoprecipitation (IP) of IL-17R from freshly isolated naive CD4$^+$ T cells or from T cells cultured for 4 d in T_H1-polarizing conditions *in vitro* with (right) or without (left and middle) amidase treatment. Actin, loading control for protein content in cell lysates. α-IL-17R, antibody to IL-17R. Results are representative of two independent experiments. (**c**) Real-time PCR analysis of gene expression in T_H0 effector cells or in T_H1 effector cells at day 4 generated *in vitro* in the presence (+ IL-17) or absence of recombinant IL-17 (20 ng/ml). Data are representative of three experiments. (**d**) Immunoblot analysis (left) of T_H1 effector cells at day 4 generated *in vitro* in the presence (+) or absence (–) of recombinant IL-17 (rIL-17) and/or IL-17-neutralizing antibody (α-IL-17). Right, differences in expression of phosphorylated STAT1 (p-STAT1), normalized to actin and presented relative to that of day-4 T_H1 cells cultured alone. Data are representative of three experiments.

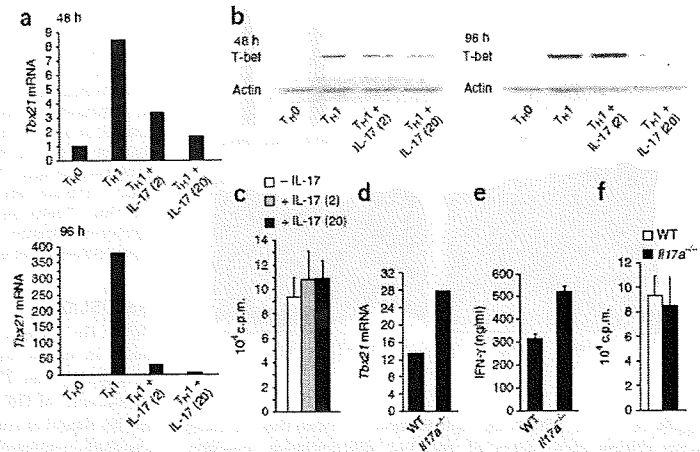

Figure 3 IL-17 suppresses the induction of T-bet in maturing T_H1 cells. (a) Real-time PCR analysis of *Tbx21* in T_H0 effector cells or in T_H1 effector cells polarized *in vitro* in the presence (+ IL-17) or absence of 2 ng/ml or 20 ng/ml (in parentheses) of recombinant mouse IL-17 after 48 h or 96 h of culture, normalized to *Hprt1* expression and presented as 'fold increase' relative to that of T_H0 cells. (b) Immunoblot analysis of T-bet in the 96-hour cultures in a. Actin, loading control. Data are representative of three or more independent experiments (a,b). (c) Proliferation of cells in T_H1 effector cultures at day 4, differentiated in the presence or absence of 2 ng/ml or 20 ng/ml (in parentheses) of recombinant mouse IL-17, assessed by incorporation of [^{3}H]thymidine. Data are representative of two independent experiments. (d) *Tbx21* expression in day-4 T_H1 effector cells generated *in vitro* from wild-type or *Il17a*$^{-/-}$ naive CD4$^+$ CD45RB^{hi} T cells, normalized to *Hprt1* expression and presented as 'fold increase' relative to that of T_H0 cells. Data are representative of three experiments. (e) Enzyme-linked immunosorbent assay of the release of IFN-γ from naive wild-type or *Il17a*$^{-/-}$ CD4$^+$ CD45RB^{hi} T cells polarized for 5 d in T_H1-type conditions before overnight restimulation. Data are representative of three experiments. (f) Proliferation of the cells in d, assessed by incorporation of [^{3}H]thymidine. Data are representative of three experiments.

Colon tissue from recipients of *Il17a*$^{-/-}$ T cells had higher expression of *Il22* mRNA at day 28 than did that of recipients of wild-type cells; however, studies with transfers of *Il17a*$^{-/-}$*Il22*$^{-/-}$ CD45RB^{hi} cells showed that increased T cell–derived IL-22 was not responsible for the accelerated wasting disease noted in the absence of T cell–derived IL-17 (Supplementary Fig. 4 online). These results demonstrate that cohorts that received *Il17a*$^{-/-}$ CD45RB^{hi} T cells, which developed an accelerated wasting disease, had higher expression of T_H1-associated cytokines in inflamed colon tissue. Larger amounts of T_H1-associated cytokines may have resulted in the observed lower overall body mass. These data therefore suggest that the accelerated colitis in recipients of *Il17a*$^{-/-}$ T cells may have been due at least in part to the acceleration of a T_H1 differentiation program *in vivo*.

IL-17A signaling in T cells suppresses T_H1 differentiation

On the basis of our *in vivo* observations, we hypothesized that T cells may be directly responsive to IL-17A. The IL-17 receptor (IL-17R) was nearly undetectable on naive CD4$^+$ T cells (Fig. 2a). In contrast, IL-17R was upregulated at late stages during the T_H1 differentiation program, with moderate amounts of cell surface IL-17R detectable by day 4 (Fig. 2a). We confirmed our flow cytometry results by immunoprecipitation and immunoblot analysis, which showed that total cellular IL-17R protein quantities were low in naive T cells (Fig. 2b) but were modestly induced during T_H1 development *in vitro* (Fig. 2b). The IL-17R can be visualized as a protein of approximately 120 kilodaltons, a value much larger than the predicted molecular weight based on amino acid analysis (98 kilodaltons). *In silico* analysis of the protein sequence of IL-17R showed that extensive *N*-glycosylation was predicted with high probability (five asparagine residues predicted by the NetNGlyc 1.0 Server (Technical University of Denmark); seven predicted sites reported in the UniProtKB/Swiss-Prot entry). To determine if glycosylation accounted for the shift in molecular weight, we deglycosylated the immunoprecipitated IL-17R with peptide *N*-glycosidase F and found that after treatment, the doublet migrating at approximately 120 kilodaltons resolved to a single band visualized by immunoblot at the predicted 98 kilodaltons (Fig. 2b).

We next sought to determine whether purified IL-17 could exert appreciable effects on the development of wild-type T_H1 cells *in vitro*. We cultured sorted naive CD45RB^{hi} CD4$^+$ T cells for 4 d in T_H1-polarizing conditions in the presence or absence of recombinant IL-17. Treatment with IL-17 resulted in much lower expression of the T_H1-associated mRNA transcripts *Ifng*, *Spp1* and *Il12rb2* (Fig. 2c), which showed that recombinant IL-17 exerted broadly suppressive effects on the T_H1 developmental program. Transcripts encoding SOCS3, a known inhibitor of IL-17 production[25], were also lower in abundance after treatment with recombinant IL-17, whereas IL-17 had no effect on SOCS1 mRNA (Fig. 2c). IL-17 also led to a 78% lower abundance of phosphorylated STAT1 (Fig. 2d). The addition of neutralizing antibody to IL-17 completely reversed the IL-17-mediated suppression of phosphorylated STAT-1 (Fig. 2d). Expression of T-bet, a transcription factor essential for T_H1 differentiation, was also much lower in IL-17-containing cultures (Fig. 2d). As with STAT1, the effect on T-bet was reversed by IL-17-neutralizing antibody (Fig. 2d). Recombinant IL-17 did not substantially affect the expression of genes encoding IL-9, granulocyte-macrophage colony-stimulating factor, the chemokine CCL3, IL-1β or the chemokine CXCL1 in developing T_H1 cells or induce the expression of T_H17-specific genes in developing T_H17 cells (Supplementary Figs. 5 and 6 online and data not shown).

To determine if IL-17 affected expression of *Tbx21*, which encodes T-bet, we again cultured sorted naive CD45RB^{hi} CD4$^+$ T cells in T_H1-polarizing conditions in the presence or absence of recombinant IL-17. Recombinant IL-17 potently inhibited *Tbx21* expression as early as 48 h after stimulation (Fig. 3a). By day 4 of *in vitro* polarization, *Tbx21* expression in IL-17-containing T_H1 cultures was less than 10% of that in cultures without recombinant IL-17 (Fig. 3a). Lower *Tbx21* expression preceded the diminished T-bet protein; we first noted the latter after 4 d of culture (Fig. 3b). The diminished T-bet was not due to lower rates of cellular proliferation, as assessed by incorporation of [^{3}H]thymidine (Fig. 3c). Notably, the addition of IL-17 to wild-type T_H1 cultures on day 4 did not lower the already robust T-bet expression (data not shown). Therefore, whereas IL-17 does not seem to extinguish T-bet expression in mature T_H1 cells, our studies

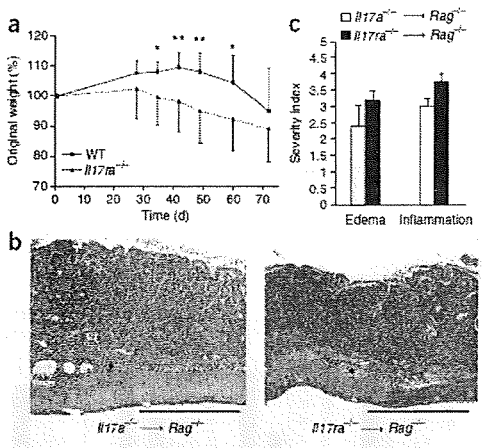

Figure 4 *Il17ra*$^{-/-}$ CD45RB^{hi} donor T cells elicit an accelerated wasting disease in *Rag1*$^{-/-}$ recipients. (a) Composite weight-loss curves of recipients of adoptive transfer of wild-type or *Il17ra*$^{-/-}$ CD45RB^{hi} CD4^{+} T cells (number of mice with weight loss, Table 1). $P \leq 0.01$, time; $P \leq 0.01$, experimental group; $P \leq 0.01$, time-group interaction (repeated-measures ANOVA). *, $P < 0.05$, **, $P \leq 0.01$, days 35–60 after transfer of cells (post-hoc *t*-test); means at day 35: recipients of wild-type T cells ($n = 8$), 108.1; recipients of *Il17ra*$^{-/-}$ T cells ($n = 8$), 99.59. Data are representative of two independent experiments with similar results (error bars, s.d.). (b) Hematoxylin and eosin–stained sections of *Rag1*$^{-/-}$ recipient colons obtained at day 28 after adoptive transfer *Il17a*$^{-/-}$ or *Il17ra*$^{-/-}$ T cells. Scale bars, 500 μm. Results are representative of two experiments. (c) Histological quantification of edema and inflammation in colons from *Rag1*$^{-/-}$ recipient mice, obtained at day 28 after adoptive transfer of *Il17a*$^{-/-}$ or *Il17ra*$^{-/-}$ T cells, presented as 'severity scores'. There were no statistically significant differences among the groups for any criteria. Data are representative of two experiments (error bars, s.e.m.).

suggest IL-17 can inhibit the upregulation of T-bet that normally occurs during early stages of the T_H1 differentiation program. Consistent with involvement of IL-17A in repressing T_H1 development, purified *Il17a*$^{-/-}$ CD45RB^{hi} CD4^{+} T cells cultured in standard T_H1-type conditions had higher expression of *Tbx21* (Fig. 3d). After restimulation, *Il17a*$^{-/-}$ T_H1 cells also secreted more IFN-γ protein (Fig. 3e). However, the presence or absence of IL-17A did not substantially affect cellular proliferation (Fig. 3f). These data, which show that IL-17 antagonizes the T_H1 differentiation program *in vitro*, further support our *in vivo* data demonstrating a more rapid T_H1-associated disease course in the absence of IL-17.

IL-17R-deficient T cells elicit an aggressive wasting disease

On the basis of our findings demonstrating that T cells can respond to IL-17, we hypothesized that the accelerated inflammation noted *in vivo* may have been due to T cell–intrinsic IL-17 acting in an autocrine way. To test our hypothesis, we did additional colitis experiments with IL-17R-deficient donor T cells. Like *Il17a*$^{-/-}$ cells, the *Il17ra*$^{-/-}$ CD45RB^{hi} CD4^{+} T cells also elicited an accelerated wasting disease in *Rag1*$^{-/-}$ recipients (Fig. 4a). Accelerated weight loss was first evident at day 35, and at days 42 and 49, differences in body mass were even more apparent (Fig. 4a, Table 1 and Supplementary Fig. 7 online). Histologically, scores for all criteria were the same for recipients of *Il17a*$^{-/-}$ or *Il17ra*$^{-/-}$ cells, with a consistent loss of crypts in the mucosa and inflammatory infiltrates both in the mucosa and submucosa readily observable by day 28 (Fig. 4b,c). These data collectively demonstrate that in this experimental system, the transferred CD4^{+} CD45RB^{hi} T cells were both the source and the relevant target of IL-17 *in vivo*.

Table 1 Accelerated disease course in *Rag1*$^{-/-}$ recipients of *Il17ra*$^{-/-}$ T cells

	Day 35	Day 42	Day 49	Day 60	Day 72
Recipients of WT T cells	0/9	0/9	0/9	3/9	6/9
Recipients of *Il17ra*$^{-/-}$ T cells	3/8	4/8	5/8	6/8	6/8

Disease incidence (mass loss) in recipients of wild-type or *Il17ra*$^{-/-}$ T cells at five time points, presented as mice with disease/total mice in group. Data are from one experiment representative of two independent experiments with similar results.

DISCUSSION

Higher IL-17 expression in the gut during intestinal inflammation, found in mouse models and in human disease, led us to begin investigating how IL-17 contributes, if at all, to the initiation and pathogenesis of IBD. Our results have shown that in the CD45RB^{hi} transfer model of colitis, an accelerated wasting disease resulted when adoptively transferred T cells were unable to produce IL-17 or failed to express IL-17R. Although the function of IL-17 in the initiation and pathogenesis of IBD has been controversial, our findings demonstrate a protective function for IL-17 in this experimental system. Notably, our results are in agreement with a report suggesting IL-17 could serve a protective function in the gut, albeit in a T cell–independent model of wasting disease[21]. Those findings were subsequently supported by studies done elsewhere[26].

Several studies have identified IL-23, one of the most potent inducers of IL-17, as being critical for IBD in mouse models assessing intestinal inflammation in the absence of IL-10 or in response to helicobacter infection[15,16]. Investigators have explored the relative importance of the contributions of IL-23 and IL-12 in intestinal inflammation with genetically deficient T cells in adoptive-transfer studies and measuring tissue infiltration and inflammation by assigning scores for histological critera. IL-23 is inarguably critical for tissue inflammation in those models and furthermore, although the results are not statistically significant, daily administration of antibody to IL-17 (anti-IL-17) during the disease course does seems to provide some benefit, diminishing intestinal inflammation scores[16]. These results are not unexpected, given that the proinflammatory properties of IL-17 and of IL-17-producing cells are well established. Indeed, it remains possible that IL-23-mediated IL-17 contributes, perhaps in a nonessential way, to the recruitment of cells of the immune system to the inflamed colon during intestinal inflammation. It is important to note that in our studies, the extent of cellular infiltration did not correlate with the wasting aspect of the disease. In recipients of wild-type T cells, *Il17a*$^{-/-}$ T cells or *Il17ra*$^{-/-}$ T cells, we noted extensive cellular infiltration, organ thickness, loss of crypts, loss of glands and edema; the greatest differences we noted were differences in recipient mouse weight loss during the ensuing wasting disease, after accumulation of cellular infiltrate in the recipient colon tissue. We conclude from our observations that infiltration of cells of the immune system is probably only one of several important components that direct the pathogenesis seen in this wasting disease and that perhaps there might be considerable differences in the functional abilities of the infiltrating cells. Our results showing higher expression of genes encoding T_H1-type cytokines in colon tissue of recipients of *Il17a*$^{-/-}$ T cells suggest this may be the case. At day 28 after

adoptive transfer, we detected elevated expression of the genes encoding IFN-γ and osteopontin in the inflamed colons of recipients of *Il17a*$^{-/-}$ T cells, concomitant with notable epithelial cell death and, in some cases, exposure of entire regions of the lamina propria to the gut lumen, as noted histologically.

One possibility is that IL-23-induced IL-17 is one of many factors that does contribute, in certain circumstances (such as in the absence of IL-10), to intestinal inflammation, whereas other IL-23-induced factors are responsible for tissue damage, in an IL-17-independent way. The idea that IL-23 uses 'downstream' effectors other than IL-17 in mediating inflammatory events is consistent with our findings and with published results of intestinal inflammation studies[15,16]. In addition, IL-23 has been shown to specifically use IL-22 to mediate dermal acanthosis[27]. Notably, in our studies, *Il22* mRNA was substantially upregulated in colon tissue in recipients of *Il17a*$^{-/-}$ T cells. We tested the hypothesis that IL-22 might mediate the exacerbated wasting disease observed in recipients of *Il17a*$^{-/-}$ T cells by using T cells deficient in both IL-17 and IL-22 in additional transfer experiments and found IL-22 was in fact not responsible for the accelerated IBD.

The precise underlying mechanisms driving the wasting disease in this model, in general, remain unidentified so far. As IL-17R is expressed nearly ubiquitously[28], it remains possible that IL-17 may influence nearly every cell type present in the gut microenvironment. Indeed, IL-17 may positively affect epithelial cell survival or otherwise aid in maintaining the integrity of the epithelial barrier; however, our data suggest that IL-17 may exert its suppressive effect in this model at least in part by suppressing T_H1 differentiation. Our results suggest that this is probably mediated through the suppression of the induction of T-bet, the 'master regulator' of T_H1 differentiation. It is important to note that the IL-17-mediated suppression of T-bet expression in our studies was not absolute and was eventually overcome by the T_H1 differentiation program. As T_H1-associated cytokines, including IFN-γ, potently inhibit IL-17 expression[29,30], it is plausible that, physiologically, IL-17 must repress early T_H1 differentiation to fulfill its critical function in promoting the recruitment of neutrophils to sites of inflammation. Of course, during late-stage chronic disease, persistent IL-17 expression may also participate in ongoing tissue damage through the recruitment of neutrophils or through other mechanisms. Indeed, we found that IL-17 induced the expression of certain target genes, including the gene encoding the chemokine receptor CCR6, in mature T_H1 effector cells *in vitro* (W.O., unpublished observations).

In the studies presented here, we have demonstrated the following: IL-17 was not required for cellular infiltration and inflammation of colon tissue in this experimental system; the transferred T cells were responsive to IL-17 *in vivo*, as shown by the wasting disease that resulted from the absence of IL-17R in T cells; despite similar cellular infiltration in the recipient colon tissues at the onset of weight loss, the severity of wasting disease was regulated by IL-17; and the absence of IL-17A or IL-17R in T cells led to an accelerated and severe wasting disease accompanied by higher expression of genes encoding T_H1-type cytokines. The proinflammatory nature of IL-17, in the context of the environment-specific anti-inflammatory effects of IL-17 we have reported, raises several questions. Is IL-17 (or are IL-17-producing cells) by default proinflammatory and simply held 'at bay' in the gut microenvironment by IL-10 and/or other immunoregulatory factors? Alternatively, perhaps a signaling mechanism exists, a biological 'switch' of sorts that controls many factors in the IL-17-producing cells themselves or in the stromal compartment in mediating the 'pathogenicity' of IL-17-producing cells.

Our data, although paradoxically at odds with some of the literature describing proinflammatory functions for IL-17, are consistent with the idea of a pleiotropic, environment-specific protective function for IL-17 in the gut. Intraepithelial γδ T cells protect the intestinal mucosa during chemically induced epithelial damage and aid in maintaining intestinal homeostasis by inhibiting exacerbated inflammatory responses to both foreign antigens and autoantigens[31]. Notably, at steady state, γδ T cells are the main IL-17-producing lymphocyte subset in mice[32]. Additional studies are needed to determine if in the absence of observable immunopathology, IL-17 expression indeed aids in maintaining intestinal homeostasis.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureimmunology/.

Note: Supplementary information is available on the Nature Immunology website.

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AUTHOR CONTRIBUTIONS

W.O. and R.A.F. designed the study and wrote the manuscript; M.K. provided flow cytometry data, advice and technical guidance; W.O. did all other *in vitro* and *in vivo* experimental work; C.J.B. did histopathological scoring analyses; T.T. provided assistance with statistical analyses; Y.I. and S.N. provided *Il17a*$^{-/-}$ mice; and J.K.K. provided the *Il17ra*$^{-/-}$ mice.

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Natural Occurring IL-17 Producing T Cells Regulate the Initial Phase of Neutrophil Mediated Airway Responses[1]

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Effector Th17 cells are a major source of IL-17, a critical inflammatory cytokine in autoimmune diseases and in host defenses during bacterial infections. Recently, splenic lymphoid tissue inducer-like cells have been reported to be a source of T cell independent IL-17. In this study, we report that the immune system contains a unique set of natural occurring IL-17 producing cell, "natural" Th17 (nTh17), which are a memory-like T cell subset. The nTh17 cells can develop in the absence of the IL-6/STAT3 signaling axis required by inducible Th17 cells. The nTh17 cell population is distinct from conventional inducible Th17 cells, since nTh17 cells express substantial amounts of IL-17A (IL-17), but not IL-17F, under the control of the master regulator, RORγt. The nTh17 cells simultaneously produce IFN-γ. DO11.10 transgenic mice with a $Rag^{-/-}$ background (DO11.10 $Rag^{-/-}$) lack nTh17 cells, and, following intranasal administration of OVA, IL-17-dependent neutrophil infiltration occurs in DO11.10 transgenic mice, but not in DO11.10 $Rag^{-/-}$ mice. The impaired neutrophil-dependent airway response is restored by adaptive transfer of nTh17 cells into DO11.10 $Rag^{-/-}$ mice. These results demonstrate that a novel T cell subset, nTh17, facilitates the early phase of Ag-induced airway responses and host defenses against pathogen invasion before the establishment of acquired immunity. *The Journal of Immunology*, 2009, 183: 7523–7530.

Beginning with their initial characterization more than 20 years ago, two major subsets of helper T cells, Th1 and Th2, have been defined based on their cytokine profiles and immune regulatory functions. Th1 cells secrete IL-2, IFN-γ, and TNF-α during cell-mediated immune responses against intracellular pathogens and viruses, whereas Th2 cells produce IL-4, IL-5, IL-6, IL-10, and IL-13, and mediate humoral immunity and allergic responses. Recently, a new subset of helper T cells, Th17, which produces IL-17A, IL-17F, IL-10, IL-21, and IL-22, but not the cytokines produced by Th1 or Th2 cells, has been identified. Th17 cells appear to be responsible for the regulation of adaptive immunity against Dectin-mediated fungal infections, such as *Pneumocystis carinii* (1–3), and several TLRs regulates the development of Th17 cells (4). IL-17 is also known to be a critical cytokine for regulating inflammatory responses, and its expression is tightly associated with pathogenesis in autoimmune disorders, such as multiple sclerosis and collagen-induced arthritis (5, 6).

IL-23 was originally identified based on its ability to exacerbate experimental autoimmune encephalitis (EAE)[3] (7) and later was shown to maintain pathogenic IL-17 producing $CD4^+$ T cells (5). IL-23 is thought to control the development of IL-17 producing $CD4^+$ T cells from naive $CD4^+$ T cells (8, 9). However, recent studies have suggested that IL-6 and/or IL-21 in conjunction with TGF-β are sufficient to control de novo Th17 development (10–12). This model is supported by the finding that Th17 development is attenuated in *Stat3*-deficient mice (10), since both IL-6 and IL-21 activate a STAT3-mediated signaling pathway. Retinoic acid related orphan receptor (ROR)γt has been identified as the master regulator controlling the lineage commitment of Th17 cells (11). Recently, another ROR family member, RORα, has been reported to be essential for the regulation of Th17 development in conjunction with RORγ (12). Expression of *Ror* family genes is strongly induced by the combination of STAT3 and TGF-β signaling (11, 13, 14). In contrast, the IFN-γ/IRF1/STAT1/t-bet, IL-27/STAT1/3, IL-4/STAT6, and IL-2/STAT5 pathways are potent negative regulators of IL-17 production and Th17 development (15–18). Recently, splenic lymphoid tissue inducer-like cells have been reported to produce IL-17 independently of Th17 cells (19). TLRs are important mediators of Th17 development. An immunization protocol using zymosan, which is recognized by TLR2, preferentially induces Th17 cells (20). TLR4-mediated LPS stimulation also enhances Th17 development by induction of IL-23 expression in dendritic cells (21). In contrast, polyinosinic-polycytidylic acid recognized by TLR3 may induce dendritic cells to produce IL-27, leading to negative regulation of Th17 development (22).

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[3] Abbreviations used in this paper: EAE, experimental autoimmune encephalitis; MP, memory phenotype; Treg, regulatory T cell; COPD, chronic obstructive pulmonary disease; ROR, retinoic acid related orphan receptor; nTh17, natural occurring IL-17 producing cell; BALF, bronchoalveolar lavage fluid; iTh17, inducible Th17; WT, wild type; SP, single positive; DO, DO11.10 transgenic mice.

IL-17 (IL-17A) is the prototypical member of the IL-17 family, which consists of six related proteins, IL-17A-F. Among family members, IL-17F has the highest homology with IL-17A. Th17 cells are the major source of IL-17A and F, although neutrophils, eosinophils, and CD45RO$^+$ CD8$^+$ T cells express IL-17A to a lesser extent. IL-17F is expressed by Th17 cells, basophils, and cord blood mast cells, as well as by liver, lung, and ovary cells. IL-17A has pleiotropic activities, including the induction of proinflammatory cytokines and chemokines such as TNF-α, IL-1α, IL-6, IL-8, and MCP-1, which control neutrophil attraction and the development of inflammatory autoimmune diseases (23). However, the factors regulating production of IL-17, which induces neutrophil accumulation early in an immune response, are poorly understood.

CD4$^+$ T cells can be categorized as naive or effector/memory cells based on the expression of CD44 (24). The CD44^{high} population contains NKT, memory phenotype (MP) CD4$^+$ T cells, and regulatory T cells (Treg). We recently established a transgenic *il4* promoter GFP reporter system and demonstrated that conserved noncoding sequence-2 in the *il4* locus regulates initial IL-4 expression by MP cells, and that the MP cell is a naturally occurring memory-like subset that differentiates in the thymus without Ag prepriming (25). Because the GFP$^+$ MP CD4$^+$ cells coexpress IL-4 and IFN-γ, the Tg reporter system allowed us to distinguish the MP CD4$^+$ subset from the effector-derived memory T cell subset. IL-17 was originally characterized as a proinflammatory cytokine, and robust IL-17 production is detected in CD4$^+$ T cells with the effector/memory phenotype (26). However, it is unclear whether the IL-17 producing memory CD4$^+$ T cells belong to the effector-derived memory T cell or the naturally occurring MP CD4$^+$ subset.

Little is known about the possible role of IL-17-producing CD4$^+$ T cells in asthma or chronic obstructive pulmonary disease (COPD), but a high concentration of IL-17 has been found in the sputum of asthma patients (27). COPD is an obstructive airway disease that involves chronic neutrophilic inflammation of the respiratory tract with airway narrowing caused by fibrosis (28). Biopsies of bronchial airways from patients with COPD contain an infiltration of T cells and neutrophils that produce matrix metalloproteinases and elastolytic enzymes, such as neutrophil elastase, that induce airway mucin production (29–31). Neutrophil accumulation in the sputum has been correlated with disease severity (32). In a mouse model, intranasal Ag treatment induced IL-17-dependent neutrophilia and airway pulmonary inflammation (33). Therefore, IL-17 is a critical cytokine for regulating the neutrophilia associated with asthma and COPD.

In the present study, we demonstrate that a peripheral unprimed population of CD4$^+$ T cells contains natural occurring IL-17 producing cells (nTh17) before the development of Th17 cells. These IL-17-expressing cells acquire the potential to express robust IL-17, but not IL-17F, and some cells coexpress IFN-γ. We propose that the IL-17 producing memory CD4$^+$ T cells are the naturally occurring MP CD4$^+$ subset, which differentiates in a STAT3-independent pathway in the thymus, and that cells with this unique phenotype play a functional role in the attraction of neutrophils.

Materials and Methods

Construction and animals

Distal 3′ GFP reporter (d3′) Tg mice, *Il17*$^{-/-}$ mice (34) and *Il23*$^{-/-}$ mice (35) were established as previously described (24). *Stat6*$^{-/-}$ mice were a gift from Dr. S. Akira (Osaka University, Osaka, Japan) (36). *Il6*$^{-/-}$ mice, which were generated as described previously (37), were provided by Dr. T. Hirano (Osaka University, Osaka, Japan) and crossed with OT2 Tg mice (38). CD4 *Cre* Tg mice (39), provided by Dr. C. Wilson (University of Washington, WA), were crossed with *Stat3 f/f* mice (40). DO11.10 Tg mice were provided by Dr. K. Murphy (Washington University, MO) (41), and *Rorγt*$^{+/GFP}$ mice were originally generated by Dr. D. Littman (New York University, NY) (11) and provided by Dr. I. Taniuchi (RCAI, RIKEN, Yokohama, Japan). The *Stat1*$^{-/-}$ mice were originally generated by Dr. Schreiber (Washington University School of Medicine, St. Louis, MO) (42). All mice used in this study were maintained in specific pathogen free conditions. Animal care was conducted in accordance with the guidelines of the RIKEN Yokohama Institute.

Cytokines and Abs

The reagents for ELISA, anti-IFN-γ (R4–6A2 and XMG1.2 biotin), anti-IL-2 (JES6–1A12 and JES6–5H4 biotin), and anti-IL-4 (BVD4–1D11 and BVD6–24G2 biotin) were purchased from BD Biosciences. Mouse IL-17 and the IL-17F ELISA development set was purchased from R&D Systems. The anti-CD28 mAb (PV-1) was a gift from Dr. R. Abe (TUS, Chiba, Japan). For FACS analysis, anti-CD44 (IM7) and anti-IL-17A-PE (TC11–18H10) were purchased from BD Biosciences. Anti-IL-17F-Alexa Fluor 647 and mIL-21R/Fc fusion protein were purchased from eBioscience and R&D Systems, respectively. Measurement of cytokines was also performed using a cytokine array Bio-Plex assay system (Bio-Rad).

Preparation of CD4$^+$ T cells for cytokine assay and cell transfer, and induction of Th cells

CD4$^+$ T cells were isolated from spleen cells using magnetic beads (MACS, Miltenyi Biotec). D3′ Tg mice-derived GFP$^-$ CD44^{high}, GFP$^+$, and CD44^{low} CD4 T cells were prepared by cell sorting with a FACSVantage instrument using the CellQuest software (BD Biosciences). CD4$^+$ thymocytes were enriched using CD4 magnetic beads (MACS), and CD44^{low} and CD44^{high} CD4SP cells were isolated from the CD8$^-$NK1.1$^-$ fraction. For NK T cell stimulation, whole spleen cells were stimulated with α-GalCer (Dr. M. Taniguchi, RIKEN, Yokohama, Japan). Memory type CD4$^+$ T cells were isolated using microbeads (MACS) conjugated with anti-CD62L and anti-CD25 mAbs. Inducible Th17 cells were prepared from sorted CD44^{low} CD4$^+$ T cells isolated from DO11.10 Tg or BALB/c mice by stimulation with anti-TCR and anti-CD28 in the presence of IL-6 (20 ng/ml; PeproTech), TGF-β (5 ng/ml; R&D Systems), anti-IL-4 (11B11), and anti-IFN-γ (XMG1.2). For transfer experiments, 1×10^6 cells were i.v. transferred into DO11.10 Tg *Rag*$^{-/-}$ mice or *Rag*$^{-/-}$ mice. Spleen cells were prepared from OVA challenged mice, and for measurement of cytokine production, 1×10^5 cells were was conducted by activation with 1 or 10 μM OVA peptide (Loh15)-loaded APCs (5×10^5 cells) or plate-bound anti-TCR plus anti-CD28 mAbs. Cytokines were measured by ICS, ELISA, or the Bio-Plex system.

Induction of Ag-specific and nonspecific airway responses

For the non Ag-specific airway response, mice were intranasally challenged with LPS (10 μg/mouse). For the OVA specific airway response, DO11.10 Tg mice were administered 50 μg of OVA (grade V, Sigma-Aldrich). At 24 h after the last challenge, total cells were collected from the bronchoalveolar lavage fluid (BALF), and Giemsa staining was conducted for differential cell counting of lymphocytes and neutrophils.

Results

Impaired early neutrophil accumulation in DO11.10 Tg Rag$^{-/-}$ *mice*

Previous studies have shown that intranasal Ag administration of OVA to TCR Tg mice promoted infiltration of IL-17 producing CD4$^+$ T cells in the airway, where they regulate neutrophil and macrophage attraction to inflammatory sites (32). To determine whether effector Th17 cells migrate into the airway, we compared the accumulation of neutrophils and IL-17-producing CD4$^+$ T cells in DO11.10 Tg BALB/c mice to that in DO11.10 Tg *Rag*$^{-/-}$ mice (DO11.10 and DO *Rag*$^{-/-}$). DO11.10 and DO *Rag*$^{-/-}$ mice showed comparable T cell independent neutrophil accumulation following LPS stimulation (Fig. 1*A*). DO11.10 mice showed a marked accumulation of lymphocytes and neutrophils into the BALF and lung. However, DO *Rag*$^{-/-}$ had a significant reduction of infiltrating cells at 24 and 48 h (Fig. 1, *B* and *C*). These results suggested that DO *Rag*$^{-/-}$ mice are missing an undefined IL-17 producing T cell subset responsible for the early phase of the airway inflammatory response.

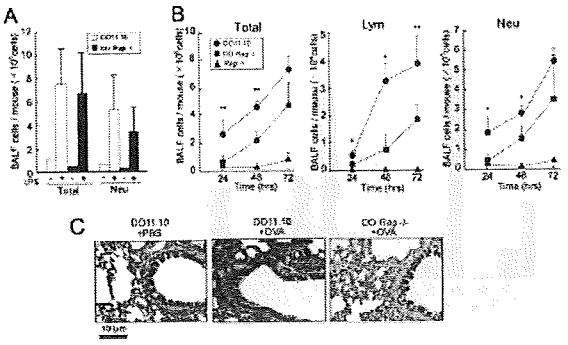

FIGURE 1. Neutrophil accumulation in the airways of DO11.10 and DO11.10 $Rag^{-/-}$ mice. *A*, DO11.10, and DO $Rag^{-/-}$ mice were infused intranasally with 10 μg LPS or PBS. After 24 h, BALF cells were obtained and analyzed by Giemsa staining. The total cells (Total) and neutrophils (Neu) infiltrating the BALF were quantified. The data are the means of four independent experiments and the error bars indicate the SEM. *B*, DO11.10 (n = 3 at 24 h, n = 5 at 48 h, n = 4 at 72 h), DO $Rag^{-/-}$ mice (n = 3 at 24 h, n = 3 at 48 h, n = 6 at 72 h) and $Rag^{-/-}$ mice (n = 3 each, at 24, 48, and 72 h) received 50 μg OVA intranasal infusions every 24 h. BALF cells were isolated at the indicated time points. Total cells (Total), lymphocytes (Lym), and Neutrophils (Neu), were quantified microscopically. Statistical significance was determined using Student's t test (*, $p < 0.05$; **, $p < 0.01$). *C*, DO11.10 and DO $Rag^{-/-}$ mice were nasally challenged with OVA twice. Histological analysis of lung tissue in the challenged mice was performed using H&E staining of formalin fixed sections. Scale bar = 100 μm. The data are representative of three independent experiments.

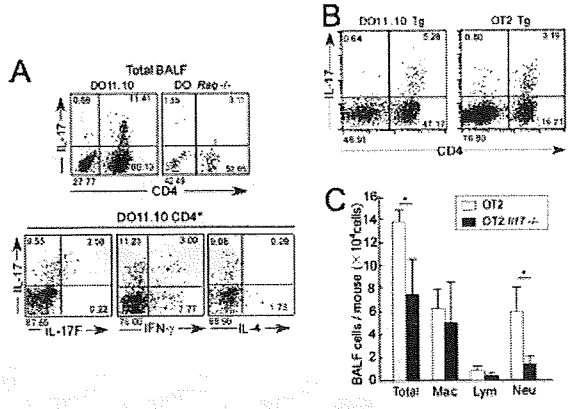

FIGURE 2. Infiltration of IL-17 producing CD4$^+$ T cells into the BALF. *A*, DO11.10 and DO $Rag^{-/-}$ mice were immunized twice by intranasal OVA administration. BALF cells (1×10^5 cells) isolated from immunized mice were stimulated with anti-TCR and anti-CD28. After 48 h, IL-17A, IL-17F, IL-4, and IFN-γ expression was analyzed by ICS of CD4$^+$ cells. Similar results were observed in four independent experiments and representative data are shown. *B*, DO11.10 and OT-2 Tg mice were OVA immunized, and IL-17A expression was analyzed by ICS as described in *A*. The data are representative of three independent experiments. *C*, OT2 Tg and OT2 Tg $Il17^{-/-}$ mice were immunized with OVA twice. After 24 h, the number of total cells (Total), lymphocytes (Lym), and neutrophils (Neu) in the BALF were quantified. Data are the means of three independent experiments and the error bars indicate the SEM.

We therefore compared the cytokine profile of CD4$^+$ T cells migrating into the BALF after OVA immunization of DO11.10 and DO $Rag^{-/-}$ mice. The infiltrating CD4$^+$ T cells clearly produced IL-17 in DO11.10, but not in DO $Rag^{-/-}$ mice, and the IL-17 producing cells had a unique cytokine profile, producing IL-17A, but not IL-17F or IL-4. Approximately one-third of the cells simultaneously expressed IFN-γ (Fig. 2*A*). To study the significance of IL-17A in OVA-induced neutrophil accumulation, we used the OT-2 TCR transgenic (OT-2 Tg) system, because *Il17a* deficient mice have the B6 background. Nasal OVA administration to OT-2 Tg mice showed clear accumulation of IL-17 producing cells and neutrophils into the BALF, whereas *Il17a*-deficient OT-2 mice exhibited a significant reduction in neutrophil accumulation (Fig. 2, *B* and *C*). These results support the general concept that Th17 cells are the major source of IL-17, however, the cytokine profile of the BALF infiltrating IL-17 producing T cells was quite distinct from that of canonical Th17 cells.

The nTh17 cells are a memory type CD4$^+$ T subset

We further defined the different T cell subsets in DO11.10 vs DO $Rag^{-/-}$ mice and found a clear difference in the memory/activated CD4$^+$ population. Splenic KJ1$^+$ CD4$^+$ T cells from DO11.10 mice, but not DO $Rag^{-/-}$ mice, had a substantial number of memory/activated phenotype cells capable of secreting IL-17 in response to a primary OVA stimulation (Fig. 3*A*). We have defined these cells as nTh17 cells and, next, asked whether the nTh17 CD4$^+$ T cells were present in nontransgenic animals. Similar IL-17 expression was observed in freshly isolated splenic CD4$^+$ T cells derived from normal BALB/c mice when the cells were stimulated by TCR cross-linking (Fig. 3*B*).

Using GFP reporter Tg mice (d3′ Tg) in which the activity of the conserved noncoding sequence-2 enhancer in the *Il4* gene could be monitored (25), we previously demonstrated that unprimed memory CD4$^+$ T cells expressed relatively high amounts of IFN-γ and IL-4. We next asked whether the nTh17 cells were part of the memory CD4 T$^+$ subset. Three distinct subsets were isolated from d3′ Tg mice based on GFP and CD44 expression (Fig. 3*C*, *left*), and their cytokine expression profiles were assessed at 48 h after TCR stimulation using ELISA and ICS. IL-17 was selectively expressed in the GFP$^-$ CD44^{high} subset and the majority of IL-17 producing cells produced IFN-γ simultaneously (Fig. 3*C*).

GFP$^+$ NK T cells produced relatively low levels of IL-17 after cognate recognition of α-GalCer-loaded CD1d (Fig. 3*C*, *right*, and Fig. 3*D*). GFP$^-$CD44^{high} cells contain CD25$^+$ Treg, but the IL-17 expressing cells did not express CD25 (S.T. and M.K., unpublished data). These data indicate that nTh17 cells are a population distinct from the IL-4 producing memory T cells, NK T cells, and Tregs.

IL-17 production by nTh17 cells is regulated by STAT3-independent RORγt

Because nTh17 cells and canonical Th17 cells share a common feature of IL-17 production, we next asked whether nTh17 cells are derived from canonical effector-type inducible Th17 (iTh17) cells. To induce iTh17 cells, Ag priming was performed under restricted cytokine conditions, namely the combination of IL-6 and TGF-β, and the iTh17 cells were found to coexpress IL-17 (IL-17A) and IL-17F (41). However, as observed in DO11.10 mice, the nTh17 cells exhibited a cytokine profile distinct from iTh17 cells. The nTh17 cells produced IL-17A, but not IL-17F, after primary stimulation and coexpressed IFN-γ (Fig. 4*A*). This cytokine profile was also confirmed by ELISA (Fig. 4*B*). We further asked whether nTh17 cells are the main source of IL-17 in primary Ag stimulation. DO11.10 transgenic mice (DO) $Rag^{-/-}$-derived naive T cells and DO11.10-derived naive and memory CD4$^+$ T cells were stimulated with OVA peptide loaded APC, and IL-17

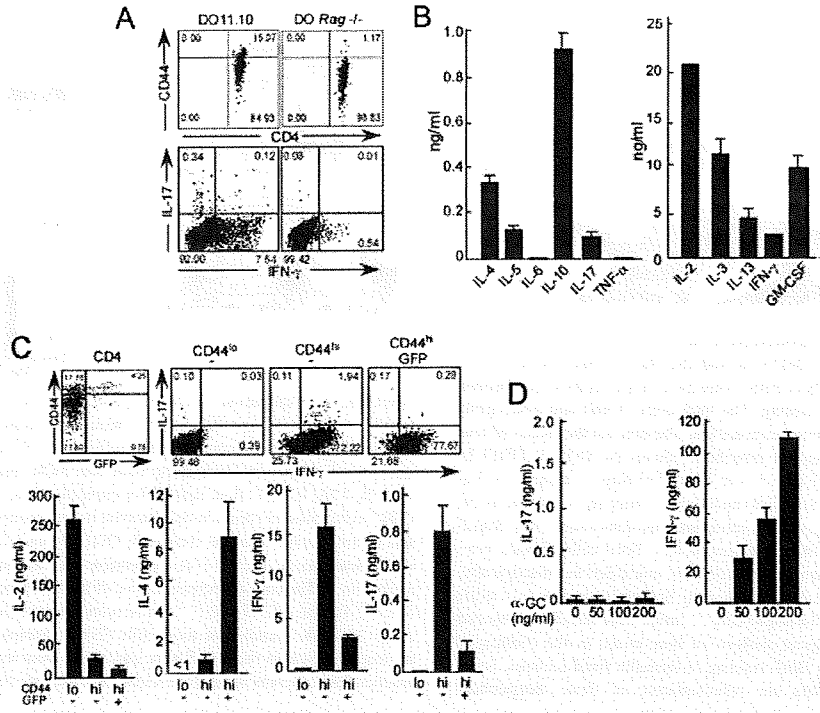

FIGURE 3. Primary IL-17 production by memory CD4$^+$ T cells. *A*, Splenic CD4$^+$ T cells enriched from DO11.10 and DO *Rag*$^{-/-}$ mice were analyzed for CD44 and CD4 expression in TCR transgenic T cells (KJ-1.26$^+$) (*top*). CD4$^+$ T cells (1×10^5 cells) were stimulated with anti-TCR and anti-CD28. After 24 h, intracellular IL-17 and IFN-γ in the KJ-1$^+$ gated CD4$^+$ T cells were assessed by ICS (*bottom*). *B*, Splenic CD4$^+$ T cells (5×10^5 cells) from unprimed BALB/c mice were stimulated with plate-adsorbed anti-TCR and anti-CD28 for 3 days. Cytokines in culture supernatants were measured using a BioPlex cytokine assay system. Data are the means of three independent experiments and the error bars indicate the SEM. *C*, CD44^{low} naive, CD44^{high} GFP$^-$, and CD44^{high} GFP$^+$ populations (*left*) were isolated by sorting from distal 3′ IL-4 Tg mice on a BALB/c background. Each sorted population (1×10^5 cells) was stimulated with anti-TCR and anti-CD28. After 48 h, IL-17 and IFN-γ expression was analyzed by ICS in three fractions, and IL-2, IL-4, IL-17, and IFN-γ in the culture supernatants were quantified by ELISA. *D*, Whole spleen cells (5×10^5 cells) from BALB/c mice were stimulated with 0, 50, 100, or 200 ng/ml α-GalCer. After 48 h, IL-17 and IFN-γ were measured by ELISA.

expression was examined. IL-17 expression was only found in KJ1$^+$ memory type nTh17 cells, which secreted IL-17 after peptide stimulation (Fig. 4*C*). Memory type CD4$^+$ T cells also contained large numbers of IL-21 producing cells, however nTh17 cells were clearly distinct from the follicular helper T cell-like memory T cells that expressed IL-21, because the majority of nTh17 cells did not express IL-21 (Fig. 4*D*).

IL-6/IL-21/IL-23-mediated STAT3 activation plays an essential role in generating iTh17 cells. In contrast, the IFN-γ/IRF-1/STAT1 and IL-4/STAT6 axis negatively regulate this differentiation pathway (9). However, *Il6* deficiency did not affect primary IL-17 production, whereas IL-17 production was partially impaired in *Il23*-deficient mice (Fig. 5*A*). Furthermore, primary IL-17 production from nTh17 cells was maintained at detectable levels in *Stat3* deficient CD4$^+$ T cells, and significant augmentation was observed in *Stat1*- and *Stat6*-deficient T cells. Primary IFN-γ expression was comparable between wild-type (WT) and *Stat3*-deficient T cells (Fig. 5*C*). Therefore, the generation and/or maintenance of nTh17 cells are IL-6/STAT3 independent, but partially IL-23/STAT3 dependent, indicating that nTh17 cells and iTh17cells have distinct developmental pathways. However, both nTh17 cells and iTh17 cells are negatively regulated by IL-4/STAT6 and IFN-γ/STAT1 during their development.

The mechanisms by which DO11.10 mice, but not DO11.10 *Rag*$^{-/-}$ mice, are able to generate Ag specific nTh17 cells that exhibit a memory phenotype, CD44^{high}, and CD62L^{low}, without prepriming, are unclear. Therefore, we examined whether nTh17 cells are generated in the absence of prepriming using DO11.10 vs DO *Rag*$^{-/-}$ mice and OT-2 vs OT-2 *Rag*$^{-/-}$. The CD44^{high} nTh17 subset was found among CD4 single positive (SP) cells in both DO11.10 and OT-2 thymus. KJ1$^+$ CD4 SP cells in DO11.10 mice or Vα2$^+$ CD4 SP cells in OT-2 mice were produced IL-17 in the stimulation with a TCR mAb, indicating that nTh17 appeared among thymic CD4$^+$ SP cells without priming (Fig. 5*C*). nTh17 cells were readily detectable even in *Stat3*-deficient and *Il6*-deficient mice and these cells had a cytokine profile similar to nTh17 cells in the periphery (Fig. 5, *D* and *E*). Therefore, nTh17 cells were generated in the thymus without the prepriming and STAT3 signal, which required for iTh17 differentiation.

RORs are critical transcription factors that regulate IL-17 production by Th17 cells (11, 12), and the expression of RORγt is tightly regulated by IL-6. Therefore, we next asked whether there was a requirement for RORs in IL-17 production by nTh17 cells, because nTh17 cells could be generated in conditions where iTh17 cells could not. The number of nTh17 cells from *Rorγt*$^{+/GFP}$ reporter knock-in (*Rorγt*^{GFP}) mice was approximately half that of WT B6 mice. Moreover, one-third of IL-17 expressing memory T cells coexpressed GFP in *Rorγt*^{GFP} mice (Fig. 5*F*). These results indicate that RORγt expression is essential for the production of

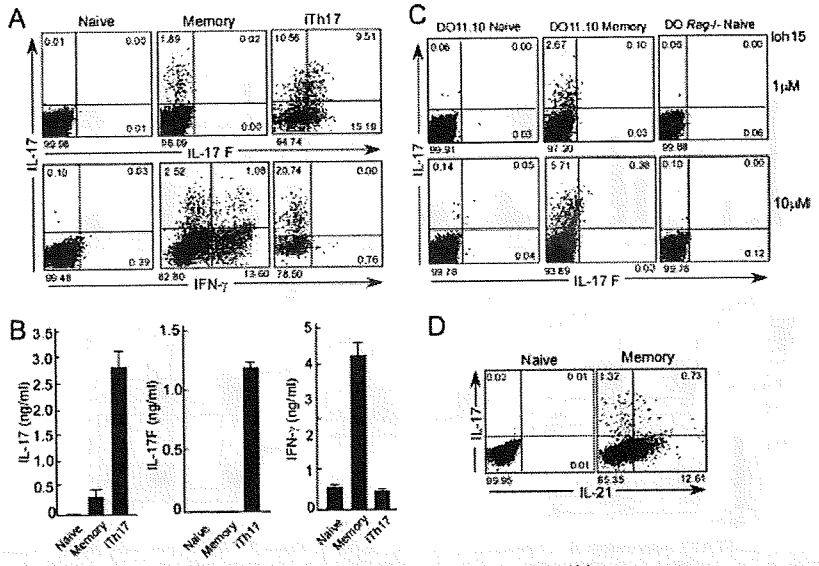

FIGURE 4. Unique cytokine expression pattern of nTh17 cells. *A*, Freshly isolated, naive, $CD44^{high}$ memory $CD4^+$ T cells or Th17 cells (1×10^5 cells) from unprimed BALB/c mice were stimulated with anti-TCR and anti-CD28, and IL-17A, IL-17F, and IFN-γ production was assessed by ICS after 24 h. The data are representative of three independent experiments. *B*, Cytokine concentrations in supernatants measured by ELISA. Data are the means of three independent experiments and the error bars indicate the SEM. *C*, $CD44^{high}$ memory and $CD44^{high}$ memory $CD4^+$ T cells (1×10^5 cells) were derived from DO11.10 Tg mice, and $CD44^{low}$ naive $CD4^+$ T cells were isolated from DO $Rag^{-/-}$ mice. Cells (1×10^5 cells) were stimulated with OVA peptide Ioh-15 (1 and 10 μM) in the presence of APC (5×10^5 cells). IL-17A and IL-17F production was analyzed by ICS after 48 h of anti-TCR and anti-CD28 stimulation. *D*, Intracellular IL-17 and IL-21 were analyzed in $CD44^{high}$ memory and $CD44^{low}$ naive $CD4^+$ T cells prepared in *A*.

IL-17 by nTh17 cells, and that nTh17 cells express RORγt in an IL-6-independent manner.

Memory Th17 cells attract neutrophils and macrophages to inflammatory airway sites

To further define the in vivo role of nTh17 cells, we tested whether the DO11.10-derived nTh17 cells could overcome the neutrophilia defect observed in DO $Rag^{-/-}$ mice. The DO11.10-derived memory $CD4^+$ T and in vitro differentiated iTh17 cells were adoptively transferred into OVA treated DO $Rag^{-/-}$ mice, respectively. DO $Rag^{-/-}$ mice and $Rag^{-/-}$ mice exhibited quite low levels of neutrophil attraction (Fig. 1*B*). Reconstitution of nTh17 completely restored the accumulation of neutrophils at levels equivalent to that of DO11.10 cell injections (Fig. 6*A*), indicating that nTh17 cells are indispensable for establishment of the neutrophil-mediated airway response and has about half level of the ability to induce neutrophil accumulation compared with iTh17 cells.

Induction of neutrophilia was further tested in an *Il6*-deficient background after intranasal OVA administration to determine whether the nTh17-mediated Ag-induced neutrophilia could occur in the absence of iTh17 cells. $Il6^{-/-}$ OT2 Tg mice had a normal accumulation of neutrophils and macrophages (Fig. 6*B*). Finally, we tested whether nTh17 cells were sufficient to induce neutrophil accumulation. Injection of DO nTh17 cells into $Rag^{-/-}$ mice enhanced neutrophil accumulation compared with untreated $Rag^{-/-}$ mice. The numbers of infiltrating neutrophils were comparable to that in DO11.10 mice (Fig. 6*C*). These data demonstrate that nTh17 cells are sufficient to induce the initial phase of neutrophil-mediated inflammatory responses.

Discussion

IL-17 is a pivotal cytokine regulating inflammation by recruiting inflammatory cells such as neutrophils and macrophages. In this study, we first used TCR transgenic DO11.10 mice crossed with $Rag^{+/+}$ or $^{-/-}$ mice to demonstrate that a noncanonical $CD4^+$ T cell subset, nTh17, regulates the early phase of Ag specific airway responses through the IL-17 mediated infiltration of neutrophils into the BALF. The nTh17 subset, which robustly secretes IL-17 but not IL-17F, is a distinct population from the effector-type of iTh17 cells. We further found that considerable IL-17 production was induced from nTh17 cells in unprimed mice. Unlike iTh17 cells, IL-6 signaling was not required for the development of the nTh17 cells, although the IL-23/STAT3 signaling pathway was partially involved in their expansion. RORγt was essential for IL-17 production by nTh17 cells, as well as by iTh17 cells. Therefore, we propose that nTh17 cells are a naturally occurring memory-type $CD4^+$ subset that is appeared in the thymus independently of prepriming and IL-6-STAT3 signaling. The nTh17 cells play an important role in regulating the early phase of IL-17 mediated inflammatory responses.

IL-17 was originally reported to be produced by activated/memory T cells (26). The present data demonstrate that $CD4^+$ T cells derived from unprimed mice are capable of producing IL-17. The source is a unique $CD44^{high}$ $CD4^+$ T cell subset, nTh17. $NK1.1^-$ invariant NK T cells have been proposed as a source of IL-17 (43), however IL-17 producing invariant NK T cells do not express IFN-γ (44, 45). In contrast, the nTh17 subset produces both IL-17 and IFN-γ, and nTh17 cells do exist as $KJ\text{-}1^+$ $CD44^{high}$ $CD4^+$ T cells in the spleen and thymus (Figs. 4*C* and 5*C*), suggesting that this nTh17 subset is a distinct population from the invariant NK T or γδ T cells. We previously reported that similar memory $CD4^+$ T cell subsets produced IL-4 or IFN-γ after primary stimulation (25). The present study indicates that the nTh17 subset is capable of secreting large amounts of IL-17 without Ag prepriming. A T cell subset similar to nTh17 cells has been reported in the spinal cord of mice with EAE, and these $CD4^+$ T cells simultaneously

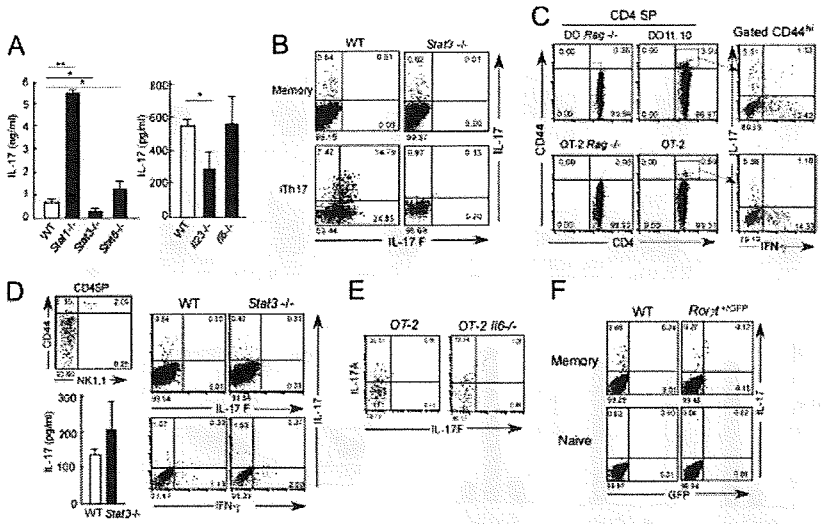

FIGURE 5. ROR dependent and STAT3 independent IL-17 production by nTh17 cells. *A*, CD44^{high} nTh17 cells were freshly isolated from *Stat1*$^{-/-}$, *Stat6*$^{-/-}$, CD4^{-}*cre stat3* f/f mice (−/−, ■), and C57BL/6 (B6) control mice (WT, □), and cells (1×10^5 cells) were stimulated with anti-TCR and anti-CD28. After 48 h, the concentration of IL-17 in the culture supernatant was determined by ELISA. Data are the means of three independent experiments and the error bars indicate the SEM. Statistical significance was determined using Student's *t* test. *, $p < 0.05$; **, $p < 0.01$. *B*, Splenic naive T cells and nTh17 cells were isolated from control CD4$^+$-*cre* Tg (WT) and CD4$^+$-*cre stat3* f/f mice (*Stat3*$^{-/-}$). Naive CD4$^+$ T cells were cultured under Th17 skewing conditions and then restimulated with anti-TCR. Freshly isolated nTh17 cells were stimulated with anti-TCR and anti-CD28. Costaining for IL-17A and IL-17F was performed by ICS. *C*, Thymic CD4 SP cells enriched from DO11.10, DO *Rag*$^{-/-}$, OT-2, and OT-2 *Rag*$^{-/-}$ mice were analyzed for CD44 and CD4 expression (*left*). CD4 SP cells (1×10^5 cells) were stimulated with anti-TCR and anti-CD28. After 48 h, intracellular IL-17 and IFN-γ in the KJ-1$^+$ (for DO) or Vα2$^+$ (for OT-2) gated CD4$^+$ T cells were assessed by ICS (*right*). ICS data are representative of three independent experiments. *D*, Magnetic sorted thymic CD4$^+$ SP cells were stained with CD44 and NK1.1 (*top left*). CD44^{high} cells (1×10^5 cells) were sorted from CD4$^+$ SP thymocytes of CD4$^+$-*cre* Tg (WT) and CD4$^+$-*cre stat3* f/f mice (*Stat3*$^{-/-}$). Following anti-TCR and anti-CD28 stimulation for 48 h, IL-17A and IL-17F were detected by ICS (*right*) and ELISA (*lower left*). ICS data are representative of three independent experiments. ELISA data are the means and SEM of three independent experiments. *E*, CD44^{high} cells (1×10^5 cells) were sorted from CD4$^+$ SP thymocytes of OT-2 Tg and OT-2 *Il6*$^{-/-}$ mice. Following anti-TCR and anti-CD28 stimulation for 48 h, IL-17 and IFN-γ were detected by ICS (*lower*). *F*, Naive CD4$^+$ T cells and CD44^{high} memory cells were freshly isolated from *Rorγt*$^{+/GFP}$ or B6 mice (WT). After stimulation with anti-TCR and anti-CD28, IL-17 and GFP expression was assessed by FACS analysis.

produce both IL-17 and IFN-γ (11). Similar double producers were also found in a colitis model induced by *Helicobacter hepaticus* (46).

Numerous previous studies have indicated that coordination of TGF-β and IL-6 and/or IL-21 signaling is critical for development of effector type iTh17 cells (13, 14, 47). Both IL-6 and IL-21 share a STAT3 mediated signaling pathway, and disruption of the *stat3* gene completely abolished iTh17 differentiation (10). However, nTh17 can develop in the absence of IL-6 and/or STAT3, an environment in which iTh17 cells fail to develop. Therefore, the existence of nTh17 cells may explain previous observations that *Il17* deletion has a stronger impact than *Il6* deletion on the EAE response (14).

RORγt and RORα are essential transcriptional factors that regulate IL-17 expression in iTh17 cells (12). *Rorγ* and *Rorα* gene expression is induced by the combination of STAT3 and TGF-β signaling pathways (11, 13, 14). Our data indicate that nTh17 cells also express RORγt, although STAT3 is dispensable for the generation of nTh17 in both the thymus and periphery. These observations suggest the possibility that nTh17 cells, in contrast to the canonical effector type Th17 cells use alternative induction mechanisms for RORγt expression.

Stat1 and *Stat6* deficient mice have augmented IL-17 expression by nTh17 cells, and a similar augmentation was also observed in *Ifnγ* deficient and *Irf1* deficient mice. A previous report has indicated that IFN-γ/STAT1-mediated T-bet expression negatively regulates the development of iTh17 cells. The IL-4/STAT6 signaling axis is also known to negatively regulate the development of iTh17 cells (9). The nTh17 cells are similarly negatively regulated by the IFN-γ/STAT1 and IL-4/STAT6 signaling axes. Additionally, we found that IFN-γ mediated IRF-1 activation is preferentially involved in nTh17, but not iTh17, cell development (15).

Although IL-23 was originally reported to be an inducer of iTh17 cells (5, 8, 9), IL-23 does not appear to be directly required for Th17 differentiation (17). In our studies, IL-17 production by nTh17 cells was partially impaired in *Il23* deficient mice, a finding consistent with the data derived from *Stat3* deficient mice. Furthermore, IL-23 augmented IL-17 production by nTh17 cells (data not shown), indicating that nTh17 cells may express the receptor for IL-23. In contrast, IL-17 producing cells in the thymic CD4$^+$ SP population were intact even in a *Stat3*$^{-/-}$ background. Therefore, the central role of IL-23 may be in the expansion and maintenance, but not in the generation, of nTh17 cells in the periphery.

A previous study indicated that Th17 cells played a critical role in recruiting neutrophils into airway inflammatory sites of an Ag induced hypersensitivity model in OVA-TCR transgenic mice (33). In our studies, the neutrophil attraction was initiated within 24 h after intranasal administration of OVA (Fig. 1*B*). However, it is very unlikely that iTh17 cells could be induced within such a short time frame, because Ag priming should be necessary for the induction of iTh17 cells. Moreover, after intranasal OVA administration into DO11.10 Tg mice, infiltration of the IL-17-producing

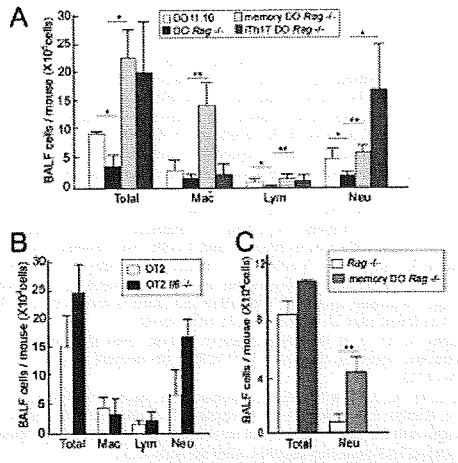

FIGURE 6. Restoration of airway infiltrating neutrophils and macrophages by nTh17. *A*, $CD44^{high}$ memory T cells were isolated from unprimed DO11.10 mice and iTh17 cells were derived from DO11.10 $CD44^{low}$ $CD4^+$ T cells. Cells (1×10^6) of both cell types were injected into DO $Rag^{-/-}$ mice, memory DO $Rag^{-/-}$, and iTh17 DO $Rag^{-/-}$, respectively. DO11.10 (white; $n = 4$), DO $Rag^{-/-}$ (black; $n = 3$), memory DO $Rag^{-/-}$ (gray; $n = 5$), and iTh17 DO $Rag^{-/-}$ (dark gray; $n = 3$) mice were intranasally challenged with OVA (50 μg/mouse) twice. After 48 h, BALF cells were isolated from each group. Total cells, lymphocytes (Lym), macrophages (Mac), and neutrophils (Neu) in the BALF were counted for each group. Data represent the means and the error bars indicate the SEM. Statistical significance was determined using Student's *t* test. *, $p < 0.05$; **, $p < 0.01$. *B*, OT2 Tg mice, WT (white), or $Il6^{-/-}$ (black) were treated with OVA twice. At 24 h after the last injection, the numbers of total cells, macrophages, lymphocytes, and neutrophils in the BALF were quantified. Data are the means of three independent experiments and the error bars indicate the SEM. *C*, $Rag^{-/-}$ mice were injected with (memory DO $Rag^{-/-}$) or without ($Rag^{-/-}$) DO11.10-derived $CD44^{high}$ memory $CD4^+$ T cells, and mice were intranasally infused with OVA twice. The data represent the numbers of neutrophils infiltrating the BALF. Statistical significance: **, $p < 0.01$.

$CD4^+$ T cells was observed in the absence of iTh17 cells. Furthermore, OVA treatment did not result in the appearance of IL-17 producing $CD4^+$ T cells in the BALF of DO $Rag^{-/-}$ mice (Fig. 1*C*). Collectively, these results indicate that the infiltrating IL-17-producing $CD4^+$ T cells correspond to nTh17 cells, which may be generated in the thymus. This model is consistent with the results of the AHR experiment in *Il6* deficient OT2 Tg mice (Fig. 6*B*). The nTh17 cells can induce the early phase of neutrophil accumulation in an unprimed situation. However, the efficiency of the neutrophil infiltration was lower in nTh17-injected $Rag^{-/-}$ mice than in DO11.10 Tg mice (Fig. 6, *A* and *C*). These results suggest that both nTh17 and naive T cells are required for maximum neutrophil attraction to the airway.

We have provided a novel insight into STAT3-independent generation of natural occurring memory-type Th17 cells, as well as the function of this T cell subset in vivo and in vitro. The nTh17 subset is required for initiating the early phase of an inflammatory response and understanding their function may aid in forming a more precise understanding of IL-17-mediated host defense and inflammatory responses in Ag-unprimed conditions.

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Disclosures

The authors have no financial conflict of interest.

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REVIEW ARTICLE

Biomarkers for Allergen Immunotherapy in Cedar Pollinosis

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ABSTRACT

To initiate, monitor, and complete effective immunotherapy, biomarkers to predict and visualize the immune responses are needed. First, we need to identify the right candidate for immunotherapy. Secondly, the immune responses induced by immunotherapy should be monitored. For the first objective, analysis of polymorphisms of candidate genes may be helpful, but still be in development. Regarding biomarkers for immune responsese, there are numerous reports that evaluate immunotherapy-induced immune changes such as suppression of effector cells, deviation to Th1 cytokine production, and induction of regulatory T cells. No standardized methods, however, have been established. Among them, a functional assay of blocking IgG activity, the IgE-facilitated allergen binding assay, may be useful. We quantitated induced expression of an activation marker, CD203c, on basophils and found that the assay efficiently predicts sensitivity to particular allergen and severity of the allergen-induced symptoms. In patients who received rush immunotherapy for Japanese cedar pollinosis, reduction in CD203c expression after the therapy was observed, suggesting the utility of the test for monitoring immunotherapy.

KEY WORDS

basophils, CD203c, cedar pollinosis, IgG4, immunotherapy

INTRODUCTION

The incidence of Japanese cedar pollinosis (JCP) is increasing at an astonishing pace, which was first recognized in early 1960s and now affects around one fourth of the population in Japan.[1-3] Effective pharmacotherapy including non-sedating antihistamines, leukotriene receptor antagonists, and topical corticosteroids, has evolved and quality of life of the patients has been improving.[4,5] Yet, the remedies merely control symptoms and do not change natural history of the disease. Further, social burden of the disease is still significant.[6] On the other hand, allergen immunotherapy generally not only alleviate allergic symptoms but has potential to modify the disease since clinical benefits are reported to be maintained at least for 3 years, even for 12 years after discontinuation.[7,8] In children, immunotherapy prevents new sensitizations[9,10] and reduces progression of rhinitis to asthma for up to 10 years.[11] Long-term efficacy of immunotherapy in Japanese cedar pollinosis has also been reported.[12]

Although immunotherapy confers a multitude of benefits, there still exist issues to be addressed; the present form of immunotherapy is still bound to IgE-mediated side effects, some patients may not benefit from the treatment, long periods for treatment are required and the timing of stopping therapy is not well defined. Along with various efforts to improve the therapy, effective biomarkers have to be developed to tailor the existing therapy and to evaluate new forms of the therapy. The markers should identify right patients with favorable therapeutic responses without adverse events, monitor the efficacy based on immunological responses to particular allergen, and identify the right timing of discontinuation. Although "ideal" biomarkers are yet to be established, prospects for the biomarkers in allergen immunotherapy will be discussed in this article. We also describe quantification of allergen-induced CD203c expression

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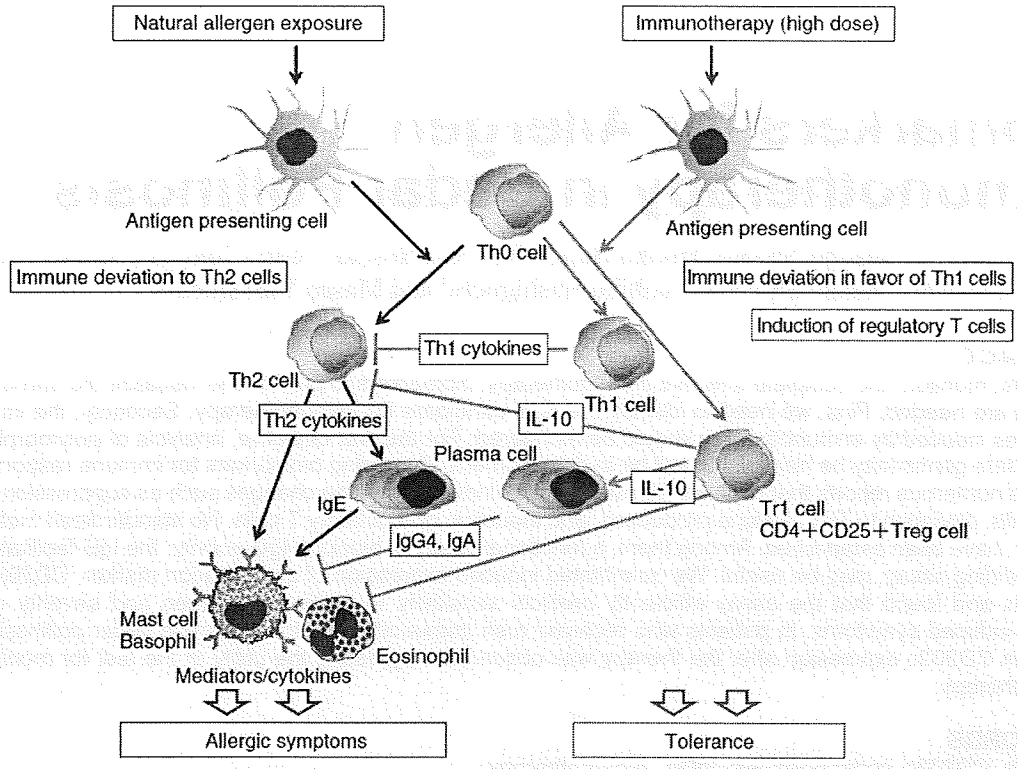

Fig. 1 Mechanisms of allergen immunotherapy.

on basophils as a possible biomarker for Japanese cedar pollinosis. Basophils are important effector cells in the pathogenesis of allergic diseases[13] because they infiltrate in the nasal mucosa of patients with allergic rhinitis[14] and produce a number of mediators and cytokines involved in immediate and late allergic responses.[15] In addition, the fact being circulating cells easily enables us to test the cells *ex vivo* by utilizing a flowcytometry. Here, we show that the basophil activation test utilizing CD203c expression may measure "blocking" activity induced by immunotherapy.

IMMUNOLOGICAL MECHANISMS IN ALLERGEN IMMUNOTHERAPY

THE ALLERGIC RESPONSE

Before discussing biomarkers in allergen immunotherapy, the putative immunological mechanisms are summarized (Fig. 1). The exposure of cedar allergen in the nose, eyes, or bronchi of genetically susceptible individuals causes Th2-deviated immune responses. Cytokines such as IL-4, IL-5, IL-9, and IL-13 derived from Th2 cells are responsible for specific IgE production, differentiation and activation of effector cells such as mast cells, basophils, and eosinophils, and direct stimulation of responder organs including mucus glands and vascular cells in the affected organ. Upon re-exposure to the allergen in the season, IgE-dependent activation of mast cells and basophils results in release of numerous mediators including histamine, cysteinyl leukotrienes, prostaglandins, and platelet activating factor, leading to sneeze, pruritus, waterly discharge, stuffy nose, and sometimes bronchospasm. In addition, mast cells and basophils, are large producers of Th2 and proinflammatory cytokines including IL-4 and TNF-α to potentiate chronic Th2-deviated inflammation in the tissue.

Allergen immunotherapy has potential to inhibit or reverse each step of the above allergic responses and to confer tolerance to the allergen (Fig. 1). Significantly higher amount of allergen is administered in immunotherapy compared to natural exposure. Because it has been shown that deviation to Th2 as expressed by IgE production depends on the allergen dose used to prime the corresponding experimental systems,[16-18] where low allergen doses favor and high allergen dose suppress IgE production. In fact, clinical efficacy is related to the allergen dose,[19,20] higher doses results in better protection.

MECHANISMS OF IMMUNOTHERAPY IN THE EFFECTOR PHASE

Recently, time course analysis of clinical and immunologic measurements during the first year of grass

pollen immunotherapy[21] has been reported, which could substantiate a number of partial information previously observed. The first change was reduction of late phase responses (LPR) to intradermal challenge testing that was observed as early as after the first 2 weeks during up-dosing stage of the conventional injection immunotherapy. Then, elevation of specific IgG4, inhibition of basophil histamine release, and inhibition of binding of allergen-IgE complex to B cells were observed during 6 to 8 weeks at maintenance allergen doses. Reduction of early skin responses, which usually associates with clinical efficacy, was accompanied with these later immunological changes. The investigators also found that allergen-induced IL-10 production from peripheral blood mononuclear cells was a very early event accompanied with LPR suppression. They concluded that IgG responses may be necessary for clinical protection, inhibition of histamine release and allergen/IgE binding to B cells, but that the preceding IL-10 production could contribute to this process.

MECHANISM OF IMMUNOTHERAPY IN T CELL DIFFERENTIATION

The important upstream events that immunotherapy bring about in immune responses to allergen is T cell differentiation, a critical step in regulating downstream effector mechanisms. Cumulative evidence revealed that Th1 cells and T regulatory cells are the key cells in this context.

First, in patients who received grass pollen immunotherapy, increase in cells expressing IFN-γ mRNA were found in the nasal mucosa during allergen-induced late responses and the number of the cells and symptoms scores were inversely correlated.[22] IL-12 is known to be a major cytokine to induce IFN-γ-producing Th1 cells and significant increases in allergen-induced IL-12 mRNA+ cells in cutaneous biopsy specimens was observed in the immunotherapy-treated patients and IL-12+ cells correlated positively with IFN-γ+ cells, inversely with IL-4+ cells.[23] In terms of Th2 cells, seasonal increases in IL-5 and IL-9-expressing cells in the nasal mucosa were significantly inhibited in immunotherapy patients.[24,25] Collectively, Th1 cells are induced and Th1/Th2 balance is altered in favor of Th1 cells by immunotherapy.

There are several subsets of T regulatory cells[26] and there exists inappropriate balance between allergen activation of regulatory T cells and effector Th2 cells in allergy. It was reported that CD4+CD25+ T cells, so-called naturally occurring regulatory T cells (nTreg), from non-allergic donors suppressed proliferation and IL-5 production by their own allergen-stimulated CD4+CD25- cells while the inhibition by CD4+CD25+ T cells from allergy patients were significantly reduced.[27] For these conditions, immunotherapy induces regulatory T cells in the treated patients, so called inducible regulatory T cells (Tr1 cells) and many studies have constantly identified induced expression of IL-10.[21,28-30] One report demonstrated that local increases in IL-10 mRNA and protein-positive cells were observed in the nasal mucosa from patients after 2 years of grass pollen immunotherapy. The changes were observed in treated patients only during the pollen season, not during off-season, nor in placebo-treated subjects and healthy controls.[30] These results suggest that IL-10 responses are allergen-specific, inducible phenomenon. IL-10 acts on B cells to induce production of IgG4.[31] IL-10-induced "blocking" IgG4 inhibits mast cell histamine release and IgE-facilitated allergen-binding to B cells. IL-10 also directly blocks IgE-mediated mast cell activation.[32] Further, IL-10 blocks T cell activation by inhibiting costimulatory molecule CD28 signaling pathway,[33] leading to reduction in cytokines such as IL-5[34] and reduction in inflammatory cell recruitment such as eosinophils.[24]

Table 1 Development of biomarkers for allergen immunotherapy

- Patient selection
  - ✧ Prediction of therapeutic responses
  - ✧ Prediction of serious adverse reactions
  - ✧ Identification of candidates for secondary prevention
- Maintenance
  - ✧ Monitoring of "protective" immune responses
    - "Blocking" antibodies
    - Regulatory T cells, IL-10 and other inhibitory cytokines
    - Suppression of effector cells: mast cells, basophils, eosinophils
  - ✧ Prediction of serious adverse reactions
- Completion
  - ✧ Identification of "normalized" immune responses to allergen
  - ✧ Prediction of recurrence after discontinuation

BIOMARKERS TO MONITOR ALLERGEN IMMUNOTHERAPY

To initiate, monitor, and complete effective immunotherapy, biomarkers to predict and visualize the immune responses are needed (Table 1). First, we need to identify the right candidate for immunotherapy. Although the present form of immunotherapy is effective, some patients may not respond to well the therapy and some may suffer from serious adverse events. We have to select ones who will benefit most. It has been shown that immunotherapy for children with rhinitis prevented "atopic march" from advancing to asthma.[11] We have to select the right child for the intervention since not all children with rhinitis develop asthma. Recent progress in genetics has led to the identification of several candidate genes that are associated with various phenotypes of allergic diseases.[35] It is hopeful in the future that novel genetic

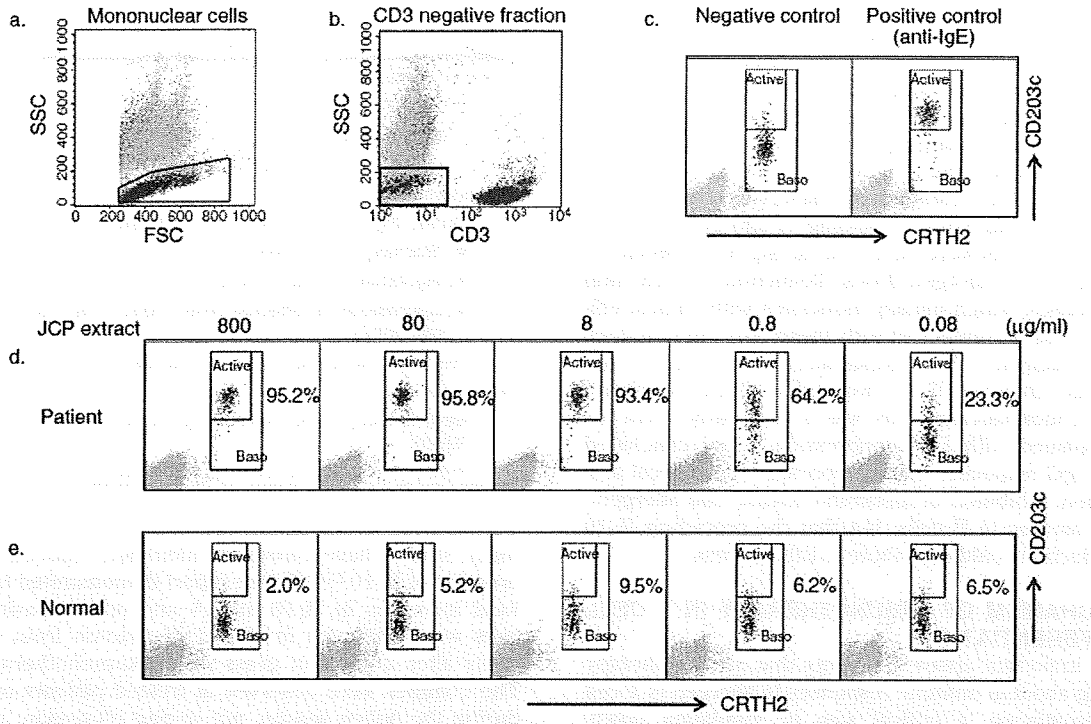

Fig. 2 Flowcytometric analysis of allergen-induced expression of CD203c. EDTA-containing whole blood was incubated with various concentrations of the Japanese cedar pollen (JCP) extract (Torii Pharmaceutical, Tokyo, Japan) for 15 min after addition of sufficient amount of calcium solution to override chelating capacity of EDTA. Anti-IgE antibody as a positive control and PBS as a negative control were also used for stimulation (**c**). PC7-conjugated anti-CD3, FITC-conjugated anti-CRTH2, and PE-conjugated anti-CD203c antibodies were also added during the reaction. The samples were analyzed on a FC500 flow cytometer (Beckman Coulter, CA, USA). Basophils were detected on the basis of forward side scatter characteristics (**a**) and expression of negative CD3 (**b**) and positive CRTH2 (**c**). Up-regulation of CD203c on basophils was determined using a threshold that was defined by the fluorescence of unstimulated cells (negative control) and expressed as $CD203c^{high}$% (**c**). JCP extract induced concentration-dependent enhancement of CD203c expression in a patient with JCP pollinosis (**d**) and no enhancement was observed in a normal control (**e**).

biomarkers identify patients who respond to the therapy without risk of developing side effects.[36]

Secondly, the immune responses induced by immunotherapy need to be evaluated. Based on the knowledge of the mechanisms of immunotherapy, several assays have been reported. Studies of peripheral blood mononuclear cells from patients receiving immunotherapy have identified reductions in proliferative responses to allergen, shifts from Th2 to Th2 cytokine production, and enhanced inhibitory IL-10 production.[25,28,31,37] Some investigators, however, did not reproduce these findings in assays using peripheral blood although changes in the local tissue were demonstrated.[38] Variations in methodology in the peripheral T cell assays may be responsible for the discrepancies and standardization is necessary. Elevation of serum allergen-specific IgG or IgG4 antibodies after immunotherapy have been clearly demonstrated but again correlation between IgG or IgG4 titers and clinical responses to immunotherapy still to be established. Instead, functional assay of blocking IgG activity have been developed. Among them, the IgE-facilitated allergen binding (IgE-FAB) assay is reported to be a validated assay for monitoring allergen immunotherapy.[39] Receptors for IgE, expressed on the surface of antigen presenting cells, B cells in this assay system, facilitate the presentation of allergens in the presence of specific IgE resulting in effective T cell activation at low concentrations of allergen. "Blocking" IgG antibodies interfere with the interaction and the assay simulates the process *in vitro*. Allergen-IgE complexes are incubated with an EBV-transformed B-cell line and complexes bound to CD23 on the surface of cells are detected by flow cytometry. Inhibition of allergen-IgE complex binding to CD23 on B cells by addition of serum from patients who have received allergen-specific immunotherapy is then quantitated. They have demonstrated that the

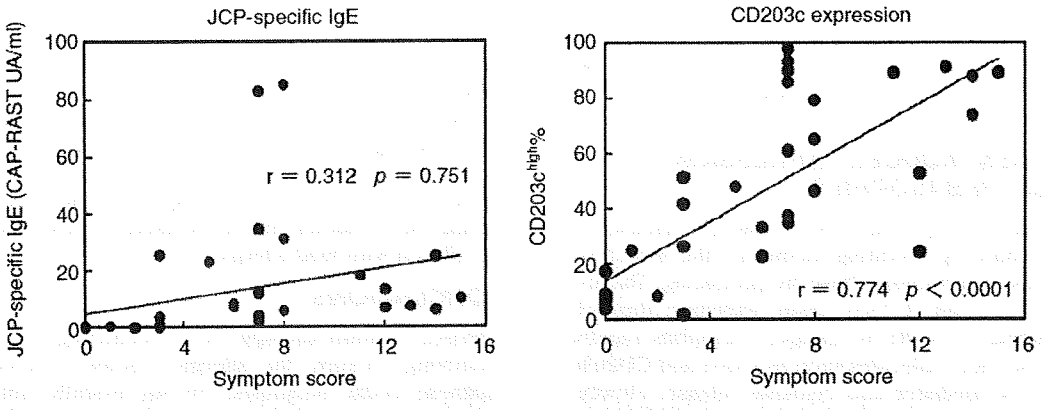

Fig. 3 Correlation of symptom score and JCP-specific IgE levels, CD203c expression by JCP extract. Thirty patients with JCP pollinosis were evaluated. Relationships between symptom score[54] and CAP-RAST titer to JCP, symptom score and JCP allergen-induced CD203c^{high}% were analyzed. Significant correlation was found in the latter.

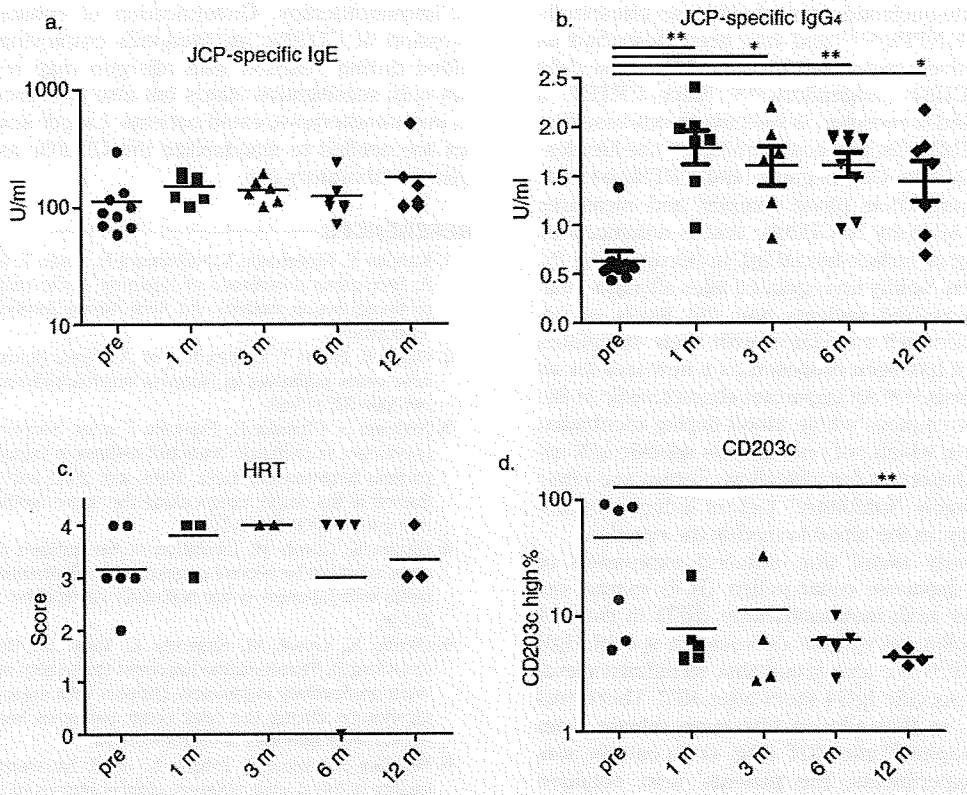

Fig. 4 Changes in JCP-specific IgE levels (**a**), JCP-specific IgG$_4$ levels (**b**), JCP-induced basophil histamine release score in HRT (**c**), and JCP-induced CD203c^{high}% in basophils (**d**) after rush immunotherapy in patients with JCP pollinosis. * $P < 0.05$, ** $P < 0.01$, Dunn's multiple comparison test (adapted from reference 49 with permission). Two subjects in whom basophils did not respond to stimulation with an anti-IgE antibody (non-responders) were excluded from the analysis for HRT and CD203c.

IgE-FAB assay have high specificity and sensitivity to diagnose clinical responses to immunotherapy. Recently, several other studies utilize the method to monitor efficacy of immunotherapy.[21,40]

ALLERGEN-INDUCED EXPRESSION OF CD203c ON BASOPHILS

Basophils play important roles in allergic diseases in effector phase by liberating mediators like histamine as well as in induction phase by producing Th2 cytokines, IL-4 and IL-13.[41] Upon activation through cross-linking of FcεRI by allergen, basophils rapidly express surface molecules such as CD63 and CD203c prior to the mediator and cytokine release. Flowcytometry-based tests for peripheral blood basophils can easily quantify these *in vitro* reactions, which presumably represent their *in vivo* activity. We utilized a commercial kit, Allergenicity Kit (Beckman Coulter, Fullerton, CA, USA), to detect expression of a basophil activation marker, CD203c. CD203c belongs to a family of ecto-nucleotide pyrophoshatase/phosphodiesterases (E-NPPs)[42,43] and has been described as being selectively expressed on basophils, mast cells and their $CD34^+$ progenitors.[44,45] Since CRTH2, a prostaglandinD2 receptor, is selectively expressed on basophils, Th2 cells, and eosinophils,[46,47] the kit identifies basophils as CD3-negative and CRTH2-positive fractions from whole blood samples and measures fluorescent intensity of CD203c that is enhanced by cross-linking of surface-bound IgE molecules (Fig. 2). As CD203c is rapidly up-regulated after allergen challenge in sensitized patients and the levels of up-regulation are well correlated with their symptoms (Fig. 2, 3), it has been proposed as a new tool for allergy diagnosis.[44,48] An important characteristic of the kit is that it employs whole blood during incubation with allergen, which not only detects specific IgE antibodies on basophils but also allows serum and other factors, possibly "inhibitory" factors induced by immunotherapy, in the blood to modify the reaction.

We recently found that induced expression of CD203c by Japanese cedar pollen (JCP) extract decreased after rush immunotherapy (RIT) in patients with JCP pollinosis without decrease in specific IgE levels to JCP.[49] We also found that significant elevation in JCP-specific IgG4 titers after RIT. There was no changes in JCP-induced histamine release from purified basophils[50] after RIT (Fig. 4). In passive sensitization experiments, the patients' sera obtained both before and after RIT showed essentially similar sensitizing capacity for basophils, corroborating the fact that specific IgE did not change. In contrast, basophil degranulation in response to the pollen extract was effectively suppressed by addition of post-RIT serum samples, which correspond with the elevation of specific IgG4 in the serum.[51] These results suggest that the CD203c test can detect blocking activity of IgG antibodies and other factors induced by immunotherapy. We also extend application of the assay to diagnosis of food allergy, especially of tolerance. Although specific IgE levels roughly predict sensitivity to food allergens,[52] markers that represent tolerance levels during outgrow phase of food allergy in childhood are not well-known. We found that the CD203c test effectively predicts sensitivity as well as tolerance to egg, milk (manuscript in preparation), and wheat[53] in children with food allergy.

CONCLUSIONS

Allergen immunotherapy is a promising disease-modifying therapy for allergic diseases including Japanese cedar pollinosis. To successfully initiate, maintain, and complete immunotherapy, predictive biomarkers have to be developed. Some prospects of biomarkers in the mechanisms of immunotherapy were reviewed in this article. Measurement of "blocking" activity of IgG such as IgE-facillitated allergen binding assay may efficiently monitor treatment effect of immunotherapy. Quantification of enhanced expression of CD203c on basophils employing whole blood during reaction with allergen may represent not only sensitization status but also tolerance levels in immunotherapy-treated patients. Larger scale studies are needed to standardize the CD203c assay for general laboratory use.

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