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

Shinya Tanaka,* Takayuki Yoshimoto,† Tetsuji Naka,§ Susumu Nakae,§ Yo-ichi Iwakura,§ Daniel Cua,¶ and Masato Kubo#*

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.

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eginning 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 autoimmune 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) (5) 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 RORγt (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/a-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).

References

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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; iTTh17, inducible Th17; WT, wild type; SP, single positive; DO, DO11.10 transgenic mice.

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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 CD44high 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, but not IL-17F, and some cells coexpress IFN-γ (29–31). Neutrophil accumulation in the respiratory tract with airway narrowing caused by fibrosis (28).

### 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). Stubs−/− 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 flox mice (40). D011.10 Tg mice were provided by Dr. K. Murphy (Washington University, MO) (41), and RorcGFP mice were originally generated by Dr. D. Littman (New York University, NY) (11) and provided by Dr. T. Taniguchi (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 XMGL1.2 biotin), anti-IL-2 (JES6–1A12 and JES6–5H4 biotin), and anti-IL-4 (BVD4–1D11 and BV6D4–24G2 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 (TC11–9H9) were purchased from Bio-Rad. 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+ CD4+ T cells, and CD4+ T cells were prepared by cell sorting with an FACSVantage instrument using Cell Quest software (BD Biosciences). IL-17A and CD4+ Th cells were isolated from D011.10 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-11 (XMG1.2), and anti-IFN-γ (XMGL1.2). For transfer experiments, 1 x 106 cells were i.v. transferred into D011.10 Tg Rag−− mice or Rag−− mice. Spleen cells were prepared from OVA-challenged mice, and for measurement of cytokine production, 1 x 106 cells were was conducted by activation with 1 or 10 μM OVA peptide (Loh15)-loaded APCs (5 x 105 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, D011.10 Tg mice were administered 50 μg of OVA (grade V, Sigma-Aldrich, keto 24 h after the last antigen 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 D011.10 Tg Rag−− mice (D011.10 and DO Rag−−). D011.10 and DO Rag−− mice showed comparable T cell independent neutrophil accumulation following LPS stimulation (Fig. 1A). D011.10 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.

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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 IL-17 producing cells had a unique cytokine profile, producing IL-17A and IL-17F, but not IL-4. We next asked whether the nTh17 cells are the main source of IL-17 in primary responses to a primary OVA stimulation (Fig. 3A). 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/reactivated 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. 3A). 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. 3B).

Using GFP reporter Tg mice (d3' Tg) in which the activity of the conserved noncoding sequence-2 enhancer in the H4 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. 3C, right), 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-CD44high subset and the majority of IL-17 producing cells produced IFN-γ simultaneously (Fig. 3C).

GFP+ NK T cells produced relatively low levels of IL-17 after cognate recognition of α-GalCer-loaded CD1d (Fig. 3C, right, and Fig. 3D). GFP+ CD44high 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. 4A). This cytokine profile was also confirmed by ELISA (Fig. 4B). 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...
expression was examined. IL-17 expression was only found in KJ1^+ memory type nTh17 cells, which secreted IL-17 after peptide stimulation (Fig. 4C). 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. 4D).

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. 5A). 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. 5C). 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 iTh17 cells 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, CD4^+ cd44^+^ and cd62l^low, without prepriming, are unclear. Therefore, we examined whether nTh17 cells are generated in the absence of prepriming using DOI11.10 vs DO Rag^-/- mice and OT-2 vs OT-2 Rag^-/- mice. The CD4^+ cd44^+ nTh17 subset was found among CD4 single positive (SP) cells in both DOI11.10 and OT-2 thymus. The CD4^+ CD4 SP cells in DOI11.10 mice or Var2^+ 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. 5C). 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 ROBs in IL-17 production by nTh17 cells, because nTh17 cells could be generated in conditions where Th17 cells could not. The number of nTh17 cells from Rorγt^ Knock-in (Rorγt^Off) mice was approximately half that of WT B6 mice. Moreover, one-third of IL-17 expressing memory T cells coexpressed GFP in Rorγt^Off mice (Fig. 5F). These results indicate that RORγt expression is essential for the production of...
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. 1B). Reconstitution of nTh17 completely restored the accumulation of neutrophils at levels equivalent to that of DO11.10 cell injections (Fig. 6A), indicating that nTh17 cells are dispensable 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. 6B). 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. 6C). 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 CD44high 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-1+ CD44high CD4+ T cells in the spleen and thymus (Figs. 4C and 5C), 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<sup>hi</sup> nTh17 cells were freshly isolated from Stat1<sup>−/−</sup>, Stat6<sup>−/−</sup>, CD4<sup>cre</sup> cre stat3<sup>fl/fl</sup> mice (<--/--), and C57BL/6 (B6) control mice (WT, □), and cells (1 x 10<sup>6</sup>) 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 naïve T cells and nTh17 cells were isolated from control CD4<sup>+</sup> from Ror<sup>g</sup> and anti-CD28 stimulation for 48 h, IL-17 and IFN-γ (46). Also found in a colitis model induced by a STAT3 mediated signaling pathway, and disruption of the signaling pathways (11, 13, 14). Our data indicate that nTh17 cells produce both IL-17 and IFN-γ (for DO) or Va2<sup>+</sup> (for OT-2) gated CD4<sup>+</sup> T cells were assessed by ICS (right). ICS data are representative of three independent experiments. D. Magnetic sorted thymic CD4<sup>+</sup> SP cells were stained with CD44 and NK1.1 (top left). CD44<sup>hi</sup> cells (1 x 10<sup>6</sup>) were sorted from CD4<sup>+</sup> SP thymocytes of CD4<sup>+</sup>cre Tg (WT) and CD4<sup>+</sup>cre stat3<sup>fl/fl</sup> mice (Stat3<sup>−/−</sup>). Following anti-TCR and anti-CD28 stimulation for 48 h, IL-17A and IFN-γ in the KJ-1<sup>+</sup> (for DO) or Va2<sup>+</sup> (for OT-2) gated CD4<sup>+</sup> T cells were assessed by ICS (right). ICS data are representative of three independent experiments. E. CD44<sup>hi</sup> cells (1 x 10<sup>6</sup>) cells were sorted from CD4<sup>+</sup> SP thymocytes of OT-2 Tg and OT-2 I<sup>h<sub>2</sub></sup>−/− mice. Following anti-TCR and anti-CD28 stimulation for 48 h, IL-17 and IFN-γ were detected by ICS (lower). F. naïve CD4<sup>+</sup> T cells and CD44<sup>hi</sup> memory cells were freshly isolated from Rorγt<sup>−/−</sup> or B6 mice (WT). After stimulation with anti-TCR and anti-CD28, IL-17 and GFP expression was assessed by FACS analysis.

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 Ifng 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<sup>−/−</sup> 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<sup>+</sup> SP population were intact even in a Stat3<sup>−/−</sup> 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. 1B). 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...
were intranasally challenged with OVA (50 μg/mouse) twice. The 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. Statistical significance was determined using Student’s t-test. *p < 0.05; **p < 0.01. C, memory DO Rag−/− mice, memory DO Rag−/−, and iTH17 DO Rag−/− 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.

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. 1C). 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. 6B). 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-infected 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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References


