A novel subset of CD4+ T_{H2} memory/effecter cells that produce inflammatory IL-17 cytokine and promote the exacerbation of chronic allergic asthma

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The inflammatory cytokine interleukin (IL)–17 is involved in the pathogenesis of allergic diseases. However, the identity and functions of IL–17–producing T cells during the pathogenesis of allergic diseases remain unclear. Here, we report a novel subset of T_{H2} memory/effecter cells that coexpress the transcription factors GATA3 and RORγt and coproduce T_{H17} and T_{H2} cytokines. Classical T_{H2} memory/effecter cells had the potential to produce IL–17 after stimulation with proinflammatory cytokines IL–1β, IL–6, and IL–21. The number of IL–17–T_{H2} cells was significantly increased in blood of patients with atopic asthma. In a mouse model of allergic lung diseases, IL–17–producing CD4+ T_{H2} cells were induced in the inflamed lung and persisted as the dominant IL–17–producing T cell population during the chronic stage of asthma. Treating cultured bronchial epithelial cells with IL–17 plus T_{H2} cytokines induced strong up–regulation of chemokine eotaxin–3, IL8, Mip1b, and Groa gene expression. Compared with classical T_{H17} and T_{H2} cells, antigen–specific IL–17–producing T_{H2} cells induced a profound influx of heterogeneous inflammatory leukocytes and exacerbated asthma. Our findings highlight the plasticity of T_{H2} memory cells and suggest that IL–17–producing T_{H2} cells may represent the key pathogenic T_{H2} cells promoting the exacerbation of allergic asthma.

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of the IL-17 cytokine family and the analysis of IL-23–mediated immune pathogenesis have led to the delineation of a new CD4+ T helper cell population termed T\textsubscript{H17} (Yao et al., 1995b; Aarvak et al., 1999; Cua et al., 2003; Murphy et al., 2003; Harrington et al., 2005; Park et al., 2005a). The retinoic acid–related orphan receptor (ROR\textgamma) is the master transcription factor for the development of T\textsubscript{H17} cell lineage, which can be characterized by their secretion of the proinflammatory cytokines IL-17, IL-17F, and IL-22 (Ivanov et al., 2006). Studies in vitro have observed that in the absence of IL-4 and IFN-\gamma, TGF-\beta, and IL-21 or IL-23 are important for the induction of ROR\textgamma expression, and that the proinflammatory cytokines IL-1\beta or IL-6 can trigger IL-17 cytokine production (Mangan et al., 2006; Veldhoen et al., 2006; Wilson et al., 2007; Manel et al., 2008; Volpe et al., 2008; Yang et al., 2008). During Th cell differentiation, transcription factors T-bet and GATA-3 are mutually inhibitory for T\textsubscript{H2} and T\textsubscript{H1} differentiation, respectively. Although T-bet is a negative regulator for T\textsubscript{H17} differentiation, enforced expression of GATA-3 does not restrain the differentiation of IL-17–producing T cells, despite the loss of T\textsubscript{H17}-mediated pathology (van Hamburg et al., 2008). Additionally, an indispensable transcription factor for T\textsubscript{H2} differentiation, IFN regulatory factor 4 (IRF-4), is also required for T\textsubscript{H17} cell development, suggesting that plasticity between the development and maintenance of T\textsubscript{H2} and T\textsubscript{H17} cells may exist (Brüstle et al., 2007).

The discovery of IL-17–producing T cells has added an additional layer of complexity to the regulation of allergic inflammation. In asthmatic patients, IL-17 expression is increased in the lungs, sputum, bronchoalveolar lavage (BAL) fluids, or sera, and the severity of airway hypersensitivity in patients correlates with IL-17 expression level (Molet et al., 2001; Chakir et al., 2003). IL-17 and IL-17F can provoke neutrophil infiltration in mouse models of asthma in an antigen-specific fashion (Hellings et al., 2003), probably by inducing lung structural cells to secrete proinflammatory cytokines and chemokines such as TNF, IL-1\beta, G-CSF, and IL-6 and CXCL1/Gro-\alpha, CXCL12, and CXCL8/IL-8, respectively (Jovanovic et al., 1998; Laan et al., 1999; Ye et al., 2001; Jones and Chan, 2002). Importantly, IL-17–deficient mice exhibit both reduced neutrophil and eosinophil recruitments (Ye et al., 2001), whereas IL-17A–/– mice exhibited reduced IL-12 responses to antigen sensitization (Nakae et al., 2002). Although these studies demonstrate the importance of IL-17–producing cells in driving the exacerbation of allergic inflammation, the identity and characteristics of these cells during type-2 dominant immune response remain unclear. Herein, we demonstrate that a subset of T\textsubscript{H2} cells in both mice and humans is capable of producing large amounts of the proinflammatory cytokines IL-17 and IL-22, in addition to classical T\textsubscript{H2} cytokines. We suggest that IL-17–producing CD4\textsuperscript+ T\textsubscript{H2} cells may be a unique subset of lung resident T\textsubscript{H2} memory/effector cells with additional inflammatory properties and contribute to the exacerbation of chronic allergic asthma.

**RESULTS**

A novel subset of human T\textsubscript{H2} memory/effector cells produces IL-17

CCR6 was a useful marker for the identification of IL-17–producing cells in the human memory T cell pool (Acosta-Rodriguez et al., 2007). CCRTH2 were reported to be the most reliable marker to identify human CD4+ T\textsubscript{H2} memory cells (Cosmi et al., 2000; Wang et al., 2006). The characterization of human CCRTH2+CD4+ T\textsubscript{H2} cells led us to identify a distinctive subset of T\textsubscript{H2} cells expressing a high level of CCR6 (Fig. 1 a).

To investigate whether the CCR6\textsuperscript+ subset of CCRTH2+CD4+ T\textsubscript{H2} cells also display features of T\textsubscript{H17} cells, freshly purified T\textsubscript{H2} cells from the peripheral blood were stimulated with anti-CD3/CD28 mAbs for 24 h, and their secreted cytokines were examined using ELISA. Both subsets of CCRTH2+CD4+ T\textsubscript{H2} cells produced classical T\textsubscript{H2} cytokines IL-4, IL-5, and IL-13, but not IFN-\gamma; notably, only the CCR6\textsuperscript+ subset of T\textsubscript{H2} cells could produce T\textsubscript{H17} cytokines IL-17 and IL-22 (Fig. 1 b). Both subsets exhibit a memory T cell phenotype featuring the expression of CD45RO, CCR7, CD27, and CD62L, as well as CCR4 and CXCR4, the chemokine receptors expressed by T\textsubscript{H2} cells (Fig. 1 c and not depicted). Notably, both T\textsubscript{H2} cell subsets express low levels of surface IL-1RI, but do not express IL-23R; additionally, very few of them express CD161 (>2%), which is the marker of human T\textsubscript{H17} precursors (Fig. 1 c; Acosta-Rodriguez et al., 2007; Annunziato et al., 2007; Cosmi et al., 2008). These findings reveal a novel subset of human T\textsubscript{H2} cells that are capable of producing inflammatory IL-17 cytokine and expresses chemokine receptors for homing to the skin and other mucosal tissues.

Because ROR\textgamma expression is essential for the generation of the classical IL-17–producing CD4\textsuperscript+ T cells (Ivanov et al., 2006), we analyzed the expression of ROR\textgamma transcript in the following cell populations: CCR6\textsuperscript+CCRTH2\textsuperscript+ IL-17–producing T\textsubscript{H2} cells, classical CCR6\textsuperscript−CCRTH2\textsuperscript+ T\textsubscript{H2} cells, CCR6\textsuperscript+ CCRTH2\textsuperscript− T\textsubscript{H17} cells (Acosta-Rodriguez et al., 2007), in vitro-generated T\textsubscript{H1} cells, and CD4\textsuperscript+CD45RO\textsuperscript− naive T cells. Both T\textsubscript{H17} and IL-17–producing T\textsubscript{H2} cells were found to express a significant level of ROR\textgamma transcript; in contrast, classical T\textsubscript{H2}, T\textsubscript{H1}, or naive T cells did not (Fig. 1 d). Although both IL-17–producing T\textsubscript{H2} and classical T\textsubscript{H2} cells expressed the master transcription factor for T\textsubscript{H2} development, GATA3, these T\textsubscript{H2} cell subsets did not express T-bet, the transcription factor for T\textsubscript{H1} development (Fig. 1 d). These data demonstrate that CCR6\textsuperscript+CCRTH2\textsuperscript+ IL-17–producing T\textsubscript{H2} cells can concurrently express ROR\textgamma and GATA3, which are the master transcription factors for T\textsubscript{H17} cells and T\textsubscript{H2} cells, respectively.

To further test whether IL-17 and IL-4 are concomitantly produced at the single-cell level, IL-17–producing T\textsubscript{H2} cells, classical T\textsubscript{H2} cells, and T\textsubscript{H17} cells were isolated and maintained with homeostatic cytokines (IL-7 and IL-15) for 3 d before intracellular cytokine analyses. We found that a significant fraction of IL-17–producing T\textsubscript{H2} cells produced cytokines IL-4 and IL-17 concurrently, whereas classical T\textsubscript{H2} cells produced mostly IL-4, but little IL-17 (Fig. 2 a). Conversely, the classical T\textsubscript{H17} cells...
produced mostly IL-17, but little IL-4 (Fig. 2 a). To examine whether GATA3 and RORγt can be coexpressed at single-cell level, the three aforementioned T helper cell subsets were activated by anti-CD3 mAb before immunofluorescence analyses for expression of GATA3 and RORγt. Notably, IL-17–producing T\(_{h2}\) cells were found to coexpress GATA3 and RORγt in the nucleus (Fig. 2, b–e). Corresponding to their cytokine production pattern, classical T\(_{h2}\) cells expressed only GATA3 and not RORγt (Fig. 2 f), whereas classical T\(_{h1}\) cells expressed only RORγt and not GATA3 in their nucleus (Fig. 2 g). These results demonstrate that IL-17–producing T\(_{h2}\) cells express both GATA3 and RORγt transcription factors.

**Proinflammatory cytokines induce classical T\(_{h2}\) memory cells to produce IL-17**

Several studies demonstrated that T\(_{h1}\)-polarizing signals could reprogram committed T\(_{h2}\) memory/effector cells to produce IFN-γ, suggesting the existence of plasticity within committed T\(_{h2}\) cells (Brugnolo et al., 2003; Filì et al., 2006; Hegazy et al., 2010). To test whether classical T\(_{h2}\) cells have the potential to produce IL-17 cytokine under T\(_{h17}\)-polarizing signals, purified classical CCR6\(^-\)CRTH2\(^+\) T\(_{h2}\) cells were cultured with homeostatic cytokines (IL-7 and IL-15) plus anti–IL-4 and anti–IFN-γ mAbs in the absence or presence of select T\(_{h17}\)-polarizing cytokines for 6 d. As shown in Fig. 3 (a and b), we found that the T\(_{h17}\) polarizing cytokine IL-1β, IL-6, or IL-21, but not IL-23, is capable of inducing the classical T\(_{h2}\) cells to produce significant amounts of IL-17 as determined by ELISA and intracellular cytokine analyses. Notably, the treatment combination of IL-1β, IL-6, and IL-21 together is most effective at inducing the classical T\(_{h2}\) cells to produce IL-17 and IL-22 cytokines (Fig. 3, a and b), possibly via up-regulation of the expression of T\(_{h17}\)-associated transcription factors IRF4 and RORγt, as well as CCR6 transcripts (Fig. 3 c). Collectively, these results suggest that committed T\(_{h2}\) memory/effector cells possess the plasticity to become IL-17–producing cells after stimulation with proinflammatory cytokines.

**IL-17 and T\(_{h2}\) cytokines synergistically induce chemokine production**

Severe asthma is often associated with elevated IL-17 expression and intense infiltration of neutrophils and eosinophils in
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mAbs can block the up-regulation of select chemokine genes induced by treatment with supernatant from IL-17–producing TH2 cells (Fig. 4 b). These results suggest that the IL-17 and TH2 cytokines produced concurrently by IL-17–producing TH2 cells can selectively enhance the expression of pro-allergic chemokine genes in lung epithelial cells, particularly eotaxin-3.

Increased frequency of IL-17–producing T\textsubscript{h}2 cells in patients with atopic asthma

Previous studies have shown that CRTH2\textsuperscript*CD4\textsuperscript+ T\textsubscript{h}2 cells circulate in the peripheral blood of all healthy subjects tested, ranging from 2–4% of total CD4\textsuperscript+ T cells, and that the frequency of these cells is elevated in patients with atopic dermatitis (Cosmi et al., 2000). To examine whether an increased frequency of circulating IL-17–producing T\textsubscript{h}2 cells is associated with patients with atopic asthma, 39 subjects were recruited for the study (23 subjects with atopic asthma and 16 healthy control subjects). Subject characteristics are summarized in Table I. The number of total CD4\textsuperscript+CRTH2+ TH2 memory/effector cells and of CCR6 + and CCR6– of CD4\textsuperscript+CRTH2+ TH2 cell subset cells from the peripheral blood of subjects were analyzed and compared by flow cytometry. Consistent with a previous study (Cosmi et al., 2000), the number of circulating total CD4\textsuperscript*CRTH2* T\textsubscript{h}2 memory/effector cells in subjects with the airway of atopic patients (Kolls et al., 2003). To address whether the combination of IL-17 and T\textsubscript{h}2 cytokines can synergistically induce chemokine production, which would enhance the recruitment of inflammatory cells, we treated normal human bronchial epithelial cells or bronchial epithelial cell lines (BEAS-2) with various combinations of IL-17 and T\textsubscript{h}2 cytokines. Compared with the treatments of IL-13, TNF, IL-17, IL-13 with TNF, or IL-17 with TNF, the combination of IL-17, T\textsubscript{h}2 cytokines, and TNF induced the greatest increase in gene expression of eotaxin-3 (>500-fold), IL-8 (>200-fold), MIP-1\textbeta (>40-fold), and Gro-\alpha (>15-fold), but not MCP-1 or eotaxin-1, in the normal human bronchial epithelial cells and bronchial epithelial cell lines (BEAS-2; Fig. 4 a and not depicted). In a parallel experiment, we examined the effect of supernatants collected from activated IL-17–producing CD4\textsuperscript+CCR6\textsuperscript*CRTH2* T\textsubscript{h}2, classical CD4\textsuperscript*CCR6\textsuperscript–CRTH2* T\textsubscript{h}2, or CD4\textsuperscript*CCR6\textsuperscript*CRTH2– T\textsubscript{h}17 cells on the induction of chemokine gene expression in normal human bronchial epithelial cells. Compared with the supernatants collected from activated classical T\textsubscript{h}2 or T\textsubscript{h}17 cells, we found that the cytokine milieu secreted by IL-17–producing T\textsubscript{h}2 cells was the most effective in inducing the up-regulation of eotaxin-3 (>1,000-fold), IL-8 (>30-fold), Gro-\alpha (>12-fold), MIP-1\textbeta (>20-fold), and MIP-1\textbeta (>10-fold), but not eotaxin-1 gene expression in normal bronchial epithelial cells (Fig. 4 b). Notably, neutralizing anti–IL-17 mAbs can block the up-regulation of select chemokine genes induced by treatment with supernatant from IL-17–producing T\textsubscript{h}2 cells (Fig. 4 b). These results suggest that the IL-17 and T\textsubscript{h}2 cytokines produced concurrently by IL-17–producing T\textsubscript{h}2 cells can selectively enhance the expression of pro-allergic chemokine genes in lung epithelial cells, particularly eotaxin-3.
suggest that patients with atopic asthma may have increased frequency of inflammatory IL-17–producing TH2 cells in their blood.

**IL-17-producing TH2 cells are induced in inflamed lung in vivo**

To further establish the function of IL-17–producing TH2 cells in the pathogenesis of asthma in vivo, we used a previously described mouse model of allergic lung diseases by challenging IL-4-eGFP knock-in (4GET) mice six times intranasally with Aspergillus oryzae or papain (Henderson et al., 1996). 1 d after the last challenge, CD4+CD62L−CD44hi memory/effector T cells isolated from lung or other lymphoid tissues were stimulated with PMA and ionomycin for intracellular cytokine analysis. As shown in Fig. 6 a, two populations of the infiltrating CD4+ cells

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**Figure 3. Proinflammatory stimuli induce classical CD4+ TH2 cells to produce IL-17 cytokine.** Sorted CCR6−CRTH2−CD4+ TH2 cells were maintained with homeostatic cytokines IL-7/IL-15 plus anti–IL-4 and anti–IFN-γ mAbs in the presence or absence of the indicated proinflammatory cytokines for 6 d. Expanded cells were restimulated with PMA plus ionomycin for the analysis of intracellular cytokine production (a) or anti–CD3/CD28 for measurement of cytokines in the culture supernatants by ELISA (b) or were used as cDNA templates for the indicated gene expression analysis by real-time PCR (c). Fold differences in gene expression level between cell/treatment groups marked in the horizontal axis are indicated in the left panel. Data represent the mean (±SD) of five experiments (b). Data are from one of three independent experiments (a and c).
inflamed lung, but not other lymphoid organs, produced large amounts of IL-17A, IL-17F, IL-22, IL-21, and IFN-γ, but very little of IL-4, IL-5, and IL-13, indicative of the classical TH17 or TH1 cell subsets (Fig. 6b). These data suggest that the IL-17/IL-4–double-producing TH2 cells were induced selectively in inflamed lung, but not in the draining lymph nodes or other lymphoid tissues in this mouse model of asthma.

IL-17–producing TH2 cells are increased in inflamed lung during chronic phase

Allergen-specific TH2 memory cells that reside in lung during disease remission are the principle cell type responsible for the exacerbation of allergic asthma (Epstein, 2006). To characterize lung-resident memory T cells after remission, we analyzed intracellular cytokine production by CD4+CD62L−memory/effector T cells in the lung and other lymphoid tissues from mice that rested for different periods of time after the last allergen challenge. 3 h after the last challenge, the majority of lung CD4+CD62L−memory/effector T cells were found to be the conventional IL-17+/IL-4−(GFP−) TH17 cells (20%) or the classical IL-17−IL-4+(GFP+) TH2 cells (16%); only 3% of lung memory/effector CD4+ T cells were IL-17−producing TH2 cells (IL-17+GFP+; Fig. 7a). Notably, the frequency of conventional TH17 cells that reside in the lung declined rapidly to ≤10% 1 d after the last challenge. Conversely, the frequency of the IL-17−producing TH2 cells accumulated to ≥20% of total lung resident CD4+CD62L−memory/effector T cells in mice that rested for

Table 1. Characteristics of the study population

<table>
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<tr>
<th>Characteristics</th>
<th>Atopic asthma subjects</th>
<th>Healthy control subjects</th>
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<tbody>
<tr>
<td>n = 23</td>
<td>n = 16</td>
<td></td>
</tr>
<tr>
<td>Age, yr</td>
<td>50 (27–65)</td>
<td>34 (24–46)</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>9/14</td>
<td>10/7</td>
</tr>
<tr>
<td>Onset of asthma (childhood/adulthood)</td>
<td>8/17</td>
<td>–</td>
</tr>
<tr>
<td>FEV1, percentage predicted</td>
<td>68 (31–98)</td>
<td>107 (102–121)</td>
</tr>
<tr>
<td>Percentage of PEF variability</td>
<td>20 (12–69)</td>
<td>ND</td>
</tr>
<tr>
<td>Skin test positive</td>
<td>23 (100%)</td>
<td>ND</td>
</tr>
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Data are presented as median with interquartile ranges. FEV1, forced expiratory volume in the first second; PEF, peak expiratory flow; ND, not done.
Anti-CD3/CD28 mAbs for 24 h before measurement of the secreted cytokine analyses. (c) Sorted CCR6+ or CCR6- subsets of CRTH2+CD4+ T4GET mice carrying OVA-specific DO11.10 TCR transgenic mice were first isolated from OVA/alum adjuvant–sensitized mice and then further induced by co-culturing splenic CD4+ T4GET cells with APCs pulsed with OVA peptides in the presence of IL-1β, IL-6, IL-23, and anti–IFN-γ or IL-4 and anti–IFN-γ, respectively. OVA-specific T417 cells were generated by co-culturing splenic CD4+ T cells from naive DO11.10X4GET mice with APCs pulsed with OVA peptides in T417-polarizing conditions. BALB/c mice were transferred intravenously with a single OVA-specific, IL-17–producing T412, classical T42, or T417 cell subset, with both the classical T412 and T417 cell subsets, or with saline only; mice were then challenged with OVA intranasally once a day for 2 d. As shown in Fig. 8 a, bronchoalveolar lavage fluid (BALF) from mice transferred with OVA-specific, IL-17–producing T412 cells or both the classical T412 and T417 cell subsets contained about threefold more infiltrating cells than those from mice transferred with only OVA-specific classical T412 or T417 cells or saline alone. Moreover, we found that mice transferred with OVA-specific IL-17–producing T412 cells or both the classical T412 and T417 cell subsets exhibited markedly enhanced recruitments of eosinophils, neutrophils, macrophage, and lymphocytes into the airway, whereas mice transferred with only OVA-specific classical T412 or T417 cells showed a moderate influx of eosinophils or neutrophils and macrophages into the airway, respectively, after intranasal OVA challenges (Fig. 8 b). Interestingly, a significant increase of inflammatory cytokines, IL-1β and IL-6, and a moderate increase of IL-5 and IL-13, but not IFN-γ production, were detected in the BALF of mice transferred with OVA-specific, IL-17–producing T412 cells or both the classical T412 and T417 cell subsets compared with those in BALF of mice transferred with OVA-specific classical T412 or T417 cells or saline only (Fig. 8 c). Histological analyses of lungs from mice transferred with OVA-specific, IL-17–producing T412 cells or both the classical T412 and T417 cell subsets exhibited markedly enhanced peribronchial inflammation with infiltrated eosinophils and neutrophils, more prominent mucin production, and goblet cell hyperplasia compared with lungs from mice transferred with conventional T412 or T417 cells or saline only (Fig. 8 d). These results suggest that during the allergen recall response, antigen–specific IL-17–producing T412 cells may have additional inflammatory properties, similar to those of the combined T412 and T417 cells, which promote the infiltration of heterogeneous leukocytes and exacerbate the immunopathology of allergic asthma.

**DISCUSSION**

Severe asthma is a heterogeneous disorder with distinct types of inflammatory processes. Although the discovery of IL-17–producing T cells has shed light on the understanding of the underlying mechanisms that contribute to the heterogeneity and severity of asthma, the identity of IL-17–producing T cells in allergic diseases, including asthma remain elusive. In this study, we identified a distinct population of human IL-17–producing T412 cells characterized by (a) the capability of concomitantly producing the classical T412 cytokines IL-4, IL-5, and IL-13, and the inflammatory T417 cytokines IL-17 and IL-22; (b) dual expression of the T417-transcription factor RORγt and the T412-transcription factor GATA3; and...
Figure 6. The induction of IL-17–producing T_{H}2 cells occurred in the inflamed lung after allergen exposure. 4GET mice (n = 4) were challenged with indicated allergens plus OVA intranasally every other day for a total of six times before sacrifice. Sorted total (a) or GFP+ and GFP– subsets (b) of CD4+CD62L–CD44hi memory/effector T cells from lung or indicated lymphoid tissues were restimulated with PMA plus ionomycin for the analysis of intracellular cytokine production (a) or with anti-CD3/CD28 mAbs for 24 h before measurement of cytokines in the supernatants by ELISA (b). Data are from one of three independent experiments (a and b). Data represented as the mean (±SD); four mice per group. LLN, lung draining LNs; MLN, intestine mesenteric LNs.

Figure 7. Inflammatory IL-17–producing T_{H}2 cells persist in the inflamed lung during the chronic phase of allergic asthma. 4GET mice (n = 4) were challenged with Aspergillus oryzae plus OVA intranasally every other day for a total of six times (a and b). After the last challenge, mice rested for the indicated time frame (a and b; horizontal axis) before sacrifice. Purified CD4+CD62L–CD44hi memory/effector T cells from lung or other lymphoid tissues were restimulated with PMA plus ionomycin for the analysis of intracellular cytokine production (a). The percentage of indicated cytokine producing cells within the total CD4+ memory/effector T cell pool were numerated as shown on the left axis (b). Data are representative of two independent experiments. Data represented as the mean (±SD); four mice per group.
that the TGF-β, IL-21, or IL-23, as well as the proinflammatory cytokines IL-1β or IL-6, are important for the induction of IL-17 cytokine production (Mangan et al., 2006; Veldhoen et al., 2006; Wilson et al., 2007; Manel et al., 2008; Volpe et al., 2008; Yang et al., 2008). However, recent findings suggest that some cytokines, such as IL-1β (Tillie-Leblond et al., 1999; Nakae et al., 2003; Chung et al., 2009) and IL-21 (Fröhlich et al., 2007; Nurieva et al., 2007; Leonard et al., 2008; Yang et al., 2008), and the transcription factor IRF4 (Rengarajan et al., 2002; Brüstle et al., 2007; Honma et al., 2008) are important for the development of both TH2 and TH17 immune responses, suggesting that the plasticity between the development and maintenance of TH2 and TH17 cells may exist. Our finding of the novel subset of CD4+ TH2 memory/effector cells capable of producing IL-17 supports this hypothesis. Notably, we showed that the proinflammatory cytokine IL-1β, IL-6, and IL-21 could directly induce the up-regulation of IRF4 and RORγt gene expression and the production of IL-17 in classical TH2 memory/effector cells in vitro. At the early phase of allergic inflammation in an (c) surface expression of CD45RO, the chemokine receptors CCR4 and CXCR4, and CLA, suggesting a memory phenotype and readiness to migrate into inflammatory mucosa sites. Notably, the number of circulating IL-17–producing TH2 cells is significantly increased in patients with atopic asthma. Moreover, we demonstrated that the mouse counterpart of IL-17–producing CD4+ TH2 cells are selectively induced in the inflamed lung and are the dominant IL-17–producing T cells during the chronic phase of asthma in vivo. Mice transferred with antigen-specific, IL-17–producing TH2 cells exhibited profound immunopathological allergic inflammation after recall response. These data suggest that the IL-17–producing CD4+ TH2 cells may represent the inflammatory TH17 cells that promote the pathophysiology of asthma.

The TH17 cell lineage possesses a unique genetic program and is described as an additional branch of the T helper cell subsets (Harrington et al., 2005; Park et al., 2005b; Ivanov et al., 2006). Studies in vitro demonstrated that the absence of TH2 and TH1 cytokines is the prerequisite for TH17 cell differentiation from naive T cells (Veldhoen et al., 2006) and that the TGF-β, IL-21, or IL-23, as well as the proinflammatory cytokines IL-1β or IL-6, are important for the induction of IL-17 cytokine production (Mangan et al., 2006; Veldhoen et al., 2006; Wilson et al., 2007; Manel et al., 2008; Volpe et al., 2008; Yang et al., 2008). However, recent findings suggest that some cytokines, such as IL-1β (Tillie-Leblond et al., 1999; Nakae et al., 2003; Chung et al., 2009) and IL-21 (Fröhlich et al., 2007; Nurieva et al., 2007; Leonard et al., 2008; Yang et al., 2008), and the transcription factor IRF4 (Rengarajan et al., 2002; Brüstle et al., 2007; Honma et al., 2008) are important for the development of both TH2 and TH17 immune responses, suggesting that the plasticity between the development and maintenance of TH2 and TH17 cells may exist. Our finding of the novel subset of CD4+ TH2 memory/effector cells capable of producing IL-17 supports this hypothesis. Notably, we showed that the proinflammatory cytokine IL-1β, IL-6, and IL-21 could directly induce the up-regulation of IRF4 and RORγt gene expression and the production of IL-17 in classical TH2 memory/effector cells in vitro. At the early phase of allergic inflammation in an

Figure 8. Antigen-specific inflammatory IL-17–producing TH2 cells promote the exacerbation of allergic asthma. Five groups of BALB/c mice were intranasally challenged once a day for 2 d with OVA 24 h after being adoptive transferred with 0.9% saline as a control or OVA-specific IL-17–producing TH2, classical TH2, classical TH17, or classical TH2 and TH17 cells generated in vitro, as described in Materials and methods. BALF of individual mice of each group were collected for the measurement of total cell counts (a) and differential cell counts (b), indicating that the total numbers of individual inflammatory cells in each group or (c) concentrations of indicated cytokines by ELISA. (d) Histological analysis of representative lung bronchovascular bundles stained with hematoxylin and eosin (H&E; top) or stained with periodic acid Schiff (PAS; bottom). The insets at the corner depict higher magnification images of the airway epithelium stained with PAS, showing that much more abundant mucus-producing cells (pink cytoplasm) are lining the airway epithelium of mice receiving indicated T helper cell subsets. Data are representative of three independent experiments. Data represented as the mean (±SD); four mice per group. Bars: (capped) 100 µm; (uncapped) 10 µm.
animal model of allergic lung diseases, IL-17–producing T_{H}2 cells could primarily be found in the inflamed lung along with other T helper subsets, including classical T_{H}1 and T_{H}17 cells, and some T_{H}1 cells. Notably, these resident IL-17–producing T_{H}2 cells persist in inflamed lung as the dominant IL-17–producing T cells at the chronic stage of airway allergic inflammation. Collectively, these observations suggest that substantial plasticity exists within CD4+ T_{H}2 memory/effector cells and that this plasticity may be controlled by local inflammatory cues. In theory, naive CD4+ T cells or T_{H}17 cells may also have the potential to become IL-4/IL-17–dual-producing cells. It is possible that some naive T cells may not have to undergo the T_{H}2 or T_{H}17 differentiation pathway and may become IL-4/IL-17–double producers through subsequent regulation from unique microenvironments in vivo. Recent studies have reported that T_{H}17 cells have the plasticity to become other cell lineages (Lee et al., 2009; Zhu and Paul, 2010). To test whether T_{H}17-polarizing signals could induce T_{H}17 cells to become IL-4/IL-17–double producers, in our pilot studies, we found that T_{H}17-polarizing stimuli (thymic stroma lymphopoietin–activated DCs or IL-4) could induce the freshly isolated human CCR6+CRTTH2+ T_{H}17 cells to produce IL-4, but shut down their IL-17 production in vitro (unpublished data). However, during the revision of this study, findings from the characterizations of human CD4 T cell clones point to the possibility that T_{H}17 cells may have the potential to become IL-4/IL-17–dual-producing cells (Cosmi et al., 2010). Understanding the cellular origin and the underlying mechanisms that drive the induction of IL-4/IL-17–double producers during allergic inflammation is the basis for further investigations.

The severity of asthma is correlated with the level of IL-17 cytokine found in the lung, sputum, BALF, or serum of patients (Molet et al., 2001; Chakir et al., 2003). One of the major functions of the cytokine IL-17 during asthmatic reactions is to orchestrate the sustained neutrophilic mobilization (Kolls et al., 2003; Lindén et al., 2005). However, the mixed eosinophilic, neutrophilic, and granulocytic infiltrations with greatly increased total cell number are often observed in the sputum of patients in some subtypes of severe allergic asthma (Simpson et al., 2006), and the underlying cellular and molecular mechanisms remain unknown. One of the mechanisms involved in the IL-13–mediated pathophysiological features of asthma is the induction of chemokine production by airway structural cells (Zimmermann et al., 2003). We found that the combination of inflammatory IL-17 and T_{H}17 cytokines IL-4 and IL-13 or the use of a cytokine milieu produced by IL-17–producing T_{H}2 cells have profound synergistic effects on the induction of various chemokine genes in primary lung bronchial epithelial cells, such as MIP-1{beta}, MCP-1, Gro-{alpha}, IL-8, and eotaxin-3, which is particularly effected. The effects of IL-17–producing T_{H}2 cells on promoting the recruitment of inflammatory leukocytes were further substantiated in this animal model of asthma in vivo. Transfer of antigen–specific, IL-17–producing T_{H}2 cells triggered much stronger influx of heterogeneous leukocytes, including neutrophils, eosinophils, macrophage, and lymphocytes, which resulted in profound goblet hyperplasia as well as elevated mucin production after antigen sensitization. In contrast, mice transferred with conventional T_{H}2 or T_{H}17 cells exhibited fewer airway infiltrations of eosinophils or neutrophils, respectively, and limited pathophysiological features. The finding that the frequency of circulated IL-17–producing T_{H}2 cells is significantly elevated in atopic asthma patients further highlights the potential role of this novel cell subset in the exacerbation of allergic diseases. Future analyses on the frequency and characteristics of the inflammatory IL-17–producing T_{H}2 cells in patients with different subtypes of asthma may facilitate the understanding of the heterogeneity and severity of allergic asthma.

Patients with severe allergic asthma during remission often have elevated nitric oxide breath levels that are indicative of their persistent lung inflammation and are possibly mediated by resident allergen–specific T_{H}2 memory cells (Yurovsky et al., 1998; Bates and Silkoff, 2003). The identification of the T_{H}17 cell lineage and its confounding roles in the pathogenesis of allergic inflammation have further unveiled the complexity of atopy (Nakae et al., 2002; Schnyder-Candrian et al., 2006) and raises new questions on how T_{H}17 cells and T_{H}2 cells cooperate to mediate the severity and heterogeneity of allergic asthma. The temporal recruitments and interplay between these two T helper subsets have been suggested as the cause of the heterogeneous in the pathology of severe asthma (Larché et al., 2003; Schmidt-Weber et al., 2007). In an animal model of asthma, we showed that the influx of T_{H}17 cells could occur within the first 3 h after the last challenge; however, the majority of IL-17–producing T cells persisted in the lung from day 3 after the last challenge and were found to express low-to-high levels of GFP (IL-4) that were indicative of their T_{H}2 characteristics. Our findings suggest that the rapid influx of T_{H}17 cells may be part of the inflammatory processes triggered by the injured epithelial cells or altered innate immunity induced by environmental stimuli or invaded pathogens at the acute phase of allergic asthma. Antigen–specific classical T_{H}2 or IL-17–producing T_{H}2 cells that respond to allergen sensitization may reside in the lung and contribute to the persistence and progression of chronic allergic asthma. Designing curative therapy for chronic allergic diseases in a phase–specific manner may require not only the understanding of the factors that regulate the balance for the development of various T helper subsets, but also their temporal sequences and potential interactions in the induction of immunopathology of allergic asthma.

**MATERIALS AND METHODS**

Cell culture and isolation of human T_{H}2 and T_{H}17 memory cell subsets. This study was approved by the institutional review board for human research at The University of Texas M.D. Anderson Cancer Center (Houston, Texas). Human CD4+ T_{H}2 memory/effector T cells were enriched by the depletion of other lineage cells using microbeads and then sorted as CD4+CRTTH2+CCR6-Lineage+ or CD4+CRTTH2+CCR6 Lineage- cells. T_{H}17 cells were sorted as CD4+CRTTH2+CCR6+ Lineage+ cells with purity >99%, as previously described (Wang et al., 2006). In some experiments,
purified CD4+ CCR5+ CCR6+ T112 cells were cultured with 20 ng/ml IL-7 and 10 ng/ml IL-15 plus 2 µg/ml anti-IL-4 and 1 µg/ml anti-IFN-γ mAbs (R&D Systems) in the presence or absence of 10 ng/ml IL-1β, 25 ng/ml IL-6, 10 ng/ml IL-21, or 25 ng/ml IL-23 (R&D Systems), or in the combination of these cytokines for the induction of IL-17 production. Human primary normal bronchial epithelial cells (Lonza) were maintained in bronchial epithelial growth medium (Lonza), and the bronchial epithelial cell line (BEAS-2) was cultured following the instructions of American Type Culture Collection.

Analysis of cytokine production. Freshly isolated or expanded human T112 cell subsets or T117 cells were stimulated with immobilized anti-CD3 (OKT3; 5 µg/ml) and soluble anti-CD28 (L293.1; 1 µg/ml) for 24 h. Collected supernatants were assessed by ELISA for IL-4, IL-5, IL-13, IL-17, IL-22, IFN-γ, IL-10, and TNF production (R&D Systems). In mouse experiments, sorted GFP+ or GFP− CD4+CD62L− memory/effector T cells, isolated from 4GET mice challenged intranasally with the allergen Aspergillus oryzae 6 times were stimulated with immobilized 5 µg/ml anti-CD3 and soluble 1 µg/ml anti-CD28 for 24 h. Collected supernatants were assessed by ELISA for IL-4, IL-5, IL-13, IL-17A, IL-17F, IFN-γ, IL-10, TNF (R&D Systems), and IL-22 production (Antigenic American). Intracellular cytokine analyses were performed using PE-IL-4, FITC-IFN-γ (BD), and APC-IL-17 (eBioscience) in human studies and PE-IL-17 (ebioscience) and APC-IFN-γ (BD) in mouse experiments.

RNA isolation and real-time quantitative PCR. Total RNA samples from sorted or cultured cells were isolated by RNeasy kit (QIAGEN). The cDNA templates were synthesized using SuperScript II (Life Technologies). Oligonucleotide primers were selected using Primer Express 2.0 (Applied Biosystems). Real-time quantitative PCR was performed with the ABI Prism 7900 (Applied Biosystems) detection system. For the analysis of GATA3, c-maf, T-bet, NFOAtc1, IL-4, and IL-5 gene expression, real-time PCR probes were purchased directly from the manufacturer (Applied Biosystems). For analyses of chemokine gene expression, transcripts were amplified using an annealing temperature of 60°C and the following primers:

- AGGAGAATCACCACCAAGACAT-3′ and 5′-TATGCAGCTGACTAAGTTCTTTAGCA-3′; and
- CTGGGTGCGAAGCTATGAAT-3′ and 5′-TATGCAGCTGACATCTAAGTTCTTTAGCA-3′. For each sample, mRNA abundance was normalized to the amount of 18S rRNA or GAPDH and expressed as arbitrary units, as described previously (Wang et al., 2006).

Immunohistology. For immunofluorescent staining, cultured human T helper subsets were activated by anti-CD3 for 6 h before being cytospun onto the slides. The tested slides were fixed and incubated with goat anti-GATA3 (Santa Cruz Biotechnology, Inc.) and mouse anti-human RORγt mAb (provided by D. Littman, Skirball Institute of Biomolecular Medicine, New York University School of Medicine, New York, NY) at room temperature for 1 h, followed by Alexa Fluor 488-F(ab′)2, fragment rabbit anti-goat IgG antibody (Invitrogen). After washing and blocking, cells were counterstained with biotinylated horse anti-mouse antibodies and visualized by Alexa Fluor 594-conjugated streptavidin (Invitrogen).

Induction and analysis of a mouse model of allergic lung diseases. We used a previously well-established animal model of allergen-induced airway disease (Henderson et al., 1996). In brief, 4GET or DO11.10 X4GET mice were anesthetized with isoflurane and subjected to intranasal inhalation of 50 µl of OVA only, Aspergillus oryzae with OVA, or Papan with OVA every other day for a total of 6 challenges. Potential endotoxin contamination was removed from OVA by endotoxin-removing gel (Thermo Fisher Scientific). After the last challenge, mice were sacrificed at various time points and CD4+CD62L− memory/effector T cells were sorted from the lung, spleen, lung draining lymph nodes, and mesenteric lymph nodes.

Adoptive transfer experiments for antigen-induced airway inflammation. The IL-17–producing T112 cells, conventional T112 cells, or T117 cells were transferred intravenously into BALB/c mice (2 × 106 cells/mouse). Control mice received saline intravenously. 1 d after transfer, mice were intranasally challenged with OVA (50 µg/ml) every day for a total of two times. Mice were sacrificed 24 h after the last challenge. Total cell numbers or numbers of eosinophils, neutrophils, macrophage, and lymphocytes in the BALF were numerated, and the levels of IL-1β, IL-6, IL-13, and IFN-γ in the BALF were evaluated by ELISA. For the histological analyses, individual lungs were fixed in 10% buffered formalin. Hematoxylin and eosin, Giemsa, and periodic-acid Shiff staining were performed by Histology Consultation Services, Inc.

Subjects and study design. Study participants were recruited from patients diagnosed in the Bernstein Allergy Group, and the Clinical Research Center in the Division of Allergy and Immunology at the University of Cincinnati. The study was approved by the University of Cincinnati Institutional Review Board. Patients taking any oral topical skin medication were excluded from the study. Exclusion criteria include: (a) having had an acute viral infection within at least 1 mo before the study; (b) being a smoker or ex-smoker who has had ≥10 pack per year smoking history; (c) having any unstable chronic disease (other than asthma), or (d) being pregnant. Inclusion criteria were as follows: (a) age 18–65 yr; (b) history of allergic asthma lasting for 1 yr or longer based on previous diagnosis in our clinics; (c) positive skin prick tests (wheat diameter ≥5 mm) to one or more of the following allergens: timothy grass pollen, ragweed, cockroach, mold, house dust mite, or cat dander in the presence of positive histamine and negative vehicle control; (d) receiving inhaled corticosteroids with or without other medications for asthma, including β2-agonists, leukotriene modifying agents or sustained release theophylline for at least 2 mo. All asthmatic subjects must have a ≥21% improvement in forced expiratory volume in the first second of exhalation (FEV1) or a fall of ≥20% or more in response to a provocative methacholine dose ≤1 mg/ml, confirming airway hyperresponsiveness. All subjects underwent a thorough history (including an asthma control test), physical examination, weight to determine body mass index, and allergy skin prick testing. Atopy is defined by one or more positive skin prick tests to at least one common inhalant allergen.

Statistical analysis. Data were analyzed using GraphPad Prism 5 software. Data are presented as mean value ± SD and analyzed using Student’s t test (n = 2 groups). P values of <0.05 were considered significant.

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