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TLR-Induced Cytokines Promote Effective Proinflammatory Natural Th17 Cell Responses

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Naive CD4 lymphocytes undergo a polarization process in the periphery to become induced Th17 (iTTh17) cells. Using retinoic acid–related orphan receptor γt (RORγt)-gfp mice, we found that RORγt and the transcription factor promyelocytic leukemia zinc finger (PLZF) are valuable new markers to identify the recently described natural Th17 (nTh17) cell population. nTh17 cells are thymically committed to promptly produce large amounts of IL-17 and IL-22. In this study, we show that, in addition to responding to TCR cross-linking, nTh17 cells secrete IL-17 and IL-22 when stimulated with IL-23 plus IL-1β, either in recombinant form or in supernatants from TLR4-activated dendritic cells. This innate-like ability of RORγt+ nTh17 cells to respond to TLR4-induced cytokines was not shared by iTTh17 cells. The other distinct properties of RORγt+ nTh17 cells are their high expression of PLZF and their absence from lamina propria; iTTh17 cells are found therein. RORγt+ nTh17 cells are present in the thymus of germ-free RORγt-gfp and IL-6−/− RORγt-gfp mice, indicating that these cells do not require symbiotic microbiota or IL-6 for their generation. Finally, we found that PLZF+ RORγt+ nTh17 cells represent one of the primary IL-17–producing innate-like T cell populations in a TLR7 imiquimod model of psoriasis-like disorder, indicating their involvement in this kind of lesion. Collectively, our results reveal RORγt and PLZF as characteristic markers for identifying nTh17 cells and demonstrate one of their novel properties: the ability to respond promptly to TLR-dependent proinflammatory stimuli without TCR engagement, placing them as members of the innate-like T cell family. The Journal of Immunology, 2014, 192: 5635–5642.

Several types of functionally distinct T cell subsets develop in the thymus. They are composed primarily of conventional naive CD4 and CD8 T cells, with a highly diverse TCR repertoire recognizing peptides in the context of classical MHC class II or class I molecules, respectively (1). In peripheral lymphoid organs, naive CD4+ T lymphocytes differentiate into effector cells upon stimulation, thus enabling them to produce cytokines, such as IFN-γ, IL-4, or IL-17A (commonly referred to as IL-17) (2). The process of Th1, Th2, or Th17 differentiation requires several days and the presence of Ags, specific cytokines, and transcription factors, which render mature cells responsive to a second Ag stimulation. In contrast, some T cells leave the thymus fully differentiated and competent for cytokine secretion without further polarization. The typical representatives of these “nonconventional” T cells are invariant NKT (iNKT) and γδ T cells (3–5). The IL-17–producing iNKT (iNKT17) cell subset originates from a new thymic-differentiation pathway that is dependent on the transcription factor retinoic acid–related orphan receptor γt (RORγt) (3, 4, 6). Similar to iNKT17 cells, CD27− γδ T cells acquire the ability to secrete IL-17 during their thymic development and are distinct from their CD27+ counterparts that produce IFN-γ (3, 5, 7). More recently, a novel IL-17+ CD4+ T cell population was identified that, similar to iNKT17 and CD27− γδ T cells, acquires the ability to secrete IL-17 during its thymic development (8, 9). These thymic-selected cells are known as natural Th17 (nTh17) cells (8–10).

Natural and induced Th17 (iTTh17) cell subsets are distinctly regulated by Akt and mTOR pathways (10, 11), but they share similar functional properties because they both can produce IL-17 and IL-22 following TCR cross-linking. It is generally acknowledged that IL-17 and IL-22 can act in concert to resist bacterial infections, even though they exert potentially distinct roles in autoimmune and allergic responses (12). IL-17 is a key cytokine for psoriasis treatment, whereas IL-22 is important for epithelial cell homeostasis, intestinal defense, tissue repair, and wound healing (12–15). Thus, the characterization of T cell populations that can produce IL-17 alone or in combination with IL-22 is of crucial importance.

In this study, we used Rorc(γt)−/−Gfp TG mice to phenotypically identify thymic nTh17 cells and demonstrated that they can secrete IL-17 and IL-22 promptly in response to TLR-induced cytokines (i.e., IL-1β and IL-23) in the absence of TCR stimuli. In addition, nTh17 cells express high levels of the transcriptional repressor promyelocytic leukemia zinc finger (PLZF), a marker
that is highly expressed by innate-like T cells, such as iNKT cells (16). These functional and phenotypic characteristics also were ascribed to peripheral nTh17 cells. Our findings provide new tools to functionally and phenotypically distinguish nTh17 cells from Tfh17 cells in the periphery, as well as to better understand the contribution of nTh17 cells to inflammatory responses.

Materials and Methods

Animals
Six- to eight-week-old bacterial artificial chromosome–transgenic Rorc(γt)-Gfp TG, IL-6–/–Rorc(γt)-Gfp TG (17), and C57BL/6J male mice were bred in our facility (Hôpital Necker Enfants Malades) or purchased from Janvier (Les Genest St. Isle, France). Germ-free (GF) Rorc(γt)-Gfp TG mice were obtained from a specific pathogen–free (SPF) colony by aseptic cesarean section, followed by the adoption of the cesarean-derived pups by GF foster mothers. Animal experiments were performed according to the guidelines of the French Institutional Committee.

FACS analysis
Mononuclear cells (MNCs) were stained with CD1d–PBS57-tetramer–allophycocyanin or CD1d–PBS57-tetramer–PE (kindly provided by the National Institutes of Health Tetramer Core Facility), TCRγδ–PE, TCRβ–allophycocyanin or TCRβ–fluor 450, anti-CD4–Pacific Blue or anti-CD4–GFP, anti-CD69–PE or anti-CD69–GFP, anti-CD3–PECy7, anti-CD28–fluor 450, and/or anti-CD24–PECy7 (BD Pharmingen, ebioscience, or R&D Systems). For intracellular staining, cells were fixed and permeabilized as described (4) and then incubated with anti-IL-17–PerCP-Cy5.5 or allophycocyanin and anti-CD12–PE or PerCP–fluor 710 or isotype controls (ebioscience or R&D Systems) and anti-GFP–Alexa Fluor 488 mAb (Invitrogen) and/or anti-PLZF–allophycocyanin (R&D Systems). Cells were then washed and analyzed in a FACS Canto II or Fortessa (Becton Dickinson) using FlowJo software.

Enrichment and cell sorting
CD44–CD4+ T cells were enriched by depleting CD8+ and CD19+. CD26+ cells labeled with the corresponding mAbs (BD Pharmingen) and with anti-rat Ig-coated magnetic beads (Bio–Adebluebs; Admetech). Depleted populations were stained with CD1d–PBS57-tetramer–allophycocyanin, anti-CD4–allophycocyanin–Cy7, anti-CD8–Pacific Blue, anti-CD4–PE–Cy7, and anti–TCRγδ–PE, and distinct RORγt+ and RORγt– cell subsets were sorted using a FACS Aria cell sorter (Becton Dickinson). In others experiments, naive CD26+CD4+ T cells were electronically sorted before their differentiation into Th17 cells.

Cell culture and stimulation
Electronically sorted CD4+CD44+ CD1d–PBS57-tetramer–TCRγδ+ cells were exposed at a concentration of 105 cells/ml to coated anti-CD3 and anti-CD28 mAbs (BD Pharmingen) and with anti-rat Ig-coated magnetic beads (Bio–Adebluebs; Admetech). Depleted populations were stained with CD1d–PBS57-tetramer–allophycocyanin, anti-CD4–allophycocyanin–Cy7, anti-CD8–Pacific Blue, anti-CD4–PE–Cy7, and anti–TCRγδ–PE, and distinct RORγt+ and RORγt– cell subsets were sorted using a FACS Aria cell sorter (Becton Dickinson). In others experiments, naive CD26+CD4+ T cells were electronically sorted before their differentiation into Th17 cells.

Results

Isolation of lamina propria lymphocytes
After cleaning, cutting open lengthwise, and rinsing with PBS, the small intestine was cut into 2-in segments. Epithelial cells were removed by sequential shaking in EDTA-containing PBS solutions. The remaining tissue was digested at 37°C in T cell media containing 1 mg/ml collagenase IV (Life Technologies) and 1 U/ml DNase I (Sigma–Aldrich), and lamina propria (LP) lymphocytes were isolated by a 40/80% Percoll gradient. Isolated cells were incubated for 16 h with supernatants from BMDCs.

Th17 differentiation and stimulation
Electronically sorted naïve CD4+CD26-CD44hiCD45rom T cells were cultured with coated anti-CD3 and anti-CD28 mAbs in Th17 conditions (TGF-β [2 ng/ml], TNF-α [10 ng/ml], IL-1β [10 ng/ml], and IL-6 [20 ng/ml]; all from R&D Systems). Differenitated Th17 cells were rested overnight and further stimulated with anti-CD3 plus anti-CD28 or IL-23 plus IL-1β. Cytokine production was measured in the supernatants.

In vivo TLR stimulation
Mice were injected i.v. with 25 μg LPS or control medium. After 3 h, peripheral lymph node cells were stimulated with PMA plus ionomycin (PI) and stained for surface markers and intracellular cytokine detection. In other experiments, a daily dose of 50 mg imiquimod (5% IMQ cream; Meda AB) or control cream (Vaseline) was applied for 4 d to shaved backs of mice, as described (7).

Adoptive transfer of CD44hi CD4+ T cells
Sorted naïve CD44hiCD4+ splenocytes wild-type (WT) Ly5.2 donors were injected into Rag2–/–Ly5.1 recipients. After 10 d, thymus, spleen, and lymph node cells were incubated for 16 h with supernatants from BMDCs, as previously described, or anti-CD3 plus anti-CD28. Transferred cells were distinguished using anti-Ly5.1–PE (ebioscience), and IL-22 and IL-17 production was assessed by intracellular staining and flow cytometry.

In another set of experiments, sorted TCRβ+CD4+CD26-CD44low RORγt+ splenic T cells from Rorc(γt)-Gfp TG Ly5.2 donors were injected into Rag2–/–Ly5.1 recipients. Four days later, mice were sensitized by an i.p. injection of 100 μg OVA (grade V, Sigma) emulsified in 0.64 mg alun (Merck). Mice were challenged with 50 mg/ml OVA upon aerosol exposure on three consecutive days (8–10). Twenty-four hours after the last challenge, mice were sacrificed, and samples were collected for further analysis.

Statistical analysis
The nonparametric Mann–Whitney U test was used to calculate the significance levels for all measurements. The p values < 0.05 were considered statistically significant.

Results

RORγt expression and IL-17 production by mature CD4+ thymocytes
RORγt, the typical transcription factor associated with IL-17 production, is a particularly useful marker to distinguish IL-17–producing iNKT17 cells from IL-4–producing iNKT cells (4, 18). This raised the question of whether RORγt could also identify the recently described thymic natural CD4+ Th17 cells. To explore this issue, we used Rorc(γt)-Gfp TG mice and excluded immature Rorc(γt)-CD4+CD8+ thymocytes routinely by analyzing only the cells that had already rearranged their TCR β chain presented on the cell surface and express CD4, but not CD8, together with high levels of the CD44 marker. Knowing that iNKT cells are likely to be present among this TCRβ+CD4+CD8-CD44hi population, we systematically excluded CD1d–PBS57-tetramer+ cells from our analysis. In addition, we eliminated TCRβ+ T cells using anti-TCR pan γδ mAb. We found that up to 0.2% of mature TCRβ+CD4+CD8– CD1d–PBS57-tetramer+ thymocytes expressed RORγt (Fig. 1A). These results fit with the percentage of thymic nTh17 cells described previously (8, 9). Thymic nTh17 cells are CD24– and express CD69 at similar high levels as do recently selected TCRβ+ thymocytes (Supplemental Fig. 1).
the thymus were electronically sorted into CD1d−PBS57-tetramer 2 RORα+CD4+CD8+CD44high thymocytes from Rorc(−/−)-GfpTG mice. Data are representative of five to seven independent experiments. Percentages of each subset are indicated in the quadrants. (D) Intracellular IL-17 and IL-22 staining was performed after in vitro stimulation of freshly isolated thymocytes with PI or PI plus IL-23 for 4 h. Representative FACS profiles of IL-17 and IL-22 production by gated CD4+RORα+ or RORα− thymic T cells. These sorted RORα+ and RORα− T cells were stimulated with coated anti-CD3 plus anti-CD28 mAb for 72 h. IL-17 was measured in the supernatants. Data are mean ± SEM from three to eight individual experiments, pooling three thymuses/experiment. (E) Sorted CD1d−PBS57-tetramer + TCRβ+CD8−CD4−/CD4+CD44low RORα+ and RORα− thymic T cells from C57Bl/6 mice were stimulated with coated anti-CD3 plus anti-CD28 mAb, with or without IL-23, for 72 h. IL-17 was measured in the supernatants. No IL-22 was detected in unstimulated control supernatants (<10 pg/ml). Data are mean ± SEM from three or four individual experiments, pooling three thymuses/experiment. (F) Sorted CD1d−PBS57-tetramer + TCRβ+CD8−CD4−/CD4+CD44high RORα+ thymocytes produced IL-17 when stimulated with coated anti-CD3 plus anti-CD28 mAb (Fig. 1B). Conversely, IL-17 was not detected in any supernatant from matching RORα− fractions (Fig. 1B). Henceforth, these naturally occurring IL-17–producing thymocytes are referred to as RORα+ thymocytes. Our results show that RORα+ cells represent a useful phenotypic marker to identify nTh17 cells in the thymus.

**IL-22 production by RORα+ nTh17 thymocytes requires IL-23**

Sorted ex vivo RORα+ nTh17 thymocytes stimulated with coated anti-CD3 plus anti-CD28 mAbs do not produce any detectable IL-22, but the addition of IL-23 effectively induced the secretion of this cytokine (Fig. 1C). The most striking finding is that RORα+ nTh17 cells generated IL-22 within only 4 h when IL-23 was provided with PI stimulation (Fig. 1D), clearly showing that thymic RORα+ nTh17 cells are primed to secrete IL-22. IL-23 also enhanced the frequency of IL-17+ cells among RORα+ nTh17 cells (Fig. 1D). These findings reveal that thymic nTh17 cells can produce high levels of IL-22 and that the majority of IL-22+ cells coproduce IL-17.

**Innate-like stimulation of thymic RORα+ nTh17 cells**

Thymic differentiated innate-like T cells, such as iNKT17 and γδ T cells, can secrete IL-17 and IL-22 in response to IL-1β plus IL-23 (19, 20). Fig. 2A shows that this innate-like property also applies to RORα+ nTh17 thymocytes because they promptly produced IL-17 and IL-22 as early as 4 h after stimulation with IL-23 plus IL-1β.

We addressed the physiological relevance of our results by examining whether endogenous IL-1β and IL-23 could also target RORα+ nTh17 thymocytes to promote their production of IL-17 and IL-22. Previous reports established that dendritic cells (DCs) secrete IL-1β and IL-23 in response to various pathogen-associated molecular patterns, including TLR agonists (21, 22). Taking advantage of this finding, we stimulated BMDCs with LPS, a TLR4 agonist, and evaluated how the supernatants generated affected IL-17 and IL-22 production by thymic RORα+ nTh17 cells. As shown in Fig. 2B, the response was quite similar to that induced by exogenous cytokines. Supernatants from DCs activated via the TLR4 pathway lost their ability to induce cytokine production by RORα+ nTh17 cells in the presence of neutralizing anti–IL-23 plus anti–IL-1β mAbs (Fig. 2C), proving the specific involvement of these two cytokines.

**Peripheral RORα+ nTh17 T cells produce both IL-17 and IL-22 following IL-1β and IL-23 stimulation**

Having characterized thymic RORα+ nTh17 cells, we sought to better determine their properties in the periphery. As shown in Supplemental Fig. 2A, CD4+TCRβ− T cells expressing similar high levels of CD44 and RORα+ as thymic RORα+ nTh17 lymphocytes also are found in peripheral organs (i.e., spleen or lymph nodes) in the steady-state. RORα+ nTh17 cells express low levels of PD-1 and high levels of ICOS, CCR6, and IL-23R (Supplemental Fig. 2B), as previously ascribed to nTh17 lymphocytes (9). Similarly, we observed no biased Vβ3 expression (Supplemental Fig. 2C) in the periphery (9).

Further, we asked whether the capacity of RORα+ nTh17 thymocytes to secrete IL-17 and IL-22 in response to IL-1β plus IL-23 was a particular feature of these cells or whether it was shared by peripheral RORα+ nTh17 lymphocytes. Lymph node RORα+CD44high T cells respond strongly to supernatants from DCs stimulated with a TLR4 agonist by producing IL-17 and IL-22 (Fig. 2D). The latter effect is once again mediated specifically through IL-1β and IL-23 because it was blocked by their respective Abs (Fig. 2E).

Additionally, a TLR4 agonist was injected into mice; this brief in vivo TLR stimulation increased IL-17 production and induced IL-22 production by nTh17 cells in response to both PI or IL-1β.
plus IL-23 stimulation (Fig. 2F, 2G), indicating that in vivo TLR4 stimulation favors the effector activation of these cells.

**RORγt+ nTh17 T cells are functionally distinct from iTh17 cells**

It might be argued that this innate-like ability to secrete IL-17 and IL-22 upon IL-1β plus IL-23 stimulation is inherent to all types of Th17 cells. However, this is not the case, because Th17 cells differentiated in vitro from naive CD44^CD62L^T cells and stimulated with IL-1β plus IL-23 generated no detectable IL-17 (Fig. 3A). They are able to secrete IL-17 following anti-CD3 plus anti-CD28 stimulation, showing their functionality (Fig. 3A). These results are in contrast to previous reports showing that, under some conditions, Th17 cells differentiated in vitro were able to produce IL-17 in response to IL-1β plus IL-23 stimulation (23). A likely explanation for this discrepancy are the different in vitro protocols used. To overcome technical in vitro issues and to be as similar as possible to in vivo situations, we tested the ability of naive CD44^RORγt^ T cells to become RORγt^ in vivo and to produce IL-17 and IL-22 in response to IL-1β plus IL-23 stimulation. To address this issue, we first set up experiments using adoptive transfer of sorted naive CD44^CD62L^Ly5.1^ T cells to Rag^-/- Ly5.1^ mice. Ten days later, CD44^Ly5.1^ cells that expressed CD44 were observed in recipient mice (Supplemental Fig. 3A). Under these conditions, a small fraction of transferred cells became IL-17 producers in response to anti-CD3 plus anti-CD28 but not in response to conditioned supernatants from DCs activated via the TLR4 pathway containing IL-1β plus IL-23 (Supplemental Fig. 3B, 3C).

Additionally, we used a protocol capable of inducing in vivo Th17-immune responses. Naive CD44^CD64^CD62L^RORγt^ Ly5.1^ T cells were adoptively transferred to Rag^-/- Ly5.1^ mice; 3–4 d later recipient animals were immunized and later challenged with OVA. This protocol is used to induce allergic asthma disease, resulting in both Th2 and Th17 cell differentiation. Adoptively transferred CD44^ T cells were found in the lung (Fig. 3B). A fraction of these lymphocytes acquired the expression of RORγt (Fig. 3C) and promptly secreted IL-17 following PI stimulation (Fig. 3C). Nevertheless, these cells were unable to secrete IL-17 in response to IL-1β plus IL-23 stimulation (Fig. 3C) or to express PLZF (Fig. 3D), a characteristic marker of nTh17 cells (see later...
In vitro differentiated iTh17 cells are differentiated into Th17 cells, as described in Materials and Methods. Representative FACS profile of IL-17 and IL-22 production by TCR β+ CD4+Ly5.1+ RORγt+ iTh17 cells obtained (5 × 10^5 cells/ml) were stimulated with anti-CD3 plus anti-CD28 (positive control), IL-1β plus IL-23, or medium (negative control) for 72 h. IL-17 was measured in the supernatants. Data represent mean ± SEM from four independent experiments, pooling three mice/experiment.

Lung MNC from OVA-treated mice

At this stage, peripheral iTh17 and RORγt+ nTh17 cells could be distinguished functionally but not phenotypically because they share the expression of CD4, CD44, and RORγt molecules. We tested some markers, but the transcriptional repressor PLZF seemed to be a promising candidate. In fact, we found that the majority of thymic and lymph node RORγt+ nTh17 cells expressed high levels of PLZF (Fig. 5A), similarly to γδ T cells (data not shown). In sharp contrast, RORγt+ iTh17 cells from LP failed to express this marker (Fig. 5A), suggesting PLZF as a novel marker for nTh17 cells. To support these findings, the innate-like stimulation of nTh17 cells with TLR4 agonist–conditioned DC supernatants revealed that IL-17+ nTh17 cells expressed high levels of PLZF (Fig. 5B). Under the same stimulatory condition, iTh17 cells from LP were PLZF− and did not produce detectable levels of IL-17 (Fig. 5B).

These findings indicate that functional (IL-17 production) and phenotypic properties (RORγt and PLZF expression) are associated in nTh17 cells. In addition, these results reveal that high PLZF expression is a novel phenotypic marker to better identify RORγt+ nTh17 cells in the periphery.

PLZF is expressed by RORγt+ nTh17 cells

At this stage, peripheral iTh17 and RORγt+ nTh17 cells failed to respond to IL-1β plus IL-23 stimulation (right panel). (D) Expression of PLZF (black line) on TCRβ+CD4+Ly5.1+ RORγt+ lung MNCs freshly isolated from OVA-treated mice. Shaded graph represents isotype control. Data are representative of two independent experiments in which three to five individual mice were analyzed. (E) FACS analysis of LP MNCs isolated from Rorcγt-GfpTG mice and stimulated with anti-CD3 and anti-CD28 mAb (upper panels) or supernatants from BMDCs stimulated with TLR4 agonist (lower panels). Plots were gated first on CD1d–PBS57-tetramer TCRβ+CD4+CD8+ cells and then on CD44+RORγt+ or RORγt− cells. Data are representative of three or four independent experiments, pooling six organs/experiment.
Enhanced numbers of PLZF⁺ RORγ⁺ nTh17 cells in acute psoriasiform lesions

In light of the above results, we examined the possible contribution of PLZF⁺ RORγ⁺ nTh17 cells in a mouse model of psoriasis-like disorder (27–29). For this, mice were treated epicutaneously with the TLR7 agonist IMQ; the development of acute psoriasiform lesions is largely attributable to the expansion of IL-17-producing cells (7, 30–32). The number of thymic nTh17 cells (gated on CD1d-PBS57-tetramer +CRγ6⁺CRoβ⁺CD4⁺CD8⁻CD44⁺RORγ⁺) or CD1d-PBS57-tetramer +CRγ6⁺CRoβ⁺CD4⁺CD8⁻CD44⁺RORγ⁺ cells) from these distinct mouse strains. Data represent mean ± SEM of three to six individual mice. Representative FACS profile of IL-17 and IL-22 production by gated CD1d–PBS57-tetramer +CRγ6⁺CRoβ⁺CD4⁺CD8⁻CD44⁺RORγ⁺ cells. Data are representative of three or four independent experiments. Percentages of each subset are indicated in quadrants.

Discussion

Our findings provide strong evidences that nTh17 cells respond promptly to TLR-derived proinflammatory cytokines (i.e., IL-1β and IL-23) in the absence of TCR stimulation. Moreover, we demonstrated that RORγt associated with PLZF are characteristic markers for identifying nTh17 cells in the thymus and periphery.

nTh17 cells can promptly produce IL-17, but they require the additional stimulus provided by IL-23 to enable them to secrete IL-22. Several recent papers suggested that, although IL-17 and IL-22 cooperate, they also can mediate divergent effects. IL-17 is primarily considered to be a promoter of inflammatory responses favoring granulopoiesis and neutrophil recruitment through stimulation of IL-1β and G-CSF release (30, 31). IL-22 is primarily involved in antimicrobial immunity via induction of antimicrobial molecules, and it contributes to tissue repair by stimulating epithelial cell and keratinocyte proliferation and survival (12, 32, 33).

nTh17 cells can produce IL-17, associated or not with IL-22, following TLR4-derived cytokine stimulation. Other TLR ligands, such as zymosan or flagellin, TLR2 and TLR5 agonists, respectively, can induce IL-23 production by dendritic cells or macrophages and potentially affect nTh17 cells (34, 35). In this article, we also reported that in vivo administration of IMQ, a TLR7 agonist, enhances the number and IL-17–producing capacity of nTh17 cells. These cells represent one of the major IL-17–producing T cell subsets present in draining lymph nodes of IMQ-treated mice. Together, our findings clearly show the acti-
from iTh17 cells, and we observed that RORαb in gated CD1d–PBS57-tetramer T cells (8) is that we analyzed Th17 cells in LP in the steady-state. Earlier studies indicated that IL-6 promoted thymic Th17 differentiation in AND × PCC double-transgenic mice (B10.BR background). We found no major contribution of IL-6 or microbiota to the development of nTh17 cells. Possible explanations for this discrepancy are the distinct mouse background (B10.BR versus C57BL/6) and experimental protocols used.

nTh17 and iNKT17 cells acquire their ability to secrete IL-17 and IL-22 in the thymus. These particular TCRαβ T cell populations are functionally very similar, but they are substantially distinct with regard to their thymic development and selection. Indeed, iNKT cells are positively selected by CD1d-expressing CD4+CD8+ thymocytes and recognize glycolipids (36, 37), whereas nTh17 cells are dependent on MHC class II expression on medullary thymic epithelium for their selection and are stimulated by peptides (9). Consequently, iNKT and nTh17 cells have complementary roles based on their ability to promptly produce cytokines in response to distinct Ags. Of note, iNKT17 cells (20), such as nTh17 cells, are capable of producing IL-17 and IL-22 upon indirect TLR activation. In addition to this shared functional property, iNKT17 and nTh17 cells express RORαb, which, to our knowledge, is the most specific marker to distinguish iNKT17 cells from other iNKT cell subsets. Another typical marker of iNKT cells at large is PLZF, a member of the Broad complex, Tramtrack, Bric-à-brac, and zinc finger family that controls a wide range of biological processes (38). PLZF defines not merely the phenotypic properties of iNKT cells, it directs their effector program and is implicated in their proapoptotic propensity (16, 39, 40). Transgenic expression of PLZF during T cell development induced a T cell–intrinsic program leading to an increase in peripheral CD44high memory/effector T cell populations in mice (41, 42). These transgenic T cells could produce large amounts of several cytokines upon primary activation without further peripheral polarization, which suggests that PLZF directly turns on the effector program in thymocytes that otherwise would have been selected as naive T cells. We found that nTh17 cells express PLZF, supporting the notion that they constitute a distinct T cell population with innate-like properties and memory/effector phenotype. We showed that cytokine-producing nTh17 cells express high levels of PLZF, which indicates that functional and phenotypic properties are somehow connected and that PLZF has rheostat functions in the generation of nTh17 cells. Taken together, nTh17 and iNKT17 cells require distinct signals for their thymic selection but likely common pathways for acquisition of their ability to promptly produce IL-17 and IL-22. Further studies are required to clarify this point.

In sum, we identified RORαb and PLZF as novel markers to discriminate nTh17 cells. Our findings also reveal a new feature of nTh17 cells: their ability to respond promptly to proinflammatory factors following TLR stimulation and independently to TCR stimuli. Additionally, nTh17 cells are significantly enhanced and constitute a major IL-17–producing T cell population in an IMQ psoriasis-like model, indicating their contribution to this type of lesion. In conclusion, our findings shed new light on nTh17 cell biology and open new possibilities for the use of RORαb and PLZF as markers to better understand this particular T cell population.

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Disclosures
The authors have no financial conflicts of interest.
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tiation of IL-17-producing T helper cells in the mucosa of the small intestine. Cell Host Microbe 4: 337–349.


Supplemental Figure 1. Thymic nTh17 cells are mature cells. Histograms show the expression CD24 (left panel) or CD69 (right panel) on gated nTh17 (CD1d-PBS57 tetramer<sub>neg</sub> TCR<sub>γδ</sub>neg TCRαβ<sub>neg</sub>CD4<sub>pos</sub>CD8<sub>neg</sub>CD44<sub>pos</sub>RORγ<sub>pos</sub>) (solid black line) and gated mature CD4<sub>pos</sub> (CD1d-PBS57 tetramer<sub>neg</sub> TCR<sub>γδ</sub>neg TCRαβ<sub>pos</sub>CD4<sub>pos</sub>CD8<sub>neg</sub>) (solid gray histogram) T cells. Data are representative of seven individually analyzed mice.
Supplemental Figure 2. Phenotype of peripheral nTh17 cells. (A) Frequency of RORγt+ cells among gated CD1d-PBS57 tetramer neg TCRγδ neg TCRαβ pos CD4 pos CD8 neg CD44 high T cells recovered from lymph nodes (upper panel) or spleen (bottom panel) from Rorc(γt)-Gfp TG mice. Data are representative of five to seven independent experiments. Percentages of each subset are indicated in the gates. (B). Histograms show the expression of PD1, ICOS, CCR6 and IL-23R on gated nTh17 (CD1d-PBS57 tetramer neg TCRγδ neg TCRαβ pos CD4 pos CD8 neg CD44 high RORγt+) (solid black line) or the corresponding CD4 pos (CD1d-PBS57 tetramer neg TCRγδ neg TCRαβ pos CD4 pos CD8 neg CD44 high RORγt-) (dashed line) lymph nodes T cell subset. Isotype controls are represented as solid histograms. (C). Representative FACS analysis of Vβ3 expression by gated nTh17 (CD1d-PBS57 tetramer neg TCRγδ neg TCRαβ pos CD4 pos CD8 neg CD44 high RORγt+) (left panel) or the corresponding CD4 pos (CD1d-PBS57 tetramer neg TCRγδ neg TCRαβ pos CD4 pos CD8 neg CD44 high RORγt+) (right panel) lymph nodes T cell subset. Data are representative of six individually analyzed mice.
**Supplemental Figure 3.** iTh17 cells failed to respond to IL-1β plus IL-23 stimulation. Naïve electronically sorted CD62L<sup>pos</sup>CD4<sup>pos</sup>Ly5.1<sup>neg</sup>Ly5.1<sup>neg</sup>T cells from wild-type mice were adoptively transferred to Rag<sup>-/-</sup>Ly5.1<sup>pos</sup>mice as described in Material and Methods. Ten days later transferred cells were recovered and further stimulated in vitro for 4 h. (A to C) Representative FACS profile of IL-17 and IL-22 production (B and C) by transferred CD4<sup>pos</sup>Ly5.1<sup>neg</sup>T cells gated on CD44<sup>pos</sup> cells (A) in response to anti-CD3 and anti-CD28 mAb (B) or SN DC + TLR4 agonist (C). Data are representative of two independent experiments where 3-5 individual mice were analyzed.
Supplemental Figure 4: Peripheral RORγtPos nTh17 cells in germ-free. (A and B). The frequency of RORγtPosCD44Pos or RORγtNegCD44Pos cells is represented among gated CD1d-PBS57 tetramerNeg TCRγδNeg TCRαβPosCD4PosCD8Neg lymph nodes freshly isolated from Germ-Free (GF) Rorc(γt)-GfpTG or SPF Rorc(γt)-GfpTG (A) or IL-6+/+ Rorc(γt)-GfpTG and IL-6−/− Rorc(γt)-GfpTG (B) mice. (C). Lymph nodes cells from GF Rorc(γt)-GfpTG or SPF Rorc(γt)-GfpTG mice were further stimulated with SN DC stimulated with TLR4 agonist. Representative FACS profile of IL-17 and IL-22 production by gated CD1d-PBS57 tetramerNeg TCRγδNeg TCRαβPosCD4PosCD8NegCD44PosRORγtPos or CD1d-PBS57 tetramerNeg TCRγδNeg TCRαβPosCD4PosCD8NegCD44PosRORγtNeg cells. Data are representative of 3-4 independent experiments. Percentages of each subset are indicated in quadrants.