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Interleukin 7 (IL-7) selectively promotes mouse and human IL-17–producing γδ cells

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IL-17–producing CD27−γδ cells (γδ27−cells) are widely viewed as innate immune cells that make critical contributions to host protection and autoimmunity. However, factors that promote their over IFN-γ–producing γδ27+ cells are poorly elucidated. Moreover, although human IL-17–producing γδ cells are commonly implicated in inflammation, such cells themselves have proved difficult to isolate and characterize. Here, murine γδ27−T cells and thymocytes are shown to be rapidly and substantially expanded by IL-7 in vitro and in vivo. This selectivity owes in substantial part to the capacity of IL-7 to activate STAT3 in such cells. Additionally, IL-7 promotes strong responses of IL-17–producing γδ cells to TCR agonists, thus reemphasizing the cells’ adaptive and innate potentials. Moreover, human IL-17–producing γδ cells are also substantially expanded by IL-7 plus TCR agonists. Hence, IL-7 has a conserved potential to preferentially regulate IL-17–producing γδ cells, with both biological and clinical implications.

lymphocytes | cytokines | proliferation

Studies of IL-17 intensiﬁed with the identiﬁcation of a speciﬁc subset of CD4+ Th17 cells that upon activation primarily produces IL-17 as opposed to IFN-γ (Th1 cells), IL-4 (Th2 cells), or IL-10/TGFβ (Treg cells) (1–3). Th17 differentiation is regulated by transcription factors RORγt and STAT3, the latter in part explaining the promotion of Th17 differentiation by IL-6 and IL-23 (3). Paradoxically, more detailed studies of Th17 immunity have identiﬁed γδ T cells and/or innate-like lymphoid cells as critical initial producers of IL-17 (4, 5). At steady-state, γδ cells are only a minor subset of T lymphocytes, but upon infection by Listeria, Mycobacteria, or Plasmodium, or upon LPS administration, they expand and make critical contributions to host protection (5–9). They likewise underpin immunopathology in widely used models of inﬂammatory disease (10, 11). In humans, IL-17 protects against mucocutaneous candidiasis and is again implicated in autoimmune inﬂammation, including psoriasis, multiple sclerosis, and rheumatoid arthritis (12, 13). Hence, there is considerable interest in identifying factors that regulate IL-17–producing γδ cells in mice and humans.

Adding to this interest is the emergence of murine γδ cells as prime examples of thymic preprogramming, whereby functional distinctions between CD27+IFN-γ producers (γδ27+ cells) and CD27−IFN-γ producers (γδ27− cells) are established by developmental cues that are largely unelucidated (8, 14). For example, γδ27− cells seem largely to arise from fetal thymocytes, requiring neither engagement of cognate ligand, nor RORγt or STAT3 that are both required for TCRγδ+ Th17 cell development (15). However, despite the dispensability of RORγt and STAT3 in development, most peripheral IL-17–producing γδ cells express RORγt and respond rapidly to IL-23 that signals via STAT3 (10). Such rapid responsiveness in the absence of TCR stimulation has led many to classify γδ27− cells as innate immune cells. Indeed, they generally respond poorly to concentrations of TCR agonists that would promote robust activation of γδ27+ cells (9). Nonetheless, assigning IL-17–producing γδ27− cells to innate immunity seems premature until more is known about what regulates the cells and how that might inﬂuence their response to TCR stimulation.

Although IL-17–producing γδ cells are likewise commonly evoked in human immune responses and immunopathologies, very little is known about these cells, because they have proved particularly hard to isolate and characterize (16). Thus, it seemed logical that by elucidating stimuli for murine γδ27− cells, one might identify the means to expand their human counterparts. This study identiﬁes IL-7 as a profound and selective activator of IL-17–producing γδ cells in mouse and in human neonates.

Results

IL-7 Enriches for Lymph Node γδ27− Cells. Lymph node (LN) γδ27+ cells appear like naive conventional T cells, being primarily CD62L+CD25−CD44hi ICOS+, whereas between 50% and 75% of γδ27− cells resemble activated T cells (CD62L−CD25−CD44hi ICOS+), although they are largely CD69+ (Fig. L4 and Fig. S1 H and I). As was reported (17), γδ27− cells also express higher levels of IL-7R than do γδ27+ cells (Fig. L4). To determine whether this phenotype had functional implications, LN cells were cultured with IL-7 for 4 d. Over eight independent experiments, γδ27− cells were strikingly enriched ~five- to sevenfold relative to γδ27+ cells and ~6- to 10-fold relative to total LN cells, whereas αβT-cell numbers declined (Fig. 1B and Fig. S1 B and C). Essentially all γδ27− cells were TCRβ CD44hi, and now ~70% expressed CD69 (Fig. 1B and C). IL-7 also increased the proportion of γδ27− cells expressing CD44 and CD69 (Fig. 1C), although their numbers declined ~70% over 4 d, whereas absolute numbers of γδ27− cells increased three- to fourfold (Fig. 1D). Strikingly, this enrichment was for cells with IL-17–producing capacity, whose representation increased from ~30% to ~70% of the γδ27− subset (Fig. 1E). Consistent with this increase, IL-7 enriched for cells expressing RORγt protein but not for those expressing T-bet, a primary regulator of IFN-γ (Fig. S1D).

To probe the generality of these observations, we investigated cells from the peritoneal cavity, known to harbor IL-17–producing γδ T cells (18, 19). Ex vivo almost all γδ cells were CD44hi (Fig. S1E), and they were enriched after 4 d in IL-7, compared with total cells (Fig. S1 F and G). However, whereas IL-7 maintained γδ27+ cell numbers in vitro relative to culture in medium alone, absolute numbers of γδ27− cells were again increased: ~ninefold relative to medium alone, and ~fourfold relative to numbers harvested ex vivo (Fig. S1G). Among these cells, the proportion of IL-17 producers was again increased (Fig. S1H). Thus, IL-7 preferentially enriches for IL-17–competent γδ T cells from two distinct anatomical sources.


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To examine whether the same effects would be achieved in vivo, mice were administered recombinant IL-7 three times over 5 d and then examined on day 7. There was a conspicuous enrichment of CD44\(^{hi}\) γδ\(^{−}\) cells, with absolute numbers of LN γδ cells competent to make IL-17 upon activation increasing >fivefold, compared with two- to threefold increases in IFN-γ-competent cells (Fig. 2A–C). Note that before and after IL-7 treatment, few γδ cells coproduced IL-17 and IFN-γ, consistent with developmental preprogramming (8).

To test whether IL-7 is required for the expansion of IL17-producing γδ cells in vivo, we examined mice treated epitactically with imiquimod (IMQ) in which the development of acute psoriiform lesions is largely attributable to expansion of IL17-producing γδ cells in the skin and skin-draining LNs (11, 20). Indeed, such lesions are comparable in WT and αβ T-cell-deficient mice but dramatically reduced in TCR\(^{γδ}\)− mice (11, 20). Administration of anti-IL-7R antibody almost completely blocked the enrichment (>10-fold) in IL17 γδ cells in the skin-draining LNs of mice administered IMQ versus baseline but did not significantly limit the two- to threefold expansion of IFN-γ+ γδ cells (Fig. 2D and E). Skin erythema scores, which compose a highly reproducible marker of IMQ-induced pathology, were significantly reduced in anti–IL-7R–treated animals (Fig. 2F), as was epidermal thickening that is associated with dermal IL-17–producing γδ cell expansion (11, 20). That some reddening nonetheless occurred most likely reflects widely acknowledged nonimmunological effects of IMQ (20).

**Mechanism of Enrichment.** Directly ex vivo, few γδ\(^{−}\) cells and γδ\(^{+}\) cells were dividing as judged by Ki67 staining, but after 4 d in IL-7, >90% of γδ\(^{+}\) cells were dividing compared with only ~30% for γδ\(^{−}\) cells (black versus gray lines; Fig. 3A). Furthermore, when cells were labeled ex vivo with a membrane-intercalating dye, carboxy-fluorescein diacetate succinimidyl ester (CFSE), γδ\(^{+}\) cells showed much greater dye dilution (by cell division) than did γδ\(^{−}\) cells (Fig. 3B), and it was those dividing cells that accounted for almost all IL-17 production upon stimulation (Fig. 3C). Hence, IL-7 drives the preferential expansion of γδ\(^{+}\) cells with, by contrast, little evidence of selective survival: indeed, Bcl-2 mRNA whose up-regulation has been associated with antiapoptotic effects of IL-7 in T cells (21) was more strongly expressed by γδ\(^{+}\) cells (Fig. S2A).

IL-7 signals are primarily transduced by STAT5 and PI3-kinase (22–24). However, IL-7–dependent STAT5 phosphorylation was comparable among γδ\(^{−}\) cells and γδ\(^{+}\) cells (Fig. 3D),
Fig. 3. IL-7 promotes expansion of IL-17–competent γδ T cells via selective STAT3 activation. (A) Staining for Ki67 (cells in cycle) in gated γδ γδ (Left) and γδ γδ (Right) LN cells ex vivo (gray line) and after 4-d culture with IL-7 (black line). Shaded histograms show Ki67 isotype staining. (B) Offset histograms of γδ γδ (red) and γδ γδ (blue) LN cells labeled with CFSE and then cultured for 4 d with IL-7. Shaded gray area represents γδ T cells stained ex vivo. (C) CFSE-labeled LN cells were cultured for 4 d with IL-7, activated with PMA + ionomycin and stained for intracellular IL-17 and gated on γδ cells. (D) Flow cytometric detection of intracellular pSTAT5 and pSTAT3 in gated γδ γδ and γδ γδ cells as labeled. Open and shaded areas indicate IL-17 treatment and controls, respectively. (E) Intracellular localization by ImageStream Flow Cytometry of pSTAT3 among two representative CD44hi γδ (Upper) and CD44lo γδ (Lower) LN cells after 30-min culture with IL-7 (BF, Bright field). (F) LN cells preincubated with a specific STAT3 inhibitor and subsequently cultured with IL-7 for 72 h. Representative plots (from n = 3 experiments). (G) Staining for Ki67 and intracellular IL-17 in gated γδ T cells cultured as in F; open and shaded areas indicate STAT3 inhibitor preincubation and controls, respectively. For all plots, numbers indicate percent of cells in relevant gate or quadrant.

Fig. 4. IL-7 enriches for γδ γδ T cells. (A and B) Histograms for IL-7R staining of: adult γδ γδ (black line) and γδ γδ (gray line) thymocytes ex vivo (A); γδ γδ cells expressing IL-17 (black line) or not expressing IL-17 (gray line) after PMA + ionomycin activation (B). Gray shaded area is isotype control staining. (C) Il17 mRNA levels in sorted γδ γδ and IL-7Rhi γδ  γδ thymocytes determined by real-time RT-PCR. (D) Total thymocytes from adult mice ex vivo or activated for 4 d in vitro with IL-7 (Left); CD44 and CD27 expression among gated γδ T cells (Center Left); Intracellular staining for IFN-γ and IL-17 in all γδ T cells (Center Right) and γδ γδ cells (Right) after PMA + ionomycin activation. For all plots, numbers indicate percent of cells in relevant gate or quadrant. (E) Adult thymocytes preincubated with a specific STAT3 inhibitor (Lower) or vehicle control (Upper) and subsequently cultured with IL-7 for 72 h. Representative plots (from n = 3 experiments) of gated γδ T cells. (F) Percentage and absolute numbers of eYFP+ γδ γδ and eYFP+ γδ γδ thymocytes from adult Il17aCreR26ReYFP mice ex vivo (black bars) or after culture with IL-7 for 4 d (open bars). (G) γδ cells stained for IL-17 and IFN-γ after 7-d FTOC from embryonic day 16.5 fetal thymus in the presence (Right) or absence (Left) of IL-7 (from n = 3 experiments with ≥3 thymic lobes per condition).

IL-7 Enriches for γδ γδ Thymocytes. IL-7–producing γδ γδ cells reportedly arise during fetal thymic development (4, 18), although failing to account for the differential effects of IL-7 on γδ γδ cells. In fact, STAT5 activation antagonizes Th17 differentiation (25) in which regard the promotion of IL-17–producing cells by IL-7 seemed paradoxical. However, IL-7 may also activate STAT3 (22) which mediates the effects of cytokines known to promote IL-17–producing γδ T cells (10); indeed, after IL-7 stimulation γδ γδ cells showed threefold higher phospho-STAT3 expression than γδ γδ cells (Fig. 3D). This phosphorylation was largely limited to CD44hi cells, in which most pSTAT3 was nuclear, as illustrated by colocalization with propidium iodide (Fig. 3E and Fig. S2C). In Il17aCreR26ReYFP “fate mapping” mice, cells transcribing the Il17a locus induce cre that excises a stop codon, thereby irreversibly activating an enhanced yellow fluorescent protein (eYFP) gene in the rosa26 locus (26). LN eYFP+ γδ cells are CD44hi and RORγt+ (Fig. S2D), and after 30-min stimulation with IL-7, pSTAT3 was selectively expressed by eYFP+ γδ cells (Fig. S2E).

When LN cells were incubated for 3 d with IL-7 in the presence or absence of an inhibitor that blocks STAT3 phosphorylation but leaves STAT5 phosphorylation intact (Fig. S2F), γδ γδ cells were little affected, whereas the preferential enrichment of γδ γδ cells was reduced by >50%, with a corresponding reduction in Ki67+ cells, and very severe attenuation of cells with IL-17–producing potential (Fig. 3 F and G and Fig. S2G). This effect was not attributable to any toxicity of the inhibitor; for example, γδ γδ annexin-V profiles were equivalent with or without it (Fig. S2H). Correlating with the selective IL-7–mediated activation of STAT3 in γδ γδ cells were very low levels of the STAT3 suppressor, SOCS3, relative to CD44hi γδ γδ cells (Fig. S2I). Interestingly, the minor CD44hi γδ γδ subset also expressed low levels of SOCS3, perhaps accounting for the maintenance of these cells in IL-7 compared with the loss of bulk γδ γδ cells (Fig. 1D).
they can easily be found in the thymus of adult mice, where they express conspicuously high levels of IL-7R (Fig. 4 A and B). High levels of IL7 mRNA were consistently detected only among IL-7R−γδ T cells (Fig. 4C). IL-7 is absolutely required for γδ T cell development, and culture with IL-7 fueled the survival and expansion of all adult γδ thymocytes, as shown by a time course (Fig. S3A). Nonetheless, there was again a strong enrichment for CD44hi IL-17–competent, TCRδ− CD27+ γδ cells, with such cells transitioning from the minority to the majority by comparison to IFN-γ–competent cells (Fig. 4D and Fig. S3A).

As for LN cells, CFSE labeling and Ki67 staining of thymocytes ex vivo showed that IL-7 primarily promoted proliferation of CD44hi CD27+ thymocytes with IL-17 potential (Fig. S3 B and C). Indeed, after 4 d, >90% of CD44hi CD27+ thymocytes were cycling with >98% of IL-17–competent cells found among these. Again there was little evidence for IL-7–mediated selective survival of γδ T cells with Bcl-2 mRNA levels lower in γδ+ cells than in γδ− cells (Fig. S3D). The preferential expansion of CD44hi γδ thymocytes was reduced ≥80% by the STAT3 inhibitor (Fig. 4E and Fig. S3E). Conversely, thymic γδ− cells were not selectively enriched by other STAT5+ (IL-2, IL-15, and IL-21) and STAT3− (IL-6) activating cytokines, either alone or added to suboptimal concentrations of IL-7. IL-2 activated all γδ thymocytes, but still enriched (almost twofold) for γδ+ cells, whereas IL-15 primarily activated γδ− cells (Fig. S4A). Thus, as for LN cells, IL-7 promotes preferential STAT3-dependent expansion of IL-17–competent γδ+ thymocytes.

Further evidence that IL-7 preferentially expands IL-17–competent thymocytes within the γδ+ subset was derived from the Il17aCreR26ReYFP mice. Ex vivo ~1% of γδ thymocytes are eYFP+ CD27+, whereas ∼8% are eYFP− CD27+ (Fig. 4F), roughly consistent with ~11% of γδ thymocytes producing IL-17 upon short-term activation (Fig. 4D, Upper). Conversely, 4 d in IL-7 increased eYFP+ CD27+ cell numbers >30-fold, making the larger subset compared with eYFP− CD27− cells that had increased much less (Fig. 4F).

To demonstrate that developing γδ+ cells are a preferential target of IL-7, fetal thymocytes were examined because the fetal thymus will not be a target for peripheral T-cell recirculation. Supplemental IL-7 added to 7-d fetal thymic organ culture (FTOC) expanded absolute numbers of total γδ thymocytes by ~fivefold, but again the impact was preferential for IL-17–competent γδ thymocytes whose representation was increased two- to fivefold over IFN-γ–producing γδ thymocytes (Fig. 4G). To verify that TCR+ preferentially activates γδ+ thymocytes rather than promoting the conversion of γδ− thymocytes to γδ+ cells, IL-7 was applied to purified CD44hi γδ− CD27−, CD44− γδ+ , or CD44− γδ− thymocytes. After a 4-d culture, CD44hi γδ− cells appeared by microscopy to be highly activated, by contrast to the γδ− subsets. To normalize the number of cells in the cultures, purified subsets were admixed with thymocytes from age-matched TCRδ− mice. Strikingly, neither γδ− subset generated IL-17–competent cells over 4 d, whereas >70% of cells arising from only 5,000 CD44hi γδ− were IL-17 competent (Fig. S5). Thus, IL-7 primarily expands cells with IL-17 competence rather than differentiating cells toward IL-17 de novo.

IL-7 Promotes Adaptive Potential to Produce IL-17. IL-17–producing γδ cells are widely viewed as innate because they are rapidly activated by IL-1 and IL-23 alone and are relatively unresponsive to TCR+ agonists (29). Hence, CD107a expression provided an additional assay for the impact of IL-7 on the response of γδ T cells to TCR stimulation. Strikingly, TCR agonists provoked surface up-regulation of CD107a by γδ+ cells only after the cells’ culture in IL-7, whereas γδ− cells up-regulated CD107a expression directly ex vivo, with no requirement for IL-7 (Fig. 5E). In sum, IL-7 selectively facilitates strong responses of IL-17–producing γδ cells to TCR stimulation whether measured by expansion, activation markers, or effector function.

IL-7 Reveals IL-17–Competent Human γδ Cells. By contrast to mice, a substantive subset of IL-17–producing human γδ T cells has been hard to identify in healthy donors (16, 28). As reported (29), there was precocious production of IFN-γ by fresh human cord blood (CB) γδ cells and by adult TCRδ− peripheral blood mononuclear cells (PBMC) stimulated by PMA + ionomycin, but there was no obvious IL-17–producing subset (Fig. 6A). When PBMC were cultured for 1 wk with anti-TCRγδ + IL-7 and then activated for 6 h, IFN-γ monoproducers described ~80% of cells; a small percentage coexpressed IL-17 and IFN-γ, but there was still no IL-17 monoproducer (Fig. 6B). However, when CB cells were likewise cultured, substantial fractions of V82+ and Vδ1− cells produced IL-17 with most being IL-17-monoproducers (Fig. 6B and Fig. S7A). Unsurprisingly, the percentages of γδ cells that were IL-17–competent varied with the source of CB from ∼15% to >40%, with higher representation always being among Vδ2+ cells. Indeed, IL-17–competent Vδ2+ cells sometimes outnumbered
Although a specific cytokine may not be essential for the development and survival of a particular lymphocyte subset, its capacity to regulate such cells is important. Such is the case for IL-7: naive memory CD40-CD8αβ T cells, which are unaffected by IL-7 depletion but are nonetheless substantially expanded by IL-7, evoking an accumulation of CD44+CD8αβ T cells (32, 33). Likewise, IL-17-producing γδ T cells may not depend on STAT3 (15), but they are rapidly responsive to IL-23 that signals via STAT3 (10). In this vein, this study identifies a capacity of IL-7 to activate STAT3 preferentially in γδ T cells competent to produce IL-17, markedly expanding and activating such cells and promoting their functional responsiveness, for example, increased cytokytic potential. This activation is a selective role for IL-7 that for years has hitherto been regarded as a generic regulator of development and homeostasis for all γδ T cell subsets (17, 34). This role of IL-7 offers parallels with its reported requirement to maintain TCRαβ Th17 cells, although a key difference is that the effects of IL-7 in that case were mediated by STAT5 (35), which seems paradoxical given that STAT5 can antagonize Th17 differentiation. By contrast, the role of IL-7 described here is in large part mediated by STAT3, emphasizing the importance of a relatively poorly understood phenomenon—namely, how different cytokines use different signaling pathways to effect specific roles.

Like the effects of IL-7 on γδ T cells (36, 37), IL-7–induced γδ+ expansion in vivo occurs in the periphery, but unlike for γδ T cells, this expansion does not require prior lymphodepletion, emphasizing the capacity of IL-7 to act selectively on γδ+ cells in physiologic situations. Indeed, blocking IL-7R almost completely abrogated the expansion of LN IL-17–producing γδ cells in response to IMQ, although it left the expansion of IFN-γ–producing cells largely untouched. Correspondingly blocking IL-7R inhibited the development of inflammatory lesions. Furthermore, increased IL-7 levels are detected in vivo after TLR activation (38) and in autoimmune diseases, such as rheumatoid arthritis (39), systemic juvenile rheumatoid arthritis (40), multiple sclerosis (41), and psoriasis (42), suggesting scenarios where potential dysregulation of IL-17-competent γδ cells should be investigated. Indeed, IL-7–producing γδ cells were recently described in psoriatic skin (11, 20, 43). γδ cells are not confined to T-cell zones and may access vascular stromal cells that express IL-7 (44), potentially in the context of tissue-draining antigens. Thus, the capacity of IL-7 to promote γδ+ cell activation in response to TCR agonists provides an important perspective on cells commonly assigned to innate immunity. Additionally, we have shown that IL-7 selectively expands CD44hi γδ+ cells from the peritoneum, where CD44hi γδ T cells are strongly implicated in defense against bacterial infection (45).

Finally, IL-7 appears most abundant in neonatal mice, which is when lymphoid compartments are being filled according to homeostatic mechanisms and when murine IL-17–producing γδ cells, largely derived from fetal progenitors, are most abundant. Indeed, IL-7 may throughout life mobilize IL-17–producing γδ cells from a “self-renewing” pool set down in the fetus. It is also striking that the major evocation of human IL-17–producing γδ cells is from CB. Interestingly, two studies using cytokines established to promote Th17 cells used CB to evoke small numbers of IL-17–producing γδ cells (46, 47). γδ cells are functionally precocious relative to γδ T cells in mice and in humans, and one of their major biological contributions may be to protect neonates (29, 48, 49). Moreover, inflammatory immunopathologies may reflect inappropriate mobilization of cells laid down in the fetal/neonatal period. In pursuing this hypothesis, the major and selective potential of IL-7 needs to be considered in immunoprophylaxis and immunopathologies. This study also implies reciprocal selective roles of IL-2 and IL-15 in regulating IFN-γ–competent γδ cells, which may be germane to the regenerative clinical use of these reagents.

**Materials and Methods**

Cell preparation, flow cytometry, PCR, and cytokine measurements were performed as described in SI Materials and Methods.
Murine and Human Samples. For methods related to animals and human samples, see SI Materials and Methods. In some experiments, mice in vivo i.p. injections included PBS or recombinant mouse IL-7 (rIL-7; R&D Systems, 5 μg per mouse, every 2 d for 1 wk). In other experiments, a daily dose of 50 mg of imiquimod (5% IMQ cream; Meda AB) or control cream (Vaseline) was applied to shaved backs of mice for 3 d. Anti–IL-7R (clone A7R34) or rat IgG control treatment was performed by i.p. injection (1 mg per mouse) on days 1 and +2 relative to IMO application. A7R34 was obtained from Biologend or, for some experiments, we made A7R34 from hybridoma (50).

Cell Culture. Cells were incubated for 1, 2, 3, or 4 d with IL-2 (100 U/mL; Immunotools) or, for some experiments, we made A7R34 from hybridoma (50).