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CD27 is a thymic determinant of the balance between interferon- γ - and interleukin 17–producing $\gamma\delta$ T cell subsets

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Abstract

The production of cytokines such as interferon- γ and interleukin 17 by $\alpha\beta$ and $\gamma\delta$ T cells influences the outcome of immune responses. Here we show that most $\gamma\delta$ T lymphocytes expressed the tumor necrosis factor receptor family member CD27 and secreted interferon- γ , whereas interleukin 17 production was restricted to CD27- $\gamma\delta$ T cells. In contrast to the apparent plasticity of $\alpha\beta$ T cells, the cytokine profiles of these distinct $\gamma\delta$ T cell subsets were essentially stable, even during infection. These phenotypes were established during thymic development, when CD27 functions as a regulator of the differentiation of $\gamma\delta$ T cells at least in part by inducing expression of the lymphotoxin- β receptor and genes associated with trans-conditioning and interferon- γ production. Thus, the cytokine profiles of peripheral $\gamma\delta$ T cells are predetermined mainly by a mechanism involving CD27.

The past three decades have witnessed intense investigation of T cell generation in the mouse thymus. As a result, insight has been gained into how bone marrow–derived

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AUTHOR CONTRIBUTIONS

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Microarray. Information on this procedure is available in the Supplementary Methods

Statistical analysis. The statistical significance of differences between populations was assessed with the Student's t-test; P values of less 0.05 than were considered significant.

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progenitors seed the thymus, rearrange their T cell antigen receptor (TCR) loci, become committed to either the $\alpha\beta$ or the $\gamma\delta$ T cell lineage and proceed through TCR-mediated selection processes that ensure that 'useful' T cells are exported to exert immune functions in the periphery^{1–3}. This notwithstanding, consensus has not been reached on the degree to which intrathymic differentiation events influence peripheral T cell function. For example, although it seems clear that events in the thymus promote the generation of Foxp3+ 'natural' regulatory T cells, there is debate over which molecular interactions are involved, with the TCR, CD28 and the lymphotoxin- β receptor (LT β R) all being linked to this¹. There is likewise no clear sense of the relative influence of thymic determination versus peripheral 'plasticity' and functional 'conversion'. This is true not only for regulatory T cells but also for the differentiation of helper T cells leading to the production of important cytokines, including interferon- γ (IFN- γ), interleukin 4 (IL-4) and IL-17. In this context, it may also be argued that there has been a disproportionate focus on $\alpha\beta$ T cells, when it is increasingly acknowledged that $\gamma\delta$ T cells have pleiotropic functional potential and, in arenas such as the production of IFN- γ and IL-17, make critical contributions to host immune competence^{4,5}. Indeed, whereas TCR $\alpha\beta$ + IL-17-producing T helper cells (T_H-17 cells) are restricted mainly to the gut⁶, $\gamma\delta$ T cells are a chief source of IL-17 in lymphoid organs and peripheral tissues^{7–10}. Consequently, there are ongoing attempts to harness the functional potential of $\gamma\delta$ T cells in the clinic. Functional T cell subsets are apparently generated by the combined actions of transcription factors. Thus, the transcription factors T-bet and eomesodermin, GATA-3 and c-Maf, and ROR γ t and Runx1 determine T cell production of IFN- γ , IL-4 and IL-17, respectively^{11,12}. However, their actions can be critically modulated by other molecules, including Notch receptors¹³. Indeed, there is much biological and clinical interest in the identification of cell surface receptors whose engagement might skew T cell differentiation. In this context, it has been reported that the differentiation of $\gamma\delta$ T cells toward IFN- γ production and away from IL-17 production is regulated by specific $\gamma\delta$ TCR agonists in the thymus¹⁴. Possibly consistent with those findings, particular $\gamma\delta$ TCRs are associated with distinct cellular functions¹⁵. Those observations notwithstanding, the differentiation of $\gamma\delta$ T cells is also regulated by other intrathymic molecular interactions. For example, the differentiation of proliferating, IFN- γ -producing $\gamma\delta$ T cells requires TCR-independent interactions between early $\gamma\delta$ T cell progenitors and CD4⁺CD8⁺ (double-positive (DP)) $\alpha\beta$ T cell progenitors. These events, collectively called 'trans-conditioning', are determined in part by signals received through the LT β R^{16–18}.

On the basis of such perspectives, this paper defines discrete intrathymic progenitors of peripheral IFN- γ - and IL-17-producing $\gamma\delta$ T cell subsets, distinguished and regulated by the tumor necrosis factor (TNF) receptor 'superfamily' member CD27 (A000546), which engages CD70 (A000547), a TNF-related transmembrane glycoprotein¹⁹. CD27⁺ $\gamma\delta$ thymocytes expressed LT β R and genes associated with a T helper type 1 (T_H1) phenotype, in contrast to CD27[−] $\gamma\delta$ thymocytes, which included the progenitors of IL-17-producing $\gamma\delta$ cells. Whereas the relative proportions of the two $\gamma\delta$ subsets in the thymus and the periphery were biased toward CD27⁺ IFN- γ producers, IL-17-producing CD27[−] $\gamma\delta$ T cell populations rapidly expanded in response to acute infection such as that posed by malaria.

RESULTS

CD27 expression segregates $\gamma\delta$ lymphocyte subsets

Attempts have been made to identify markers of distinct $\gamma\delta$ T cell subsets, particularly after the realization that $\gamma\delta$ cells are key sources of IL-17 and IFN- γ and contribute to host defense and to certain inflammatory and autoimmune diseases^{14,20–22}. Although $\gamma\delta$ cells have been subcategorized by their expression of CD25 and CD122 into IL-17 and IFN- γ producers, respectively²³, as discussed below, this classification is not generally applicable beyond the peritoneal cavity, and there is no evidence that these markers actively regulate the outcome of T cell differentiation. Here we examine the validity of CD27 as a subset marker for $\gamma\delta$ T cells, as CD27 has been linked to the functional segregation of subsets of $\alpha\beta$ T cells^{19,24} and natural killer (NK) cells²⁵.

CD27 expression distinguished two $\gamma\delta$ cell subsets in the spleen and lymph nodes (axillary, inguinal, branchial and mesenteric) and in the peripheral tissues (lung or gut) of adult C57BL/6 mice. Overall, 10–30% of $\gamma\delta$ cells were CD27[–] (called ' $\gamma\delta^{27-}$ ' here) and 70–90% were CD27⁺ (called ' $\gamma\delta^{27+}$ ' here; Fig. 1a). The two subsets had very different surface phenotypes, with $\gamma\delta^{27-}$ cells generally being CD44^{hi}CD62L^{lo} and $\gamma\delta^{27+}$ cells having lower expression of CD44 and higher expression of CD62L (Fig. 1b). They also showed conspicuously little interconversion, as the CD27⁺ and CD27[–] phenotypes were maintained even after 3–5 d of continuous stimulation with antibody to CD3 ϵ (anti-CD3 ϵ) in vitro or 2 weeks of residence in lymphopenic mice deficient in the product of recombination-activating gene 2 (RAG-2; Fig. 1c).

These experiments also showed that the proliferation of $\gamma\delta^{27+}$ cells after TCR stimulation in vitro exceeded that of $\gamma\delta^{27-}$ cells (Fig. 1d) and that $\gamma\delta^{27+}$ cells had a considerable advantage over $\gamma\delta^{27-}$ lymphocytes when these cells were injected together into lymphopenic mice (Figs. 1e,f). Thus, $\gamma\delta^{27+}$ and $\gamma\delta^{27-}$ cells constitute distinct and essentially stable subsets of $\gamma\delta$ T lymphocytes in naive mice.

Cytokine production segregates with CD27

When comparing the gene expression of splenic $\gamma\delta^{27+}$ and $\gamma\delta^{27-}$ cells from naive C57BL/6 mice, we noted considerable polarization toward constitutive expression of Ifn γ and Il17, respectively (Fig. 2a). After 4 h of activation in vitro with phorbol 12-myristate 13-acetate (PMA) and ionomycin, $\gamma\delta^{27+}$ cells had typical T_H1-like characteristics, including abundant intracellular IFN- γ and TNF but no IL-17; in contrast, $\gamma\delta^{27-}$ populations had far fewer cells that expressed IFN- γ and TNF but had a conspicuous population of IL-17-producing cells (Fig. 2b and Supplementary Fig. 1a online). Neither $\gamma\delta^{27+}$ nor $\gamma\delta^{27-}$ cells had substantial expression of IL-4, IL-13 or IL-10 in the conditions used, but they had similar cytolytic capacity (Supplementary Fig. 1a,b and data not shown). Independent reports have cited an association of CD122 expression with IFN- γ -producing $\gamma\delta$ T cells^{14,23} and of the scavenger receptor SCART2 with IL-17-secreting $\gamma\delta$ T cells²⁶. Consistent with those findings, expression of CD122 and SCART2 segregated with $\gamma\delta^{27+}$ and $\gamma\delta^{27-}$ cells, respectively (Fig. 2c,d). However, the subsets did not overlap perfectly. Thus, many $\gamma\delta^{27+}$ cells were CD122[–], and a substantial percentage of CD122[–] cells also produced IFN- γ after short-term activation

(Supplementary Fig. 1c). Indeed, as most $\gamma\delta$ T cells are CD122⁻, over 50% of IFN- γ -producing $\gamma\delta$ T cells may have been CD122⁻ whereas less than 5% were CD27⁻ (Fig. 2b,d). Hence, CD27 expression seemed to segregate much more accurately with IFN- γ production by $\gamma\delta$ T cells than did CD122. The degree to which SCART2-expressing cells overlap with $\gamma\delta^{27-}$ cells cannot be assessed until reagents facilitating the detection of SCART2 by flow cytometry become generally available.

To analyze how each $\gamma\delta$ subset might respond to infection, we used *Plasmodium berghei*, because in mice and humans, $\gamma\delta$ T cells make important contributions to the response to this pathogen and other apicomplexan parasites²⁷⁻³⁰. We evaluated the spleen and peripheral lymphoid organs of C57BL/6 mice infected with *P. berghei* (strain ANKA), a model of experimental cerebral malaria that leads to death between 6 and 8 d after infection³¹. The $\gamma\delta^{27+}$ and $\gamma\delta^{27-}$ subsets each proliferated after inoculation with a fixed number of *P. berghei* ANKA-infected erythrocytes, but with different kinetics. The $\gamma\delta^{27+}$ populations expanded more rapidly (by day 3 after infection); however, by day 6 after infection, $\gamma\delta^{27-}$ cells had also increased almost fivefold in number and accounted for approximately 40% of cells in the splenic $\gamma\delta$ compartment (Fig. 2e). These findings show that whereas $\gamma\delta^{27-}$ cells proliferated relatively poorly in response to stimulation in vitro or during homeostatic expansion in vivo (Fig. 1), they were able to proliferate robustly in response to physiological stimuli in vivo. Moreover, as early as day 3 after infection, approximately 60% of $\gamma\delta^{27-}$ cells had an activated CD69⁺ surface phenotype, compared with only about 20% of $\gamma\delta^{27+}$ cells (Fig. 2e). A higher inoculum of *P. berghei* ANKA-infected erythrocytes resulted in augmented parasitemia (Supplementary Fig. 2a online) and population expansion of spleen and lymph node $\gamma\delta$ T cells that was almost exclusively attributable to $\gamma\delta^{27-}$ cells, which at high parasitemia represented over 50% of all $\gamma\delta$ T cells (Fig. 2f and Supplementary Fig. 2b). Notably, however, both $\gamma\delta$ T cell subsets showed strong conservation of the cytokine (IFN- γ versus IL-17) and surface marker (CD44 and CD62L) phenotypes of naive mice (Fig. 2b,e).

Factors that influence $\gamma\delta^{27-}$ cell IL-17 production

By analyzing cytokine production versus dilution of the cytosolic dye CFSE by total $\gamma\delta$ T cells, we confirmed that most IL-17 producers, like $\gamma\delta^{27-}$ cells, proliferated poorly after activation in vitro, whereas IFN- γ producers, like $\gamma\delta^{27+}$ cells, proliferated robustly (Supplementary Fig. 3a,b online). Similarly, IL-17 was produced by CD44^{hi}CD62L^{lo} $\gamma\delta$ T cells but not by CD44^{lo}CD62L^{hi} $\gamma\delta$ T cells (Supplementary Fig. 3c,d). Collectively, these data show that the properties of the $\gamma\delta^{27-}$ subset fully resemble those of the IL-17-producing subset of the peripheral $\gamma\delta$ T cell pool. Therefore, we considered it appropriate to use $\gamma\delta^{27-}$ cells to study the requirements for IL-17 expression. Consistent with reports that IL-17 production by $\gamma\delta$ T cells does not require stringent conditioning by transforming growth factor- β (TGF- β), IL-6 or other inflammatory cytokines^{7,23} required for $\alpha\beta$ T_H-17 differentiation³²⁻³⁴, $\gamma\delta^{27-}$ cells expressed IL-17 mRNA immediately after isolation and required only 4 h of treatment with PMA and ionomycin to express IL-17 protein (Fig. 3a,b). Neither TCR $\alpha\beta$ ⁺CD4⁺CD27⁻ effector T cells nor $\gamma\delta^{27+}$ cells produced IL-17 mRNA or protein (Fig. 3a,b). Given that elaboration of IL-17 by $\gamma\delta^{27-}$ cells occurs in different conditions than those established for $\alpha\beta$ T_H-17 cells, we also determined if the T_H-17-determining transcription factor ROR γ t was expressed in $\gamma\delta^{27-}$ cells. For this we used a

mouse in which green fluorescent protein (GFP) is expressed under the control of Rorc (which encodes ROR γ t transcriptional elements)^{6,35}. By the criterion of GFP expression, all IL-17-producing $\gamma\delta^{27-}$ cells expressed Rorc (Fig. 3c). A small percentage of $\gamma\delta^{27+}$ cells weakly expressed Rorc but did not express IL-17, consistent with data above (Figs. 2b and 3b) and with the generally accepted view that ROR γ t is necessary but not sufficient for IL-17 induction in $\alpha\beta$ T cells¹¹. Nonetheless, we investigated whether the constitutive expression of IL-17 in $\gamma\delta^{27-}$ cells could be further upregulated by particular stimuli. Indeed, IL17 transcription and IL-17 protein production was augmented in 3-day cultures by activation with anti-CD3e plus IL-2 (in the absence of any other cytokines); this augmentation was further enhanced by TGF- β treatment (Fig. 3a,b). Of note, these stimuli also induced IFN- γ production in a substantial fraction of $\gamma\delta^{27-}$ cells (although this fraction was always lower than that of the $\gamma\delta^{27+}$ cell subset; Fig. 3b), which demonstrates some degree of functional plasticity of this population, at least in vitro. That observation notwithstanding, IL-17 expression was achieved without the addition of exogenous IL-6 or IL-21.

In addition, the finding that IL-17- producing $\gamma\delta^{27-}$ cells lacked transcripts for IL-6 or IL-21 (Supplementary Fig. 4 online) excluded the possibility that these cytokines might be provided in an autocrine way. Likewise, in contrast to the need for antibody blockade of IFN- γ for the optimal polarization of CD4⁺CD25²⁷⁻ $\alpha\beta$ T_H-17 cells in vitro^{7,32} (data not shown), $\gamma\delta^{27-}$ cells produced IL-17 even in the presence of IFN- γ -producing cells (Fig. 3b). Moreover, when exogenous IL-6, IL-21 and neutralizing anti-IFN- γ were added to cultures alone or in combination, they had essentially no effect on IL-17 production by TGF- β -treated cells; the addition of IL-23 had only a very small effect (Fig. 3d). Notably, in all conditions tested, most $\gamma\delta^{27+}$ cells failed to produce IL-17 (Fig. 3b,d), which reemphasizes the functional distinction between the two $\gamma\delta$ T cell subsets. Although it has been proposed that IL-17 production is more common among cells positive for the variable region V γ 4 than among V γ 1⁺ cells, conflicting data have left this point contentious and unresolved^{21,22}. In our analyses, IL-17-expressing cells accounted for almost 50% of V γ 4⁺ $\gamma\delta^{27-}$ cells and only about 11% of V γ 1⁺ $\gamma\delta^{27-}$ cells (Fig. 3e). This finding supports the hypothesis that there is an association between the TCR and a bias in the functional differentiation of $\gamma\delta$ T cells^{4,14}.

Thymic progenitors of $\gamma\delta^{27+}$ and $\gamma\delta^{27-}$ cells

Given their essential stability in naïve and infected mice, we assessed whether the $\gamma\delta^{27+}$ and $\gamma\delta^{27-}$ subsets were developmentally established in the thymus. We found that TCR $\gamma\delta^{+}$ thymocytes had the highest expression of CD27 of all the T cell-committed subsets (Fig. 4a). However, in contrast to the homogeneous expression of CD27 on mature TCR $\alpha\beta^{+}$ thymocytes, approximately 10% of $\gamma\delta$ thymocytes conspicuously lacked CD27 (Fig. 4a). As thymocyte precursors at double-negative stages 2 and 3, from which $\gamma\delta$ cells mainly derive^{1,36,37}, express CD25, we used staining for CD25 and CD27 to segregate $\gamma\delta$ thymocytes into subsets; 5–10% were CD27⁺CD25⁺ (called ' $\gamma\delta^{27,25+}$ ' here), whereas the remaining 90–95% cells were negative for CD25, including 10–15% CD27⁻ cells ($\gamma\delta^{27-}$) and 75–80% CD27⁺ cells ($\gamma\delta^{27+}$; Fig. 4b). This representation of the subsets was completely similar in independent mouse colonies maintained in two different countries. Moreover, each subset was detectable in the thymus from the earliest embryonic stages tested

(embryonic days 14–15 (E14–E15), although the $\gamma\delta^{27+}$ subset greatly expanded between E15 and E18, by which time the relative proportions found in the adult were established (Fig. 4c). The finding that peripheral $\gamma\delta$ cells were essentially all CD25[−] (Fig. 4b) underpins the concern that the proposed use of CD25 to identify IL-17-producing $\gamma\delta$ T cells may be applicable only to the peritoneal cavity²¹ (Supplementary Fig. 5 online) and suggests that the thymic $\gamma\delta^{27,25+}$ subset might constitute a progenitor compartment. Consistent with that hypothesis, $\gamma\delta^{27,25+}$ cells were larger in size than were $\gamma\delta^{27+}$ or $\gamma\delta^{27-}$ thymocytes (Fig. 4d), had a CD24^{hi}CD25^{hi}CD62L^{hi}CD44^{hi}CD2^{lo}CD5^{lo} ‘immature’ phenotype very similar to that of progenitors at double-negative stages 2 and 3 (Fig. 4e and Supplementary Fig. 6 online), had lower surface expression of TCR than did the $\gamma\delta^{27+}$ and $\gamma\delta^{27-}$ subsets (data not shown) and had high proliferative turnover (Fig. 4f,g). In contrast, the surface phenotype and proliferative activity of $\gamma\delta^{27+}$ and $\gamma\delta^{27-}$ thymocytes closely mimicked those of their respective peripheral counterparts (Figs. 1b and 4e–g). Next we repopulated fetal thymic reaggregation organ cultures with highly purified $\gamma\delta^{27,25+}$ thymocytes. After 7 d in culture, these $\gamma\delta^{27,25+}$ thymocytes gave rise to both $\gamma\delta^{27+}$ and $\gamma\delta^{27-}$ cells (Fig. 4h). This observation confirms that $\gamma\delta^{27,25+}$ thymocytes have the potential to generate $\gamma\delta^{27+}$ and $\gamma\delta^{27-}$ cells. Another demonstration of the relationship between the thymic $\gamma\delta^{27+}$ and $\gamma\delta^{27-}$ subsets and their peripheral counterparts was evident in their constitutive expression of Ifng and Il17, respectively (Fig. 4i). Because this expression was present in E18 thymi, it seems implausible to attribute it to the recirculation of activated peripheral $\gamma\delta$ T cells back to the thymus. Likewise, $\gamma\delta^{27-}$ cells from E18 thymi already expressed Rorc and its T_H-17-driving cofactor Runx1 (ref. 11), whereas fetal $\gamma\delta^{27+}$ cells expressed Tbx21, which encodes T-bet, a chief determinant of T_H1 differentiation (Fig. 4j). The idea that such functional segregation was already established in $\gamma\delta^{27+}$ and $\gamma\delta^{27-}$ thymocytes, respectively, was also demonstrated by their corresponding expression of IFN- γ and IL-17 protein (Supplementary Fig. 7a online). Incubation for 3 d with anti-CD3 ϵ and IL-2 supplemented with a ‘cocktail’ of IL-6, IL-21 and IL-23 further increased IL-17 production by $\gamma\delta^{27-}$ cells but did not induce IL-17 production by $\gamma\delta^{27+}$ thymocytes (Supplementary Fig. 7b). Thus, the functional potential and constraints of $\gamma\delta^{27+}$ and $\gamma\delta^{27-}$ cells seem to be ‘imprinted’ as early as in the fetal thymus.

CD27 regulates $\gamma\delta$ T cell functional potential

The number of $\gamma\delta$ T cells, TCR V γ segment use and profiles of developmental markers (such as CD24, CD25, CD44 and CD62L) were similar in the thymus and peripheral lymphoid organs of wild-type and Cd27^{−/−} mice³⁸ (Fig. 5a, Supplementary Fig. 8a,b online and data not shown). However, TCR $\gamma\delta^{+}$ thymocytes from Cd27^{−/−} mice had lower expression of Ifng (Supplementary Fig. 8c), and peripheral $\gamma\delta$ cells from Cd27^{−/−} mice had lower expression of IFN- γ and TNF mRNA and protein (Fig. 5b and Supplementary Fig. 8d). The most overt difference was that the proportion of IFN- γ ^{hi} cells in Cd27^{−/−} mice was approximately 65% lower (Fig. 5b). Likewise, CD44^{lo}CD62L^{hi} $\gamma\delta$ T cells from Cd27^{−/−} mice (which in wild-type mice would also express CD27) had less IFN- γ production than their wild-type counterparts had (Supplementary Fig. 8e). Thus, CD27 is a regulator as well as a marker of the functional differentiation of $\gamma\delta$ T cells. IL-17 expression was essentially unchanged in Cd27^{−/−} mice and, as in wild-type mice, IL-17 expression was limited to the

CD44^{hi}CD62L^{lo} subset in Cd27^{-/-} mice (Supplementary Fig. 8f)

Infection with *P. berghei* ANKA elicited a similar expansion of total $\gamma\delta$ splenocyte populations in wild-type and Cd27^{-/-} mice (Fig. 5c), but the latter had 60% lower absolute numbers of IFN- γ producers and 80% fewer IFN- γ ^{hi} cells than did wild-type mice (Fig. 5d). Notably, there was a delay in the progression of cerebral malaria, a severe inflammatory syndrome partly attributable to IFN- γ ²⁸, in Cd27^{-/-} mice (Supplementary Fig. 9 online).

To investigate possible mechanisms by which CD27 alters the thymic and peripheral phenotypes of $\gamma\delta$ T cells, we considered the selective impairment of IFN- γ production in $\gamma\delta$ cells that occurs in TCR β -deficient mice. Like CD27-deficient mice, TCR β -deficient mice show no overt defect in $\gamma\delta$ T cell numbers. In TCR β -deficient mice, $\gamma\delta$ T cell functional alterations have been attributed mainly to the absence in the thymus of trans-conditioning of $\gamma\delta$ cell progenitors by TCR β ⁺ DP thymocytes^{1,17}. Both $\gamma\delta$ T subsets defined here were present in wild-type proportions in TCR β -deficient mice (data not shown), but $\gamma\delta$ ²⁷⁺ cells in TCR β -deficient mice showed impaired expression of IFN- γ and TNF mRNA (Fig. 6a). We did not find this impairment in $\gamma\delta$ ²⁷⁺ cells from TCR α -deficient mice, which had wild-type numbers of DP thymocytes; this suggests that the defective $\gamma\delta$ T cell function in CD27-deficient mice may at least in part reflect altered trans-conditioning.

To test that possibility, we compared the transcriptomes of wild-type and CD27-deficient $\gamma\delta$ T cells with whole-genome microarrays (ArrayExpress accession number E-NCMF-23; Fig. 6b). Conspicuous among CD27-dependent genes was *Ltbr*, which encodes LT β R, a mediator of trans-conditioning¹⁷, as well as four ‘signature’ genes of LT β R signaling in $\gamma\delta$ thymocytes: *Crem* (also called *Icer*), *Nr4a2* (*Nurr1*), *Rgs2* and *Rgs1* (Table 1). We confirmed differences in the expression of these genes in wild-type and CD27-deficient $\gamma\delta$ thymocytes by RT-PCR; in contrast, *Il7ra*, which encodes a factor critical for $\gamma\delta$ thymocyte development¹, was not impaired in CD27-deficient $\gamma\delta$ thymocytes (Fig. 6c,d).

Consistent with the effects of CD27, wild-type mice showed expression of *Ltbr* and *Ltbr*-dependent genes in $\gamma\delta$ ^{27,25+} and $\gamma\delta$ ²⁷⁺ thymocytes but not in $\gamma\delta$ ²⁷⁻ thymocytes (Fig. 6e). To derive further evidence that CD27 regulates the differentiation of $\gamma\delta$ thymocytes, we added to E14 fetal thymic organ cultures (FTOCs) a fusion protein of CD70 and immunoglobulin that acts as a CD27 agonist³⁹. After 1 week of culture, the recovered $\gamma\delta$ ²⁷⁺ thymocytes showed significant upregulation of *Ifng* and $\gamma\delta$ ²⁷⁻ thymocytes showed down-regulation of *Il17* (Fig. 6f). Thus, embryonic $\gamma\delta$ progenitors were effectively skewed toward IFN- γ production simply through the manipulation of CD70-CD27 signals. To assess whether CD27 might be acting together with other determinants of $\gamma\delta$ differentiation, we added to parallel organ cultures either anti-CD3e or cyclosporin A; the former enhances and the latter inhibits TCR signaling. Relative to their representation in control cultures, these treatments increased and decreased representation of the $\gamma\delta$ ²⁷⁺ subset, respectively, with reciprocal effects on the $\gamma\delta$ ²⁷⁻ subset (Fig. 6g). We conclude that the developmental preconditioning of discrete peripheral $\gamma\delta$ cell subsets may involve some form of ‘collaboration’ between TCR and CD27 signaling in the thymus.

Finally, to determine whether the classification that we have introduced might apply to other unconventional T cells, we examined invariant NKT cells, as it has been reported that IL-17

expression is restricted to the NK1.1⁺ subset of NKT cells and that this restriction is 'imprinted' in the thymus^{40,41}. After isolating NKT cells on the basis of their reactivity to tetramers of CD1d and α -galactosylceramide, we found that surface marker staining 'assigned' over 80% of NK1.1⁺ cells to the CD27⁺ compartment, whereas over 80% of CD27⁺ cells were NK1.1⁺ (Supplementary Fig. 10 online). Thus, it seems that in the NKT compartment, IL-17-producing cells are also found mainly in the CD27⁺ subset.

DISCUSSION

Here we have described mouse $\gamma\delta$ cell subsets that had distinct functional capacities and could be defined by CD27 expression. The $\gamma\delta^{27+}$ subset was more abundant and included cells that produced IFN- γ but essentially no IL-17 immediately after isolation and in all activation conditions used. In contrast, the $\gamma\delta^{27-}$ subset comprised essentially all IL-17-producing $\gamma\delta$ cells. Both subsets actively responded to infection in vivo by proliferating and producing cytokines. Although $\gamma\delta^{27-}$ cells may have produced IFN- γ after in vitro activation, very few did so ex vivo, whether from naive or infected mice. Moreover, their thymic counterparts did not produce IFN- γ , which raises the possibility that the IFN- γ and IL-17 'double-producing' cells were an artifact of in vitro activation protocols, even though 'double production' of IL-17 and IFN- γ has been described for TCR $\alpha\beta^{+}$ T_H-17 cells^{6,42}. Whereas TCR $\alpha\beta^{+}$ T_H1 and T_H-17 cells can take up to 7 d to differentiate and expand clonally under the influence of specific combinations of cytokines⁴³, the ability of the $\gamma\delta$ T cell subsets described here to express their 'signature' cytokines directly ex vivo was apparently acquired during their differentiation in the thymus, when the subsets also adopted their characteristic phenotypic markers and other properties that remained essentially stable, even during infection. These observations define a parallel with NKT cells, another 'innate-like' lymphocyte lineage that promptly secretes a panoply of cytokines, including IL-17 and IFN- γ ^{40,44}. Having found that NK1.1⁺ NKT cell populations, which are known to include 'spontaneous' IL-17-producing cells^{40,41}, were mostly CD27⁺, we propose that CD27 may influence the functional differentiation of several lineages of unconventional T cells. The thymic 'imprinting' of the CD27⁺ and CD27⁺ subsets perhaps reflects the fact that $\gamma\delta$ T cells (and other unconventional T cells) may need to provide specific effector cytokines very rapidly, to counter microbial growth and dissemination before maturation of the adaptive response. Indeed, myriad analyses have shown that $\gamma\delta$ cells are crucial providers of either IFN- γ or IL-17 in diverse physiological settings^{8-10,14,20-22}. This may be particularly true in early life, when there is a precipitous encounter with environmental antigens and when $\gamma\delta$ T cells seem precociously mature compared with $\alpha\beta$ T cells in both mice³⁵ and humans (D. Gibbons and A.C.H., unpublished observations). Rapid $\gamma\delta$ T cell responses may also determine in part the complexion and magnitude of ensuing adaptive responses^{14,45}. Developmental 'imprinting' may more strongly influence $\gamma\delta$ T cells than $\alpha\beta$ T cells, as defined sets of cytokines seem able to skew bulk populations of peripheral $\alpha\beta$ T cells toward widely different functional differentiation pathways (such as T_H1, T_H-17 and regulatory T cells). In contrast, the various conditions we used here failed to skew peripheral $\gamma\delta^{27+}$ cells toward IL-17 production. Such functional 'rigidity' and our notable failure to identify any substantial interconversion of $\gamma\delta^{27+}$ and $\gamma\delta^{27-}$ cells in vitro or in vivo suggest that these two subsets, although heterogeneous, constitute discrete lineages. Because it regulates $\gamma\delta$ T cell

functional differentiation, we believe the TNF receptor family member CD27 is a more incisive marker of $\gamma\delta$ T cell subsets than others described^{14,23,26}. CD27 controls the expression of LT β R, another TNF receptor family member and a component of $\gamma\delta$ T cell trans-conditioning that is associated with IFN- γ -biased differentiation and high in vitro proliferative potential¹⁷. Thus, CD27 may induce or sustain expression of LT β R and its 'downstream' targets in $\gamma\delta^{27+}$ thymocytes and in their $\gamma\delta^{27,25+}$ progenitors. This would be consistent with the finding that genes induced by trans-conditioning were expressed in early CD4⁻CD8⁻ progenitors and their expression was then selectively retained or lost in more mature cells. The retention of the expression of such genes in $\gamma\delta^{27+}$ cells would confer their 'signatory' proliferative, T_H1-like functional potential, which is impaired in CD27-deficient mice and LT β R-deficient mice¹⁷. Because the lymphotoxin that trans-conditions through LT β R is derived at least in part from TCR β^+ DP thymocytes, this model also readily explains the functional impairment of the $\gamma\delta^{27+}$ subset in TCR β -deficient mice but not in TCR α -deficient mice. It might also explain the notable increase in the size of the thymic $\gamma\delta^{27+}$ subset between E15 and E18, as this period corresponds to the main expansion of TCR β^+ DP thymocyte populations.

Clearly, as the impairment in IFN- γ production by $\gamma\delta$ T cells in mice lacking CD27, LT β R or TCR β is incomplete, other factors probably influence $\gamma\delta$ T cell function. Signaling by the $\gamma\delta$ TCR, which has been reported to skew $\gamma\delta$ T cells toward IFN- γ production and away from IL-17 production¹⁴ may be one such factor. Our findings of TCR bias in the $\gamma\delta^{27+}$ and $\gamma\delta^{27-}$ subsets and of the influence of TCR signal manipulation on the relative abundance of $\gamma\delta^{27+}$ and $\gamma\delta^{27-}$ subsets in FTOC are consistent with that idea. TCR signals in the thymus may upregulate CD27 (ref. 37), which may then act together with TCR signaling (and/or other differentiation factors) by providing a critical competence to $\gamma\delta$ T cells (and possibly other unconventional T cells) so they can progress through T_H1 differentiation. Although the transcription factor ROR γ t characterizes all IL-17-producing $\gamma\delta^{27-}$ cells, its method of operating would seem different from that in TCR $\alpha\beta^+$ T_H-17 cells as, consistent with other data⁷, we found that IL-17 production by $\gamma\delta$ T cells required neither 'collaborative' signaling from TGF- β , IL-6 and IL-21 receptors nor interference with IFN production. It is likewise notable that microbe-induced IL-17 production in the gut seems to derive from $\alpha\beta$ T cells⁴⁶, whereas IL-17-producing $\gamma\delta$ T cells are relatively rare in the gut compared with their presence in other tissues. Indeed, the relative over-representation of TCR $\alpha\beta^+$ T_H-17 cells in the mouse gut⁶ may represent a 'compensation' for the relative scarcity of gut $\gamma\delta^{27-}$ cells. Another contrast in the IL-17 production of $\alpha\beta$ and $\gamma\delta$ T cells lies in the augmented $\gamma\delta^{27-}$ cell production of IL-17 in response to activation by TGF- β and TCR in the presence of IL-2, a combination that upregulates the transcription factor Foxp3 and a bias toward regulatory T cell function in CD4⁺ $\alpha\beta$ T cells^{7,47}. Indeed, it was reproducibly difficult to detect Foxp3 expression in $\gamma\delta$ T cells in wild-type mice¹⁸ (data not shown). Possibly, a key feature of IL-17 production by $\gamma\delta$ cells may be the ability of ROR γ t to act without interference from Foxp3. Although $\gamma\delta^{27-}$ cells were mostly unaffected in CD27-deficient mice, as might be expected, CD27 gain of function in FTOC did result in lower IL-17 production by these cells, which indicates that CD27 may diminish IL-17 production as well as enhance IFN- γ production, at least in T cell progenitors. CD27 is expressed on a plethora of lymphoid cells and bone marrow precursors, where it has been assigned many pleiotropic

functions^{38,39,48,49}. Despite its expression on mouse and human thymocytes and its responsiveness to pre-TCR and TCR signals³⁷, CD27 seems to be not critical in the development of conventional $\alpha\beta$ T cells³⁸ (unpublished data). That is notable given the constitutive expression of CD70 on thymic epithelial cells of mice and humans⁵⁰. Thus, here we have reported a previously unknown function for CD27 expression on thymic T cell precursors. Future studies should determine which cells direct key CD70-CD27 interactions in the thymus and whether the functional and phenotypic stability of $\gamma\delta^{27+}$ cells requires sustained interactions between CD27 and CD70 in the periphery. The resolution of such issues will provide a better understanding of the balance of precommitment and the peripheral plasticity of $\gamma\delta$ T cell subsets.

METHODS

Mice

All mice used were adults 4–10 weeks of age. Embryos were obtained by the setting up of timed pregnancies. C57BL/6 (B6), B6.TCR α -deficient, B6.TCR β -deficient and B6.RAG-2-deficient mice were from Jackson Laboratories. B6.CD27-deficient mice have been described³⁸. B6.ROR γ t-GFP mice³⁵ were a gift from D. Littman. Mice were bred and maintained in the specific pathogen-free animal facilities of the Instituto de Medicina Molecular (Lisbon), Queen Mary London University (London) and Yale University School of Medicine (New Haven). All experiments involving animals were done in compliance with the relevant laws and institutional guidelines and were approved by local ethics committees.

Cell preparation

Thymuses, lymph nodes and spleens were homogenized and washed in RPMI medium containing 10% (vol/vol) FCS. In some cases, thymocyte samples were depleted of CD8⁺ and CD4⁺ cells by treatment with anti-CD8 (31.1M) and anti-CD4 (RL172; both from J. van Meerwijk) plus complement (HD Supplies), followed by gradient centrifugation in Lympholyte M (Cederlane Laboratories). Lungs were cut into pieces 5 mm² and were incubated for 2 h at 37 °C with stirring in complete RPMI medium supplemented with collagenase (0.05 mg/ml; Sigma). Supernatants were collected and live cells were isolated on a gradient of Lympholyte M (Cederlane Laboratories).

Monoclonal antibodies

Fluorescein isothiocyanate-labeled anti-V γ 1 (2.11) was a gift from P. Pereira. The following monoclonal antibodies were from BD Pharmingen: fluorescein isothiocyanate-labeled anti-TCR δ (GL3), anti-V γ 4 (UC3-10A6) and anti-CD62L (MEL14); phycoerythrin-labeled anti-TCR δ (GL3), anti-CD25 (PC61), anti-CD44 (IM7), anti-IFN- γ (XMG1.2), and anti-TNF (MP6-XT22); peridinin chlorophyll protein complex-cyanine 5.5-labeled anti-CD4 (RM4-5) and anti-CD8 α (53.6.7); allophycocyanin-labeled anti-CD25 (PC61); biotinylated anti-TCR δ (GL3); peridinin chlorophyll protein complex-cyanine 5.5-conjugated streptavidin; and purified anti-CD3e (145.2C11). The following monoclonal antibodies were from eBiosciences: phycoerythrin-labeled anti-CD27 (LG.7F9), phycoerythrin-indotricarbocyanine-labeled anti-CD3e (145.2C11), fluorescein isothiocyanate- or allophycocyanin-labeled anti-IL-17 (eBio17B7), and allophycocyanin-labeled anti-CD27

(LG.7F9). Phycoerythrin-labeled mCD1d tetramer loaded with the glycolipid PBS57 was from the National Institutes of Health Tetramer Core Facility.

Flow cytometry and cell sorting. Information on these procedures is available in the Supplementary Methods online

Cell culture—For preactivation regimens, cells were incubated for 4 d on plate-bound anti-CD3e (10 mg/ml) with IL-2 (100 U/ml; Sigma), with or without TGF- β (5 ng/ml; R&D Systems). Where indicated, IL-6 (20 ng/ml), IL-21 (100 ng/ml), IL-23 (20 ng/ml) or neutralizing anti-IFN- γ (10 mg/ml) was also added to the medium. For analysis of proliferation, flow cytometry—sorted cells were stained by incubation for 12 min at 37 °C with 5 mM CFSE (carboxy-fluorescein diacetate succinimidyl ester; Molecular Probes). Reactions were quenched by washing with ice-cold RPMI medium supplemented with 10% (vol/vol) FCS. CFSE-labeled responder cells (5×10^4) were cultured in 96-well round-bottomed plates in the presence of antigen-presenting cells (1×10^5 erythrocyte-depleted splenocyte samples treated with mitomycin C (Sigma)) and various doses of anti-CD3e. After 3 d, the proliferation of responder cells was assessed by flow cytometry.

FTOC

The medium for FTOC was RPMI-1640 medium with 10% (vol/vol) FCS, 50 mM β -mercaptoethanol, L-glutamine, nonessential amino acids, 10 mM HEPES, pH 7.2, penicillin and streptomycin. C57BL/6 fetal thymic lobes at E15 were cultured for various times in FTOC medium on nucleopore filters (Whatman) and then were analyzed by flow cytometry. Cultures were provided fresh medium every 3 d. For reaggregate thymic organ culture, E15 thymic lobes treated with 1.35 mM 2-deoxyguanosine (Sigma) were digested for 30 min at 37 °C to a single-cell suspension with 0.125% (vol/vol) trypsin (Invitrogen). Reaggregates were formed by the centrifugation of 7×10^5 stromal cells with 1×10^3 to 10×10^3 sorted $\gamma\delta^{25,27+}$ cells and pipetting of the pellet onto a nucleopore filter in FTOC medium. Cultures were incubated 7–10 d. For CD27 stimulation, a fusion protein of recombinant CD70 and immunoglobulin (10 mg/ml; provided by A. Al-Shamkhani) was added to the cultures.

Adoptive transfer—Sorted cells were stained with CFSE as described above and were injected intravenously into RAG-2-deficient mice (3×10^5 to 5×10^5 cells per mouse). Splenocytes from injected mice were collected after 3 d (for analysis of short-term division and stability of CD27 expression) or 3 weeks (for analysis of long-term stability of CD27 expression).

Malaria infection. Mice were infected as described³¹

RT-PCR. RNA was extracted with TRIzol reagent (Invitrogen) and was treated with RNase-free RQ1 DNase (Promega). RNA concentrations were measured with a Nanodrop ND 1000 (Nucliber). Superscript II reverse transcriptase was used according to the manufacturer's protocol (Invitrogen) for the production of cDNA in a reaction volume of 20 μ l. Primers for quantitative real-time RT-PCR or semiquantitative RT-PCR (Supplementary Table 1 online) were designed with the Primer3 program or with the Universal ProbeLibrary Assay Design Center (Roche Applied Science). For semiquantitative RT-PCR, cDNA was amplified as described¹⁸ in a MyCycler thermocycler (BioRad). For quantitative PCR,

cDNA was amplified in a reaction volume of 50 µl, with half the volume (25 µl) being SYBR Green PCR master mix (Applied Biosystems), in a Rotor Gene 6000 (Corbett Research). Reactions consisted of 2 min at 50 °C and 10 min at 95 °C, followed by 50 cycles of 15 s at 95 °C and 1 min at 60 °C, followed by 50 s at 95 °C. Expression was normalized to that of *Eef1a1* (encoding eukaryotic translation elongation factor 1a1; called 'Eef1' here) or *Hprt1* (encoding hypoxanthine guanine phosphoribosyl transferase) and is presented as percent of maximum expression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

CD27 expression defines distinct subsets of peripheral $\gamma\delta$ T cells. (a) Flow cytometry of cells obtained from various tissues (above plots) of adult wild-type C57BL/6 mice, stained for CD3 ϵ , CD27 and TCR $\gamma\delta$ and gated on CD3 ϵ ⁺TCR $\gamma\delta$ ⁺ cells. a+i(LN), axillary and inguinal lymph nodes; mLN, mesenteric lymph nodes. Data are representative of three to eight independent experiments with three to five mice each. (b) Flow cytometry of splenocytes stained for CD3 ϵ , CD27 and TCR $\gamma\delta$, plus CD62L or CD44 and gated on CD3 ϵ ⁺TCR $\gamma\delta$ ⁺CD27⁺ cells ($\gamma\delta$ ²⁷⁺) and CD3 ϵ ⁺TCR $\gamma\delta$ ⁺CD27⁻ cells ($\gamma\delta$ ²⁷⁻). Data are representative of three independent experiments with three to five mice each. (c) Flow cytometry of $\gamma\delta$ ²⁷⁺ or $\gamma\delta$ ²⁷⁻ T cells sorted from pooled spleens and lymph nodes and cultured separately for 3 d with antigen-presenting cells and anti-CD3 ϵ (1 mg/ml; left) or injected separately intravenously into RAG-2- deficient mice and analyzed after 2 weeks (right), then recovered, stained for CD3 ϵ , CD27 and TCR δ and gated on CD3 ϵ ⁺TCR δ ⁺ cells. Data are representative of three independent experiments. (d-f) Flow cytometry analyzing the proliferation of $\gamma\delta$ ²⁷⁺ or $\gamma\delta$ ²⁷⁻ T cells sorted from pooled spleens and lymph nodes, labeled with CFSE and cultured separately with antigen-presenting cells in presence of various doses of anti-CD3 ϵ (horizontal axis; d) or injected intravenously into RAG-2- deficient mice separately (e) or together at ratio of 1:1 (f), and assessed on the basis of CFSE-dilution kinetics at day 3. Data are representative of four independent experiments.

Figure 2.

CD27 expression segregates IFN- γ - versus IL-17-producing $\gamma\delta$ cells in naive and malaria-infected mice. (a) Quantitative real-time PCR of the expression of *Ifnγ* and *Il17* by $\gamma\delta^{27+}$ and $\gamma\delta^{27-}$ T cells sorted from pooled spleens and lymph nodes of naive C57BL/6 mice, normalized to *Ea1* expression and presented as the percent of maximum expression of the gene of interest. (b) Intracellular staining for IFN- γ or IL-17 in $\gamma\delta^{27+}$ and $\gamma\delta^{27-}$ T cells sorted from pooled spleens and lymph nodes of naive or *Plasmodium berghei* ANKA-infected C57BL/6 mice (at day 4 after infection), then stimulated for 4 h with PMA and ionomycin. FSC, forward scatter. Numbers adjacent to outlined areas indicate percent IFN- γ^+ cells (top row) or IL-17 $^+$ cells (bottom row). (c) Quantitative real-time PCR of the expression of *Il2rb* (encoding CD122) and the gene encoding SCART2 (National Center for Biotechnology Information identification number 5830411N06Rik; called 'Scart2' here) by $\gamma\delta^{27+}$ and $\gamma\delta^{27-}$ T cells, as described in a. (d) Flow cytometry of splenocytes from naive mice, stained for CD3 ϵ , CD27, TCR $\gamma\delta$ and CD122 and gated on CD3 ϵ^+ TCR $\gamma\delta^+$ cells. Numbers in quadrants indicate percent cells in each. (e) Absolute number of total $\gamma\delta$ T cells (left) and percent CD69 $^+$ cells (middle) and CD44 hi CD62L lo cells (right) among $\gamma\delta^{27+}$ and $\gamma\delta^{27-}$ T cells from splenocytes obtained from naive mice (0) and mice at days 3 and 6 after infection with *P. berghei* ANKA and stained for CD3 ϵ , CD27, TCR $\gamma\delta$ and CD69, plus CD62L or CD44. (f) Absolute number of total $\gamma\delta$ cells (left), $\gamma\delta^{27+}$ T cells (middle) and $\gamma\delta^{27-}$ T cells (right) among splenocytes obtained from naive or naive mice and mice at day 4 after infection with 'titrated' doses of *P. berghei* ANKA (5×10^4 , 5×10^5 or 5×10^6 parasites per mouse; to achieve variable parasitemia) and stained for CD3 ϵ , CD27 and TCR $\gamma\delta$, presented relative to parasitemia (percent *P. berghei* ANKA in blood). Each symbol represents an individual mouse ($n=4$ mice/group). Data are representative of three independent experiments (a,c,e; error bars, s.d.; $n=3$ samples of four to five mice, pooled) or four to five (b), three (d) or two (f) independent experiments.

Figure 3.

Constitutive expression of IL-17 and RORγt by γδ²⁷⁻ cells. (a) Quantitative real-time PCR analysis of *Il17Ela1* expression by CD4⁺CD25⁻ αβ T cells (4⁺25⁻) and γδ²⁷⁺ or γδ²⁷⁻ T cells sorted from pooled spleens and lymph nodes of C57BL/6 mice and assessed without activation (Fresh) or after activation for 4 d with plate-bound anti-CD3ε plus IL-2, with (+) or without (-) TGF-β, presented as described in Figure 2a. Data are representative of three independent experiments (error bars, s.d.; n=3 mice). (b) Intracellular staining of IFN-γ and IL-17 in CD27⁺ or CD27⁻ γδ and CD4⁺CD25⁻αβ⁻ T cells sorted from pooled spleens and lymph nodes from C57BL/6 mice, assessed without preactivation (Fresh) or after preactivation for 4 d on plate-bound anti-CD3ε and IL-2 (PreAct) with or without TGF-β, followed by stimulation with PMA and ionomycin. Data are representative of four independent experiments. (c) Intracellular staining for IL-17 and GFP in CD4⁺CD25⁻, γδ²⁷⁺ and γδ²⁷⁻ T cells isolated from pooled lymph nodes of RORγt-GFP mice. Data are representative of triplicate experiments with five mice each. (d) Intracellular staining for IFN-γ and IL-17 in flow cytometry-sorted γδ²⁷⁻ and γδ²⁷⁺ splenocytes activated in vitro for 3 d with anti-CD3ε and IL-2 in the presence (+) of various cytokines (above plots). Data are representative of three independent experiments. Numbers in quadrants (b–d) indicate percent cells in each. (e) Staining for surface Vγ1 or Vγ4 on and intracellular IL-17 in γδ²⁷⁺ and γδ²⁷⁻ cells sorted from pooled spleens and lymph nodes of C57BL/6 mice, preactivated for 4 d on plate-bound anti-CD3ε plus IL-2 and TGF-β, then stimulated with PMA and ionomycin. Data are representative of three independent experiments.

Figure 4.

Both $\gamma\delta^{27+}$ and $\gamma\delta^{27-}$ cells originate from common CD27⁺CD25⁺ thymic $\gamma\delta$ progenitors. (a) Flow cytometry of thymocytes of C57BL/6 mice (n=3) gated on single-positive stage 4 cells (SP4; CD4⁺CD8⁻) or $\gamma\delta$ cells (TCR $\gamma\delta$ ⁺CD3 ϵ). Dashed line, isotype control. (b) Flow cytometry of thymocytes (left) and splenocytes (right) from C57BL/6 mice, stained for CD3 ϵ , CD27, CD25 and TCR $\gamma\delta$, gated on CD3 ϵ ⁺TCR $\gamma\delta$ ⁺ cells. Numbers in plots indicate percent cells in each area. (c) Proportion of the subsets defined in b among total $\gamma\delta$ thymocytes from embryonic (E14–E18), newborn (days 1–9 (D0–D9)) or adult (6-week-old (W6)) C57BL/6 mice. (d) Flow cytometry of the cell size of the subsets defined in b. (e) Staining of thymocytes from newborn mice (day 2) with antibodies specific for various markers (below plots), gated on the subsets defined in b. (f) Incorporation of the thymidine

analog BrdU (5-bromodeoxyuridine) by gated subsets (defined in b) of thymocytes obtained from C57BL/6 mice given BrdU daily intraperitoneally and then stained for CD27, CD25, TCR $\gamma\delta$ and BrdU. (g) Intracellular staining of thymocyte subsets (defined in b) with 7-amino-actinomycin D (7-AAD), an indicator of DNA content. 27,25+, $\gamma\delta^{27,25+}$; 27+, $\gamma\delta^{27+}$; 27-, $\gamma\delta^{27-}$. (h) Flow cytometry of $\gamma\delta^{25,27+}$ thymocytes after sorting (Post-sort; left) and after the sorted cells were mixed with thymic stromal cell samples from E15 C57BL/6 mice, depleted of thymocytes and incubated for 7 d (RTOC; right). Numbers adjacent to or in outlined areas indicate percent cells in each. (i) Quantitative RT-PCR analysis of the expression of Ifn γ and Il17 in various cell subsets, presented as described in Figure 2a. (j) Semiquantitative RT-PCR analysis of the expression of Rorc, Runx1 and Tbx21 in various cell subsets, normalized to Actb expression (encoding b-actin). For Rorc and Runx1, cells were sorted from pooled organs of over four mice; for Tbx21, $\gamma\delta$ thymocyte subsets were purified from pooled FTOCs at day 6 (n>5 cultures). 4⁺8⁺, CD4⁺CD8⁺. Data are representative of three (a,d,e,h,j) or over ten (b) independent experiments or three (c,g,i) or two (f) independent experiments with three mice (f,g,i) or over three mice (c; error bars (c,f,g,i), s.d.).

Figure 5.

CD27 controls the functional potential of $\gamma\delta$ T cells. (a) Staining of TCR $\gamma\delta$ and CD3 surface proteins on wild-type (WT) C57BL/6 and CD27-deficient ($Cd27^{-/-}$) thymocytes (left), and absolute number of TCR $\gamma\delta$ ⁺CD3⁺ thymocytes (right). Data are representative of five independent experiments (error bars, s.d.; n=3 mice). (b) Intracellular staining of various cytokines (left margin) in sorted total $\gamma\delta$ T cells obtained from pooled spleens and lymph nodes of wild-type and CD27-deficient mice and stimulated with PMA and ionomycin. Right, percent of total $\gamma\delta$ T cells. NS, not significant. *, P<0.05 (Student's t-test). Data are representative of four independent experiments with four to five mice each. (c,d) Absolute number of $\gamma\delta$ T cells (c) or IFN- γ ⁺ or IFN- γ ^{hi} $\gamma\delta$ T cells (d) among spleen cells of naive or P berghei ANKA-infected wild-type or $Cd27^{-/-}$ mice. D4PI, day 4 after infection. *, P<0.05

(Student's t-test). Data are representative of two independent experiments (error bars, s.d.; n=5 mice).

Figure 6.

CD27 signals regulate the differentiation of $\gamma\delta$ thymocytes. (a) Quantitative RT-PCR analysis of the expression of *Ifng* and *Tnfa* in $\gamma\delta^{27+}$ cells sorted from TCR α -deficient mice (a) and TCR β -deficient mice (b), presented as described in Figure 2a. Data are representative of two independent experiments (error bars, s.d.; n=3 mice). (b) Gene expression and fluorescence intensity of microarray analysis of wild-type and CD27-deficient (KO) $\gamma\delta$ T cells. Black, genes with differences in expression of over twofold (WT/KO); gray, all other genes analyzed. $P < 0.00003$ (adjusted) for genes selected as having differences in expression. Data are representative of two independent experiments. (c) Semiquantitative RT-PCR analysis of the expression of various genes (left margin) in total $\gamma\delta$ thymocytes from wild-type or CD27-deficient mice. Data are representative of two independent experiments with triplicate samples of cells purified from four to six mice. (d) Quantitative RT-PCR analysis of the expression of *Ltbr* in total $\gamma\delta$ thymocytes sorted from wild-type and CD27-deficient mice, presented as described in Figure 2a. *, $P < 0.001$ (Student's t-test). Data are representative of two independent experiments (n=3 mice). (e) Semiquantitative RT-PCR analysis of the expression of various genes (left margin) in $\gamma\delta$ subsets from wild-type thymi. Data are representative of two independent experiments with triplicate samples of cells purified from four to six mice. (f) Quantitative RT-PCR analysis of the expression of *Ifng* and *Il7* in $\gamma\delta$ T cell subsets sorted at day 7 from FTOCs treated with a soluble recombinant fusion protein of CD70 and immunoglobulin (10 mg/ml; sCD70 +) or control human immunoglobulin G (sCD70 -), presented as described in Figure 2a. Data are representative of two independent experiments (error bars, s.d.; n=3 mice). *, $P < 0.05$ (Student's t-test). (g) Flow cytometry of FTOCs at day 7, treated with medium alone, anti-

CD3 ϵ (0.67 mg/ml), anti-keyhole limpet hemocyanin (0.67 mg/ml; isotype control) or cyclosporin A (0.5 mM). Data are representative of two independent experiments with a pool of three E14 thymic lobes each.

Table 1
Transcription in wild-type C57BL/6 and CD27-deficient $\gamma\delta$ T lymphocytes

Expression of genes linked to T cell biology, presented as expression in wild-type cells relative to expression in CD27-deficient $\gamma\delta$ T cells ('fold' change). Gene ID, identification number in the National Center for Biotechnology Information database. Full cDNA microarray data, ArrayExpress accession number E-NCMF-23. $P=0.00003$ (threshold for microarray data analysis). Data are representative of two independent experiments.

Gene ID	Symbol	Description	Function	Expression
21940	<i>Tnfrsf7</i>	CD27 (TNF-RSF7)	Activation	21.11
17000	<i>Ltbr</i>	Lymphotoxin- β receptor (TNF-RSF3)	Cytokine receptor	9.00
12477	<i>Ctla4</i>	CTLA-4 coreceptor	Activation	7.01
12916	<i>Crem</i>	CREM/ICER	Transcription	5.10
18227	<i>Nr4a2</i>	Nuclear orphan receptor	Transcription	4.66
19735	<i>Rgs2</i>	Regulator of G protein signaling	Signaling	4.17
50778	<i>Rgs1</i>	Regulator of G protein signaling	Signaling	3.86
21943	<i>Tnfsf11</i>	RANK ligand (TNF-SF11)	Cytokine	3.66
12487	<i>Cd28</i>	CD28 coreceptor	Activation	3.36
19734	<i>Rgs16</i>	Regulator of G protein signaling	Signaling	3.25
16364	<i>Irf4</i>	Interferon-regulatory factor	Transcription	2.30
16478	<i>JunD</i>	JunD	Transcription	2.20
16172	<i>Il17ra</i>	IL-17 receptor α -chain	Cytokine receptor	0.14