Adaptive Immune-like γ/δ T Lymphocytes Share Many Common Features with Their α/β T Cell Counterparts

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Adaptive Immune-like γ/δ T Lymphocytes Share Many Common Features with Their α/β T Cell Counterparts

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To better apprehend γ/δ T cell biological functions in the periphery, it appears crucial to identify markers highlighting the existence of distinct phenotypic and functional γ/δ T cell subsets. Interestingly, the expression of CD44 and Ly-6C subdivides murine peripheral γ/δ T cells into several subsets, with Ly-6C− CD44hi γ/δ T cells corresponding to the IL-17–producing CD27− γ/δ T cell subset exhibiting innate-like features. By comparing the other subsets to naive and memory CD8α/β T cells, in this study, we show that Ly-6C− or + CD44hi and Ly-6C+CD44hi γ/δ T cells greatly resemble, and behave like, their CD8α/β T cell counterparts. First, like memory CD8α/β T cells, Ly-6C+CD44hi γ/δ T cells are sparse in the thymus but largely increased in proportion in tissues. Second, similarly to naive CD8α/β T cells, CD44hi γ/δ T cells are poorly cycling in vivo in the steady state, and their proportion declines with age in secondary lymphoid organs. Third, CD44hi γ/δ T cells undergo spontaneous proliferation and convert to a memory-like Ly-6C+CD44hi phenotype in response to lymphopenia. Finally, CD44hi γ/δ T cells have an intrinsic high plasticity as, upon appropriate stimulation, they are capable of differentiating nonetheless into Th17-like and Th1-like cells but also into fully functional Foxp3+ induced regulatory T cell-like γ/δ T cells. Thus, peripheral CD27− γ/δ T cells, commonly considered as a functionally related T cell compartment, actually share many common features with adaptive α/β T cells, as both lineages include naive-like and memory-like lymphocytes with distinct phenotypic, functional, and homeostatic characteristics. The Journal of Immunology, 2015, 195: 000–000.

The γ/δ T cells are a well-conserved T cell population throughout evolution and across species. Indeed, in every vertebrate in which T cell ontogeny has been examined, γ/δ T cells appear to be the first T cells to develop (1, 2). γ/δ T cells are unique and distinct from other lymphocyte subsets, such as NK cells, B cells, and α/β T cells, in that they combine adaptive features with rapid, innate-like responses that allow them to play an important role in all phases of an immune response.

Unlike α/β T cells, γ/δ T cells are produced in the thymus in sequential waves during defined periods of fetal, neonatal, or adult life (3). Most γ/δ T cells are not restricted to the recognition of peptides bound to conventional MHC molecules, which, once again, distinguishes them from the great majority of α/β T cells. Indeed, it has been shown that γ/δ T cells are reactive either to self-MHC molecules (4), to non–peptide-binding MHC class IIb molecules such as mouse H2-T10 and H2-T22 (5, 6), or to stress-induced proteins with or without conformational similarities with MHC molecules (7, 8). However, the paucity of the present knowledge on γ/δ TCR ligands confounds the full dissection of γ/δ T cell activation processes and thus compromises the in-depth characterization of the functions of these cells in the periphery. To overcome this, a strong effort has been made recently to identify markers highlighting the existence of distinct phenotypic and functional T cell subsets within the peripheral γ/δ T cell compartment (9, 10). In secondary lymphoid organs, this distinction appears predominantly based on γ/δ T cell cytokine production profiles and the expression of specific surface markers. Indeed, peripheral γ/δ T cells are currently subdivided into two subsets depending on their expression of CD27 and according to their ability to produce IL-17 or IFN-γ (11–14). In this way, robust IFN-γ production is associated with a CD27+ phenotype, whereas secretion of IL-17 is restricted to CD27− γ/δ T cells (11). This phenotypic and functional dichotomy is established during thymic development, as γ/δ T cells that do not express the costimulatory receptor CD27 are considered to emerge by default from thymic progenitor cells receiving only weak TCR signals during development, in contrast to the strong signals required for CD27+ γ/δ T cell generation (15, 16). However, in analogy to the α/β T cell compartment, the existence of naive or memory γ/δ T cells remains to be determined.

In this study, we show that murine peripheral γ/δ T cells can be subdivided into four subsets according to CD44 and Ly-6C expression. Ly-6C− CD44hi γ/δ T cells correspond to the CD27− IL-17+ γ/δ T cell subset that exhibit innate-like features (12). By comparing the other subsets to naive and memory CD8α/β T cells, we found that the CD27+ γ/δ T cell compartment include naive-like and memory-like lymphocytes that share many phenotypic, functional, and homeostatic characteristics with their adaptive α/β T cell counterparts.

Materials and Methods

Mice

C57BL/6 mice (CD45.2) were obtained from Charles River Laboratories. C57BL/6 CD45.1 mice, C57BL/6 CD3ê−− mice (17), and C57BL/6 CD3ê−− MHC class II−− mice (18) were maintained in our own animal facilities, under specific pathogen-free conditions. C57BL/6 Foxp3-GFP CD45.2 mice were initially obtained from Dr. Bernard Malissen (Centre
d’Immunologie de Marseille-Luminy, France) (19). Donor and recipient mice were sex-matched. Six- to 8-wk-old mice were used for experiments unless otherwise indicated (Fig. 5). Animal housing, care, and research were carried out in accordance with the guidelines of the French Veterinary Department. All procedures performed were approved by the Paris-Descartes Ethical Committee for Animal Experimentation (decision CEEA34.BL.002.12).

Cell suspensions

Peripheral lymph nodes (pLNs), mesenteric LNs (mLNs), cervical LNs, Peyer’s patches (PP), spleen, and thymus were homogenized and passed through a nylon cell strainer (BD Falcon) in RPMI 1640 Glutamax (Life Technologies) supplemented with 10% FCS (Biochrom) for adoptive transfer and cell culture (LNs only) or in 5% FCS and 0.1% NaN3 (Sigma-Aldrich) PBS. Each cell-staining reaction was preceded by a 15-min incubation with a mixture of anti-CD8a (FcγRIIb; 2.4G2), 0.5 μg/ml ionomycin (Sigma-Aldrich), and 10 μg/ml brefeldin A (Sigma-Aldrich) for 2 h at 37°C. Cells were then stained for surface markers, fixed in 2% paraformaldehyde in PBS, and permeabilized with 0.5% saponin, followed by labeling with specific cytokine Abs. For Foxp3 and Ki-67 intracellular staining, the Foxp3 Staining Buffer Set (eBioscience) was used. Multicolor immunofluorescence was analyzed using BD LSRII and BD Fortessa cytometers (BD Biosciences). List-mode data files were analyzed using Diva software (BD Biosciences). Data acquisition and cell sorting were performed at the Cochin Immunobiology facility.

Adaptive transfer of γδ T cells

γδ T cells were purified from LNs (pooled superficial cervical, axillary, brachial, inguinal, and mLNs) of C57BL/6 CD45.1 mice by incubating cell suspensions on ice for 20 min with a mixture of anti-CD8a (53-6.7), anti-CD4 (GK1.5), anti-CD11b (Mac-1), and anti-CD19 (1D3) Abs obtained from hybridoma supernatants and then with magnetic beads coupled to anti-CD8ε (Dynal Biotech). Purified cells were then labeled with biotinylated anti-Ly-6C (AL21), PE-conjugated anti-NK1.1 (PKR16), anti-TCRβ (HST-597), anti-B2A2 (RA3-6B2), anti-CD11b (M-170), anti-CD11c (HL3), anti-CD8β (53-7.8), and allophycocyanin-conjugated anti-CD44 (IM7), all from BD Biosciences, and Pacific Blue-conjugated streptavidin (Invitrogen). CD44hiLy6C-, CD44hiLy6C+ (Invitrogen), CD44hiLy6C- (Invitrogen), and CD44hiLy6C+ γδ T cells were flow cytometry sorted as CD44hiLy6C- γδ T cells and then stimulated for 4 d with immobilized anti-CD3 (clone 145.2C11; 4 μg/ml; obtained from hybridoma supernatants) and anti-CD28 Abs (Thermo Fisher).

In vitro polarization assays

After magnetic enrichment as described above, purified cells from LNs of C57BL/6 Foxp3-GFP mice were then labeled with biotinylated anti-Ly-6C (AL21), PE-conjugated anti-TCRβ/γ (GL5), and allophycocyanin-conjugated anti-CD44 (IM7), all from BD Biosciences, and Pacific Blue-conjugated streptavidin (Invitrogen). CD44hiLy6C-, CD44hiLy6C+, and CD44hiLy6C+ γδ T cells were flow cytometry sorted as GFP+ TCRβ/γ+ cells and then stimulated for 4 d with immobilized anti-CD3 (clone 145.2C11; 4 μg/ml; obtained from hybridoma supernatants) and anti-CD28 (clone 37.51; eBioscience; 4 μg/ml) Abs, in the presence of LEAF-purified anti-IFN-γ neutralizing Abs (R4-6A2; BioLegend) and graded concentrations of oxazolone recombinant human TGF-β1 (Invitrogen) in the presence of 20 ng/ml recombinant mouse IL-6 (R&D Systems) (γδ Treg) to study the ability of γδ T cells to differentiate into γδIFN-γ+ effector cells, stimulation was performed in the presence of graded concentrations of recombinant mouse IL-12 (R&D Systems).

In vitro suppression assay

FACS-sorted Foxp3+ CD44hiTCRβ/γ+ cells or Foxp3+ CD25-CD44hi naive CD4 T cells from LNs of C57BL/6 CD45.1 Foxp3-GFP mice were stimulated for 3 d with coated anti-CD3 and anti-CD28 Abs in the presence of 1 ng/ml TGF-β1 and then allowed to rest for 3 more d in the presence of recombinant human IL-2 (13 ng/ml; R&D Systems). GFP-expressing cells were then flow cytometry sorted, and the suppressive capacities of these highly purified Foxp3-expressing cells were then assessed as previously described (18). Briefly, conventional CD4 T cells (GFP+ CD4+ T cells) were purified from LNs of C57BL/6 CD45.2 Foxp3-GFP mice, labeled with Cell Trace Violet (Invitrogen), and stimulated for 64 h, alone or together with Tregs at various Treg/conv T cell ratios.

TLR stimulation in vitro assay

Flow cytometry–sorted TCRβ/γ+ T cell subsets from LNs of CD45.1 C57BL/6 were stimulated for 16 h in the presence of LPS (100 ng/ml), Pam3CSK4 (1 μg/ml), polyinosinic-polycytidylic acid (Poly I:C; 5 μg/ml), or CpG (1 μg/ml) all from Invitrogen.

Intracellular Ca2+ mobilization assay

Cells were loaded for 30 min at 37°C with the membrane-permeable fluorescent Ca2+ indicator dye Indo-1 AM (Invitrogen) at a concentration of 1 μmol in RPMI 1640 medium with no FCS. Thereafter, cells were stained for surface markers and kept on ice. Before stimulation, cell aliquots were allowed to equilibrate to 37°C for 5 min and then were analyzed by flow cytometry. After acquisition of background intracellular Ca2+ concentrations for 1 min, cells were stimulated with 3 μg/ml hamster anti-CD3ε (145-2C11; obtained from hybridoma supernatants) and then were crosslinked by the addition of 20 μg/ml goat anti-hamster Abs (Thermo Fisher).

Statistics

Data are expressed as mean ± SEM, and the significance between two series of results was assessed using the Student unpaired or paired t test. The p values < 0.05 were considered significant (*p < 0.05, **p < 0.01, and ***p < 0.001).

Results

Heterogeneity of Ly-6C and CD44 expression in peripheral γδ T cells

γδ T cells as well as CD8αβ T cells from LNs of C57BL/6 mice can be subdivided into several subsets according to Ly-6C and CD44 expression (Fig. 1A). More precisely, whereas Ly-6C- and CD44 expression split the CD8+ T cell compartment into three subsets (naive Ly-6C- or Ly-6C+ CD44hi CD8+ T cells and effector/memory Ly-6C-CD44hi CD8+ T cells), these markers allow to subdivide peripheral γδ T cells into four different subsets. Indeed, in addition to Ly-6C+ or Ly-6C-CD44lo cells and Ly-6C-CD44hi cells, a subset of Ly-6C-CD44hi cells can be defined. In the literature, peripheral γδ T cells are currently dichotomized into two subsets according to CD27 and CD44 expression (CD27-CD44+ and CD27+CD44lo/hi γδ T cell subsets; Fig. 1B). We observed that Ly-6C-CD44hi γδ T cells correspond to CD27-CD44hi γδ T cells, whereas the CD27+CD44lo/hi γδ T cell compartment, commonly considered as functionally related, can be subdivided into three different subsets on the basis of CD44 and Ly-6C expression (Fig. 1B). Although the proportion of these newly defined subsets varied strongly according to the analyzed organ, this applied that Ly-6C-CD44hi γδ T cells correspond to CD27+CD44hi γδ T cells, whereas the CD27+CD44lo/hi γδ T cell compartment, commonly considered as functionally related, can be subdivided into three different subsets on the basis of CD44 and Ly-6C expression (Fig. 1B). Although the proportion of these newly defined subsets varied strongly according to the analyzed organ, this applied...
repertoire usage. We then analyzed the Vγ-chain repertoire of the four γδ T cell subsets recovered from LNs and spleen (Fig. 1F). Interestingly, a lower Vγ1.1- and a higher Vγ2-expressing cell proportion could distinguish Ly-6C<sup>−</sup>CD44<sup>hi</sup>γδ T cells from the other three subsets.

Consistent with their CD27<sup>−</sup> phenotype, Ly-6C<sup>−</sup>CD44<sup>hi</sup>γδ T cells were the only cells able to produce IL-17 in response to stimulation (Fig. 2A, 2B). By contrast, the other γδ T cell subsets produced IFN-γ rather than IL-17, with a pattern similar to that observed in their CD8<sup>+</sup>α/β T cell counterparts.

To go further, we then examined the expression of several surface markers by γδ T cells. Once again, Ly-6C<sup>−</sup>CD44<sup>hi</sup>γδ T cells seemed to stand out from all other γδ T cell subsets with high CD25 and low CD5 and CD27 expression levels (Fig. 3A). Similarly to effector/memory Ly-6C<sup>−</sup>CD44<sup>hi</sup>CD8<sup>+</sup> T cells, we found that Ly-6C<sup>−</sup>CD44<sup>hi</sup>γδ T cells expressed high surface amounts of CD122, IL-15Ra, Fas ligand, CD137, and OX40 (Fig. 3B). By contrast, all of these molecules were poorly expressed by either Ly-6C<sup>+</sup> or Ly-6C<sup>+</sup>CD44<sup>lo</sup>γδ T cells, which exhibit a phenotype comparable to that of naive Ly-6C<sup>−</sup>/CD44<sup>+</sup>CD8<sup>+</sup> T cells.

Therefore, phenotypic, Vγ-chain repertoire, cytokine production, and thymic cell distribution analysis suggest that Ly-6C<sup>−</sup>CD44<sup>hi</sup>γδ T cells could result from a T cell lineage distinct from the three other γδ T cell subsets.

**Hyporesponsive TCR signaling in CD44<sup>hi</sup>Ly-6C<sup>−</sup> γδ T cells**

We then examined the capacity of the aforementioned γδ T cell subsets to respond to TCR stimulation by measuring the intracellular calcium increase and compared them to their CD8<sup>+</sup>α/β T cell counterparts (Fig. 4A). We observed strong and similar calcium fluxes in both naive Ly-6C<sup>−</sup> or Ly-6C<sup>−</sup>CD44<sup>lo</sup>α/β T cells and Ly-6C<sup>−</sup> or Ly-6C<sup>−</sup>CD44<sup>lo</sup>γδ T cells and a weaker signal in Ly-6C<sup>−</sup>CD44<sup>hi</sup>CD8<sup>+</sup>α/β and γδ T cell subsets. By contrast, no intracellular calcium mobilization was observed in Ly-6C<sup>−</sup>CD44<sup>hi</sup>γδ T cells (Fig. 4A).

We then examined the sensitivity of γδ T cell subsets to respond to TCR-independent signals such as TLR-mediated stimulation (Fig. 4B). To do so, FACS-sorted γδ T cells were cultured for 16 h in the presence of different TLR ligands (LPS, Pam3CSK4, Poly I:C, or CpG). Cytokine production analysis revealed that the presence of TLR ligands in the culture medium potentiated the IL-17 production capacity of Ly-6C<sup>−</sup>CD44<sup>hi</sup>γδ T cells. By contrast, IFN-γ production by the other three γδ T cell subsets did not appear to be modulated by the presence of these ligands. Thus, these results suggest a heterogeneity of the peripheral γδ T cell
compartment comprising: 1) TCR-hyporesponsive innate-like γδ T cells (Ly-6C-CD44hi γδ T cells); and 2) TCR-responsive γδ T cell subsets (Ly-6C+CD44hi, Ly-6C+CD44lo, and Ly-6C+CD44hi γδ T cells), sharing phenotypic and functional features with their adaptive effector/memory CD44hi or naive CD44loCD8+ α/β T cell counterparts.

Peripheral homeostasis of γδ T cell subsets according to Ly-6C and CD44 expression

We then examined whether we could distinguish various differentiation stages (naive-like or effector/memory-like) within the peripheral γδ T cell pool. Effector/memory α/β T cells have a higher proliferation rate than naive T cells (23). As assessed by KI-67 staining (Fig. 5A, 5B), similar to their effector/memory CD8+ α/β T cell counterparts, a significantly greater proportion of Ly-6C−CD44hi γδ T cells were cycling compared with Ly-6C− CD44lo γδ T cells. Of note, although hyporesponsive to in vitro TCR stimulation, Ly-6C−CD44hi γδ T cells contained an important proportion of cycling cells, which is in agreement with the work of Haas et al. (24), suggesting that IL-17–producing γδ T cells persist in adult mice as self-renewing, long-lived cells.

We then decided to study the impact of ageing in the homeostasis of peripheral γδ T and conventional CD8+ α/β T cell subsets. Indeed, during ageing, an enrichment in the proportion of effector/memory T cells to the detriment of naive T lymphocytes can be observed in the periphery as a consequence of thymic involution.
Of note, when transferred into lymphopenic environment, naïve α/β T cells proliferate strongly and acquire a memory-like phenotype (27, 28). To study the fate of γδ T lymphocytes in response to lymphopenia, FACS-sorted γδ T cell subsets were transferred separately into C57BL/6 CD3e−/− mice (Fig. 6A). Absolute numbers of recovered γδ T cells were then calculated 2 and 3 mo after transfer (Fig. 6B, 6C). We first observed that, after Ly-6C−/− γδ T cell transfer, the absolute numbers of γδ T cells recovered from secondary lymphoid organs were extremely low, suggesting survival and/or migration defects of this γδ T cell subset in this setting. By contrast, after transfer of the three other γδ T cell subsets, the absolute numbers of γδ T cells recovered from the spleen and LNs were higher than the number of cells initially injected, suggesting a proliferation and subsequent expansion of these T cell subsets in response to lymphopenia (Fig. 6B). Of note, we observed that the proportion of Ly-6C−/− γδ T cells recovered from LNs was strongly increasing with age.

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Ly-6C and CD44 expression on recovered γδ T cells was then analyzed (Fig. 6D). First, we noticed that the phenotype of Ly-6C−/− or Ly-6C−/−CD44hi cells was stable over time. In addition, 2 mo after transfer, initially injected Ly-6C−/− CD44hi γδ T cells were still producing IL-17, whereas Ly-6C−/−CD44lo γδ T cells produced IFN-γ (Fig. 6E). By contrast, such a phenotypic stability was not observed for Ly-6C−/− α/β CD44lo γδ T cells. Indeed, all γδ T cells recovered 2 mo after the initial injection of Ly-6C−/− or Ly-6C−/−CD44hi cells were expressing high surface amounts of both Ly-6C and CD44 (Fig. 6D) and gave rise to a proportion of IFN-γ–producing cells, comparable to what can be observed after Ly-6C−/−CD44hi γδ T cell transfer (Fig. 6E).

Thus, these results indicate that, as observed for naïve α/β T cells, CD44hi γδ T cells convert to memory-like CD44hiγδ T cells in response to lymphopenia, suggesting that these three γδ T cell subsets (Ly-6C−/− α/β CD44hi and Ly-6C−/−CD44hi) would actually represent various differentiation stages of a single T cell lineage. CD44lo γδ T cells are highly plastic in vitro

Following activation by APCs in the periphery, naïve α/β T cells can differentiate into a variety of well-documented Th cell subsets, such as Th1, Th2, Th17, or induced Tregs (iTregs), characterized by their cytokine production profiles and specific effector functions (29). Thus, we assessed whether this characteristic feature could also be observed for γδ T cells and first compared the capacity of the different γδ T cell subsets to polarize into Th17-like (IL-17–producing) and Th1-like (IFN-γ–producing) cells in vitro (Fig. 7). FACS-sorted γδ T cell subsets were stimulated with anti-CD3– and anti-CD28–coated Abs in the presence of either IL-6, IL-23, and graded doses of TGF-β (Th17-like conditions, Fig. 7A) or IL-2 and graded doses of IL-12 (Th1-like conditions, Fig. 7B). In these polarization conditions, Ly-6C−/−CD44hi and Ly-6C−/−CD44lo γδ T cell subsets mainly retained their natural effector cell profile, by producing IL-17 or IFN-γ respectively, suggesting that both CD44lo γδ T cell subsets were at an early stage of differentiation. By contrast, Ly-6C−/− and Ly-6C−/−CD44lo γδ T cells were able to differentiate into both IL-17– and IFN-γ–producing cells according to polarization assays. Of note, under Th17-like polarization conditions, part of Ly-6C−/− and Ly-6C−/−CD44lo γδ T cells acquired the expression of the iTreg lineage-defining transcription factor Foxp3.

Therefore, we then compared the capacity of γδ T cell subsets to differentiate into iTreg-like cells in vitro (Fig. 7C). In contrast to CD44lo γδ T cell subsets, low doses of TGF-β were sufficient to induce the expression of Foxp3 by the vast majority of Ly-6C−/− or Ly-6C−/−CD44lo γδ T cells (Fig. 7C). Furthermore, we observed that Foxp3 expression in these cells was correlated with suppressive capacities, as induced Foxp3+ γδ T cells displayed similar suppressive capacities as CD4+ iTregs (Fig. 7D). Taken together,
our data strongly suggest that, as described for naive α/β T cells, CD44lo γ/δ T cells have an intrinsic higher plasticity, illustrated by their capacity to differentiate into Th17-like, Th1-like, or Foxp3+ iTreg-like γ/δ T cells, than their CD44hi γ/δ T cell counterparts.

Discussion
In the last decade, several teams have used the expression level of the CD27 coreceptor to subdivide peripheral γ/δ T cells (11–14). In the present paper, we found that peripheral γ/δ T cells can be subdivided into four subsets, corresponding to two distinct functional γ/δ T cell lineages (i.e., Ly-6C−CD44hi versus Ly-6C+ or + CD44lo and Ly-6C+CD44hi γ/δ T cells) according to CD44 and Ly-6c expression. We first noticed that Ly-6C+CD44hi γ/δ T cells were corresponding to the CD27+ γ/δ T cell subset previously described by others (11, 12). Consistent with their CD27+ phenotype, CD44hiLy-6C− γ/δ T cells seemed to stand out from all other γ/δ T cell subsets through their high constitutive expression of CD25 and their capacity to produce IL-17 (9) but also by their ability to proliferate strongly in secondary lymphoid organs in the steady state, a property that is in agreement with previous results suggesting that IL-17–producing γ/δ T cells persist in adult mice as self-renewing, long-lived cells (24).

Interestingly, Cai et al. (30) reported that dermal γ/δ T cells, which are capable of producing IL-17, were proliferating strongly few days after birth but exhibited a far lower proliferation rate (3%) in adult mice compared with the important proportion of cycling CD44hiLy-6C− γ/δ T cells that we observed in the secondary lymphoid organs of young adult mice. This apparent discrepancy could reflect an impact of γ/δ T cell localization (lymphoid organs versus tissues) on their functional and homeostatic properties. In addition, Shibata et al. (9) previously examined age-related changes in the frequency of IL-17–producing γ/δ T cells recovered from the peritoneal cavity. They observed that the proportion of this γ/δ T cell subset was decreasing from 1 wk
after birth to adult age (17-wk-old mice). In the present paper, we have studied age-related changes in the frequency of Ly-6C+CD44hi γδ T cells recovered from the LNs of young (6–8 wk old), adult (8–10 mo old), or elderly mice (18 mo old) and observed that the proportion of this γδ T cell subset strongly increased in elderly mice when compared with 6/8-wk- or 8/10-mo-old mice. Thus, altogether these data suggest that Ly-6C–CD44hi γδ T cell homeostasis could vary according to cell localization and ageing.

Our study emphasized subdivision in the γδ T cell lineage as analysis of Vγ-chain repertoire, cytokine production, and cell distribution at the thymic level suggest that Ly-6C–CD44hi γδ T cells would result from a distinctive T cell lineage when compared with the other γδ T cells. Functionally, as previously shown by Wencker et al. (12) for CD27+ phenotype, these γδ T cell subsets appeared skewed toward IFN-γ production ex vivo. More interestingly, by comparing these subsets to naive and memory CD8+ α/β T cells, in this study, we reveal important similarities between naive Ly-6C– or Ly-6C+CD44hi CD8+ α/β T cells and Ly-6C+ and Ly-6C–CD44hi γδ T cells as well as between memory Ly-6C+CD44hi CD8+ α/β T cells and Ly-6C+ CD44hi γδ T cells. Indeed, with respect to the expression of several phenotypic markers, Ly-6C–CD44hi γδ T cells look like memory Ly-6C+CD44hi CD8+ α/β T cells. Like memory CD8+ lymphocytes, they will perceive weak TCR signals in the steady state (16, 31).

The activation of adaptive lymphocytes depends on Ag recognition, engagement of costimulatory ligands, and the presence of cytokines. By contrast, innate lymphocytes are able to respond to innate signals alone. For instance, it has been shown that some γδ T cells can produce IL-17 in a TCR-independent manner in response to IL-1β plus IL-23 (32) and TLR2 or Dectin-1 ligands (33). Moreover, it has also been observed that innate TLR/MyD88-dependent signals selectively expand IL-17–producing CD27+ γδ T cells in vivo (13). In this study, we observed that the IL-17 production capacity of Ly-6C–CD44hi γδ T cells was potentiated in the presence of various TLR ligands, strongly supporting the innate-like hallmark of this γδ T cell subset. This result seems contradictory to the work from Ribot et al. (13) as, in their study, the authors did not observe a cis activation of CD27– γδ T cells by TLR agonists in vitro. This discrepancy could reside in the difference of culture duration between their study and ours (2 d versus 16 h). Indeed, the increased production of IL-17 by Ly-6C–CD44hi γδ T cells in response to TLR agonists could only be transitory and not detectable after an extended culture time.

Unlike CD27– γδ T cells, the CD27+ γδ T cell compartment can be subdivided into three different subsets on the basis of Ly-6C and CD44 expression. Consistent with their CD27+ phenotype, these γδ T cell subsets appeared skewed toward IFN-γ production ex vivo. More interestingly, by comparing these subsets to naive and memory CD8+ α/β T cells, we reveal important similarities between naive Ly-6C– or Ly-6C+CD44hi CD8+ α/β T cells and Ly-6C+ and Ly-6C–CD44hi γδ T cells as well as between memory Ly-6C+CD44hi CD8+ α/β T cells and Ly-6C+ CD44hi γδ T cells. Indeed, with respect to the expression of several phenotypic markers, Ly-6C–CD44hi γδ T cells look like memory Ly-6C+CD44hi CD8+ α/β T cells. Like memory CD8+ T cells, Ly-6C–CD44hi γδ T cells recovered 2 and 3 mo after transfer from pLNs, mLNs, or spleen show a progressive increase in their numbers of total γδ T cells recovered from pooled LNs (pLNs and mLNs) and spleen are also represented. Horizontal dotted line corresponds to the initial absolute number (5 × 10⁴) of transferred cells. Each symbol represents an individual mouse. (A) Diagram illustrating the experimental model. (B) Absolute numbers of γδ T cells recovered 2 and 3 mo after transfer from pLNs, mLNs, or spleen. Absolute numbers of total γδ T cells recovered from transferred mice. Each symbol represents an individual mouse. (A) Diagram illustrating the experimental model. (C) Absolute numbers of γδ T cells recovered 3 mo after transfer from PP and intestine of recipient mice. Each symbol represents an individual mouse. (E) IL-17/IFN-γ dot plots for gated TCRγδ+ recovered 2 mo after transfer from pLNs, mLNs, and spleen of representative recipient mice.
α/β T cells, they are sparse in the thymus but largely increased in proportion in tissues. Furthermore, similar to CD8+ α/β T cells, we found that within the γδ T cell compartment, Ly-6C+CD44hi γδ T cells mobilized calcium less efficiently than Ly-6C−or + CD44lo cells upon in vitro stimulation but divided more in vivo in the steady state. In both the α/β and γδ T cell compartments, the proportion of CD44lo cells (naive/naive-like) decreased with age in secondary lymphoid organs, a process that may derive in both cases from thymic involution (25, 26). Finally, as observed for naive α/β T cells (34), Ly-6C−or + CD44hi γδ T cells exhibit a high intrinsic plasticity in vitro, illustrated by their capacity to differentiate efficiently into Th17-like, Th1-like or, more strikingly, into functional Foxp3+ iTreg-like γδ T cells. Altogether, these data strongly suggest that the peripheral CD27+γδ T cell compartment comprises both naive-like and memory-like adaptive cells. Although Ag-specific memory γδ T cell responses following immunization or infection in mice and humans still need to be better characterized, our results appear consistent with previous

**FIGURE 7.** CD44hi γδ T cells are highly plastic in vitro. Flow cytometry–sorted Ly6C−CD44hi, Ly6C+CD44hi, Ly6C−CD44lo, and Ly6C+D44hi γδ T cells from LNs of C57BL/6 Foxp3-GFP mice were stimulated for 4 d with coated anti-CD3 and anti-CD28 in the presence of IL-6 (20 ng/ml), IL-23 (10 ng/ml), and graded concentrations of TGF-β1 (A) or in the presence of graded concentrations of IL-12 (B) or TGF-β1 (C). Proportions of IL-17–, IFN-γ–producing γδ T cells and the proportion of Foxp3+ cells among γδ T cells are shown as a function of TGF-β1 concentration (A and C) or as a function of IL-17 concentration (B). (D) FACS-sorted Foxp3+CD44hi TCRγδ+ T cells or Foxp3−CD25−CD44hi naive CD4 T cells from LNs of C57BL/6 CD45.1 Foxp3-GFP mice were stimulated for 3 d with coated anti-CD3 and anti-CD28 Abs in the absence of 1 ng/ml of TGF-β1 and then let to rest for 3 more d in the presence of IL-2 (13 ng/ml). GFP-expressing cells were then flow cytometry sorted and their suppressive abilities to inhibit the proliferation of Cell Trace Violet (CTV)–labeled conventional CD4 T cells (LN GFP+CD4 T cells from C57BL/6 CD45.2 Foxp3-GFP mice) in response to anti-CD3 Abs in the presence of APCs were tested at various Treg/conventional T cell (Tconv) ratios.
studies describing γ/δ T cell memory-type responses in mice after Staphylococcus aureus infection (35), in macaques following a secondary challenge with bacillus Calmette-Guérin (36), or in humans in a CMV infection context (37).

When transferred into a lymphopenic environment, naïve α/β T cells proliferate strongly and acquire a memory-like phenotype (20, 27, 38). In our study, we observed that naïve-like Ly-6C- and Ly-6C-CD44+ γ/δ T cell subsets were able to undergo lymphopenia-induced spontaneous proliferation and to convert to a memory-like phenotype (Ly-6C+CD44+) while increasing their capacity to produce IFN-γ, suggesting that these three γ/δ T cell subsets would actually correspond to various differentiation stages of a unique T cell lineage. It is well established that interactions with self-peptides or commensal bacterium-derived peptides presented by MHC molecules are required for the lymphopenia-induced spontaneous proliferation of α/β T cells (28, 39). By contrast, the homeostatic resources such as the cytokines [IL-7 and IL-15 (40)] or the TCR ligands driving this process for γ/δ T cells still need to be addressed.

We recently showed that peripheral Tregs and naïve CD4+ α/β T cells can be subdivided into two subsets according to Ly-6C expression and that these newly defined subsets were functionally not equal (34, 41). In this study, we noticed that, in both the α/β CD8+ T cell and γ/δ T cell compartments, CD44+Ly-6C- cells exhibit a greater ability to produce IFN-γ than their Ly-6C+ cell counterparts ex vivo. One can thus wonder whether, as observed for naïve and regulatory CD4+ α/β T cells (34, 41), Ly-6C expression or nonexpression within the naïve α/β CD8+ T cell and the naïve-like γ/δ T cell compartments could reflect distinct functional features.

γ/δ T cells are crucially involved in host immune defense against infections (42) but are also known to have a strong clinical association with various autoimmune diseases such as inflammatory bowel disease (43, 44), rheumatoid arthritis (or collagen-induced arthritis), the murine model of rheumatoid arthritis (45, 46), and multiple sclerosis (or experimental autoimmune encephalomyelitis, the murine model of multiple sclerosis) (32, 47). In addition, there is compelling evidence indicating that γ/δ T cells play an important role in immunity to cancer by sensing and reacting to cellular stress. This has been clearly demonstrated in murine models of spontaneous (48), chemically induced (49), transgenic (50), and transplantable tumors (51, 52). However, it seems that the activity of γ/δ T cells in response to tumors can differ radically according to tumor types or tumor environments (53, 54). This may reflect the high diversity of the γ/δ T cell compartment highlighted in this study.

γ/δ T cells hold promise for adoptive immunotherapy because of their reactivity to bacteria, viruses, and tumors. Although these cells represent a small fraction (<1%) of the peripheral T cell pool, various methodologies (such as aminophosphonates and synthetic phosphoantigens) to expand human Vγ9Vδ2 T cells or immobilized Ag, Abs, or artificial APCs to grow Vδ1 or other non-Vγ9Vδ2 T cells) have been developed to expand these cells ex vivo to achieve clinical benefit (55). These techniques have been transitioned to the clinic for investigational treatments of cancer (56, 57). It has been observed that adoptive transfer and in vivo expansions of different γ/δ T cell subsets (Vγ9Vδ2, Vδ1, or other non-Vγ9Vδ2 T cells) are safe therapeutic modalities and can result in objective clinical responses in the treatment of cancer such as renal cell carcinoma (58), colorectal cancer, and melanoma (59) or leukemia (60).

Future studies aimed at deciphering the diversity of the γ/δ T cell compartment and the molecular mechanisms that control its functional plasticity will be of major interest and would pave the way for the development of novel therapeutic requirements for γ/δ T cell–based immunotherapy.

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Disclosures

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References


