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Porcine γδ T Lymphocytes Can Be Categorized into Two Functionally and Developmentally Distinct Subsets according to Expression of CD2 and Level of TCR

Katerina Stepanova* and Marek Sinkora*

Porcine γδ T cells have two levels of TCRγδ expression. Whereas TCRγδmed cells are mostly CD2CD8− and CD2CD8+, TCRγδhi cells are highly enriched for CD2CD8−. This distribution is independent of bacterial colonization and it is already established in the thymus prior to export of γδ cells to the periphery. Sorting and cultivation experiments revealed that CD2−CD8− γδ cells are unable to acquire CD2 and CD8, whereas CD2− subsets can gain or loose CD8. There is also differential susceptibility for proliferation between CD2+ and CD2− γδ cells. Although CD2−CD8− almost do not proliferate, proliferation of CD2−CD8+ and CD2+CD8+ is substantial. Population of CD2− γδ cells is also absent in CD1+ immature thymocytes. Additionally, subpopulations of CD2+ and CD2− γδ cells in the thymus differ in expression of auxiliary surface molecules such as CD25, CD45RA/RC, and MHC class II. Moreover, TCRγδhi cells can generate TCRγδmed cells but never the opposite. The only exception is the thymus, where a few TCRγδmed cells can be induced to TCRγδhi but only under IL-2 influence. The repertoire of TCRβ is polyclonal in all subsets, indicating that there is the same extent of diversification and equal capability of immune responses. Results collectively indicate that CD2 expression determines two lineages of γδ cells that differ in many aspects. Because CD2− γδ cells are missing in the blood of humans and mice but are obvious in other members of γδ-high species such as ruminants and birds, our findings support the idea that circulating CD2− γδ T cells are a specific lineage. The Journal of Immunology, 2013, 190: 2111–2120.

**Materials and Methods**

**Experimental animals**

Animals used in the study were Minnesota miniature/Vietnam-Asian-Malaysian crossbred piglets bred in Novy Hradec (3, 20). All pigs were healthy and normal at slaughter. Germ-free piglets were kept in isolator units under germ-free conditions at all times and monitored for the unwanted appearance of bacteria. All animal experiments were approved by the Ethical Committee of the Institute of Microbiology, Czech Academy of Science, according to guidelines in the Animal Protection Act.
Preparation of cell suspensions

Cell suspensions were prepared essentially as previously described (3, 21). Briefly, heparinized (20 U/ml; LECITA-Zentiva) blood was obtained by intracardial puncture. Cell suspensions from the spleen and thymus were prepared in cold PBS by carefully teasing the tissues using forceps and then by passage through a 70-μm mesh nylon membrane. In the case of the blood and spleen, lymphocyte fractions were purified using a Histopaque-1077 (Sigma-Aldrich, St. Louis, MO) gradient centrifugation. Before staining for flow cytometry, all cell suspensions were washed twice in cold PBS containing 0.1% sodium azide and 0.2% gelatin from cold water fish skin (PBS-GEL; all chemicals were from Sigma-Aldrich), filtered through a 70-μm mesh nylon membranes, and cell numbers were determined by a hemacytometer.

Immunoreagents

The following mouse anti-pig mAbs, whose source and specificity were described earlier (3, 4, 21), were used as primary immunoreagents: anti-TCRγδ (PPT26, IgG1; PPT16, IgG2b; or PGBL22A, IgG1), anti-CD1 (76-7-4, IgG2a), anti-CD2 (MSA4, IgG2a; or 1038H-5-37, IgM), anti-CD8 (76-2-11, IgG2a; or PT36B, IgG1; VMRD, Pullman, WA), anti-CD25 (K231-3B2, IgG1), anti-CD45RA (FGZ29, IgG1), anti-CD45RB (MIL5, IgG1), and anti-swine MHC class II (MHC-II) leukocyte Ag type (SLA-DR; 1038H-12-34, IgM). Goat polyclonal Abs (pAbs) specific for mouse Ig subclasses labeled with FITC, PE, PE/Cy5 (alternatively PE/Cy7), or allophycocyanin were used as secondary immunoreagents (SouthernBiotech, Birmingham, AL). All immunoreagents were titrated for optimal signal/noise ratios. In the case of indirect subisotype staining, primary anti-swine pAbs and secondary pAbs conjugated with different fluorochromes were then added to the cell pellets in appropriate combinations. After 15 min, cells were washed three times in PBS-GEL and analyzed by flow cytometry. In some experiments, direct subisotype staining was used to elucidate the effect of direct versus indirect staining. Secondary pAbs were tested for cross-reactivity (no primary mAbs) and also for cross-reactivity with primary isotype-mismatched mouse anti-pig mAbs. No background or false staining was observed. In some cases, directly labeled anti-TCRγδ/PE-DY747 mAb (PPT16; Exbio, Prague, Czech Republic) and/or different directly labeled mAbs were used. These were labeled with Zenon labeling technology (Molecular Probes, Eugene, OR) according to a protocol recommended by the manufacturer.

Staining of cells

Staining of cells for flow cytometry was performed as described previously (3, 11, 21) by indirect subisotype staining. Briefly, multicolor staining was done using cells that had been incubated with a combination of three (three-color staining) or four (four-color staining) primary mouse mAbs of different subisotypes. Cells were incubated for 30 min and subsequently washed twice in PBS-GEL. Mixtures of goat secondary pAbs conjugated with different fluorochromes were then added to the cell pellets in appropriate combinations. After 15 min, cells were washed three times in PBS-GEL and analyzed by flow cytometry. In some experiments, direct staining was used to elucidate the effect of direct versus indirect staining. In that case, the procedure was as described above but only one 30-min incubation step was used. In the case of intracellular staining, cells that had been indirectly stained for cell surface molecules were subsequently intracellularly stained using an IntraStain kit according to a protocol recommended by the manufacturer (DakoCytomation, Glostrup, Denmark).

Flow cytometry and cell sorting

Samples were measured or sorted on standard FACSCalibur or FACSaria III flow cytometers, respectively (Becton Dickinson Immunocytometry Systems, Mountain View, CA). In each measurement, 300,000–700,000 events were collected. Sorted cells were collected into 1) inactivated FBS (PAA, Pasching, Austria) in the case of cultivation, or 2) empty tubes in the case of PCR amplification. Electronic compensation was used to eliminate residual spectral overlaps between individual fluorochromes. Forward scatter area/forward scatter width parameters were used for elimination of doublets. PClysis or FACSdiva software (Becton Dickinson Immunocytometry Systems) was used for data processing.

Proliferation assay and CFSE labeling

Proliferation history of cells was determined by CFSE (Sigma-Aldrich) using techniques described previously (22). Briefly, suspensions of 4 × 10^7/ml fresh cells or 1 × 10^7/ml sorted cells in PBS or PBS with 5% FBS, respectively, were stained by 5 μM/ml CFSE solution under vigorous mixing for 5 min. Final suspension was 10% diluted by PBS supplemented with 5% PCS, washed three times in the same diluting solution, resuspended in culture medium, and cultivated. After cultivation, suspensions were stained by indirect subisotype staining as described above and analyzed by flow cytometry.

Cell cultures and stimulation in vitro

Cell cultures were done in RPMI 1640 medium supplemented with 1-glutamine and 25 mM HEPES, 10% FBS, 100 U penicillin, and 0.1 mg/ml streptomycin (all chemicals were from PAA). Final concentration of cells was always set to 2 × 10^6 cells/ml and cells were cultivated with one of the following: 50 ng/ml PMA (Sigma-Aldrich), 100 U/ml porcine rIL-2 (RayBiotech, Norcross, GA), 10 ng/ml porcine rIL-4 (ProSpec, Ness Ziona, Israel), 5 μg/ml Con A (Sigma-Aldrich), or without any stimulation. As a control, a part of the cells was also stored at 4°C. Some combinations of the above-mentioned activators were also used and PMA was sometimes used with 1 μg/ml ionomycin (Sigma-Aldrich). Culturing times were 3, 4, or 7 d.

Confocal microscopy

Confocal microscopy was done to test the effect of cross-linking with secondary pAbs to patching and capping of stained molecules. Cell suspensions from the spleen were washed in cold PBS, followed by incubation for 30 min at 4°C or 37°C with directly labeled or unlabeled primary anti-TCRγδ, anti-CD2, and anti-CD8 mAbs. Afterward, cells were washed twice in PBS and 1) in the case of directly labeled mAbs fixed with 2% paraformaldehyde for 15 min, or 2) in the case of unlabeled mAbs incubated with the appropriate fluorescence-labeled secondary pAbs for an additional 30 min at 4°C or 37°C, washed twice in PBS, and fixed with 2% paraformaldehyde. Resulting cell suspensions were visualized by Olympus IX-81 microscope equipped with SV-1000 confocal system and analyzed by Olympus FV10-ASW 2.0 viewer software (Olympus, Tokyo, Japan). Cell suspensions were also analyzed by flow cytometry for the number of positive cells and expression level of stained molecules.

PCR amplification and CDR3 spectratyping

The diversity in the TCRβ repertoire is overwhelmingly determined by the diversity in the δ-chain third complementary region (CDR3). Thus, separation of CDR3 regions for TCRβ on polyacrylamide sequencing gels provides a clonotypic analysis of porcine γδ T cells showing their level of diversification (19). This procedure for measuring CDR3 polymorphism is called spectratyping and was performed essentially as described previously (12, 13, 19). Briefly, 50,000 or 100,000 sorted cells were immediately after sort dissolved in 0.5 ml TRI Reagent (Sigma-Aldrich). In a particular analysis, only the same amount of sorted cells was used for preparation of RNA and cDNA (by random hexamer primers). Gene segments for VDJ regions of TCRβ (TRDV1) were PCR amplified and efficiency of PCR amplification was checked on agarose gels. Amplified segments were next reamplified only for CDR3 regions using β2m-labeled primers and the product was separated on sequencing gels. Gels were dried and images were obtained by fluorescent image analyzer FLA-7000 (Fujifilm, Tokyo, Japan).

Statistical analysis

Data are expressed as the means ± SD. Differences among the median frequency values for sorted and thereafter cultivated populations at 37°C and 4°C were analyzed by one-way ANOVA and a Dunnett multiple comparison test. Difference between populations of cells originating from one sample were analyzed by a paired t test. All other comparisons were analyzed by an unpaired t test. In all analyses, GraphPad Prism4 software (GraphPad Software, San Diego, CA) was used. A p value <0.05 was considered statistically significant.

Results

γδ T cells have two levels of TCRγδ expression

Natural expression of TCR on porcine γδ T lymphocytes occurs in two densities: medium and high (Fig. 1A). These two subsets are differentially distributed among CD2/CD8 γδ T cells. Although TCRγδhi cells are preferentially CD2+ (either CD2+CD8+ or CD2+CD8−), TCRγδlo cells are mostly CD2− CD8−.

Indirect staining causes patching of molecules on the surface of positive cells but there is no capping, colocalization, induced antigenic modulation, receptor-mediated endocytosis, or activation

Indirect subisotype staining was mostly used reporting this study. Although we can easily control cross-reactivity and nonspecific binding of used secondary pAbs, we cannot rule out potential cross-
linking of key signaling molecules on the surface of cells. For this reason, an effect of direct and indirect staining on the patching and capping of stained molecules on the surface of γδ T cells was studied by confocal microscopy (Fig. 1B). Direct staining always produced uniform circumferential fluorescence of TCRγδ, CD2, and CD8 on γδ T cells independently of cultivation temperature (Fig. 1B, cells B1–B6). In contrast, indirect staining always produced patching of surface molecules (Fig. 1B, cells B7–B12). Formation of patches after cross-linking with secondary pAbs at 4˚C (Fig. 1B, cells B7–B9) indicate that TCRγδ, CD2, and CD8 does not require a dynamic cellular response and was a result of cross-linking alone. This is in agreement with the observation that there was no induced capping and no clear colocalization of TCRγδ/CD2/CD8 (Fig. 1B, cells B7–B9 and cells B10–B12). Moreover, the possibility of induced antigenic modulation and/or receptor-mediated endocytosis by indirect staining was excluded by examination of the same cells by flow cytometry (Fig. 1B). If any of these effects would have taken place, there should have been numerical and/or expression level differences between samples kept at 4˚C and at 37˚C, which were not observed.

The effect of indirect staining on the responsiveness of γδ T cells was also examined in a culture. Table I shows that behavior of γδ T cells that were cultivated either unstained or prestained by indirect staining is comparable. In agreement with confocal microscopy, there are no differences in proliferation activity (Table I, left two columns), modulation of TCRγδ expression level (Table I, right two columns), and/or alteration of CD2/CD8 phenotype (data not shown). This type of analysis also allows examination of the effect of various activators and their combinations. The results show that proliferation of γδ T cells occurs spontaneously in medium alone and can be significantly (p < 0.01) increased by IL-2 and Con A activation (Table I, left two columns). There was no synergistic effect of IL-2 with either PMA or Con A. Although ionomycin increased proliferation caused by PMA, the differences were not statistically significant. For this reason and also because of low cell viability and peculiar scatter characteristic of γδ T cells after ionomycin treatment, activation by PMA plus ionomycin was not further used in this study. Modulation of TCRγδ expression level (Table I, right two columns) occurs also spontaneously in medium alone and can be significantly (p < 0.01) increased, namely by IL-2 activation. In fact, stimulation by IL-2 was significantly different from any other treatment. Again, addition of IL-2 does not synergize with PMA or Con A.

**Sorted TCRγδmed and TCRγδhi subsets have different features**

Spleen was chosen for most sorting experiments because there is approximately the same CD2/CD8 γδ T cell subsets ratio (12). In any case, confirmation experiments done on other tissues had comparable outputs (data not shown). Sorted cells were cultivated for 2–7 d without any activators (at 4˚C or 37˚C) or supplemented with PMA, IL-2, or IL-4 (Fig. 2). Results show that sorted TCRγδmed cells (Fig. 2A) are unable to increase TCR expression and never become TCRγδhi (Fig. 2C). In contrast, there is always a part of sorted TCRγδhi cells (Fig. 2B) that spontaneously decrease TCR expression (Fig. 2D). This downregulation occurs fast and is detectable within hours. Downregulation of TCRγδ does not occur at 4˚C (Fig. 2D; see data for 4˚C) and is independent of anti-TCRγδ mAb, direct or indirect staining procedures, and the particular fluorochrome used (data not shown). Supplemented activators can enhance the downregulations so that PMA had the highest impact, followed by IL-2 and IL-4 (Fig. 2D).

Analysis of CD2/CD8 subpopulations within TCRγδmed or TCRγδhi sorted cells revealed that sorted TCRγδmed cells are mostly composed of CD2−CD8+ and CD2+CD8− γδ T cells (Fig. 2).
newly generated TCRγδmed cells (Fig. 2G) as well as cells that do not change their TCRγδhi expression (Fig. 2H) had a similar proportion of CD2/CD8 subpopulations as did originally sorted cells (compare Fig. 2G and 2H with 2F). The only exception is a slightly higher representation of CD2⁺CD8⁺ within newly generated TCRγδmed cells (Fig. 2G), which was observed in all experiments.

**Sorted CD2/CD8 subpopulations of γδ T cells alter their phenotype in vitro**

Cultivation of sorted CD2⁺CD8⁻, CD2⁺CD8⁺, and CD2⁺CD8⁻ T cells from spleen with different activators revealed that CD2⁺CD8⁻ γδ T cells are unable to acquire CD2 and/or CD8 molecules in vitro (Fig. 3A–E). However, CD2⁺ subsets of γδ T cells can change their phenotype by gain (Fig. 3F–J) or loss (Fig. 3K–O) of CD8. Whereas a gain of CD8 on CD2⁺CD8⁻ cells is mainly influenced by IL-2 (Fig. 3I), the loss CD8 can largely occur spontaneously (Fig. 3L) but can be increased by activators (Fig. 3M–O).

**CD2/CD8 subsets of γδ T cells have different proliferation activity**

Analysis of several pigs (n = 20) for spontaneous and induced proliferation of γδ T cells revealed that animals can be divided into two groups. The first group is nonproliferative where there is negligible spontaneous proliferation of γδ T cells in RPMI 1640, and where induced proliferation by PMA or IL-4 is also minimal (Fig. 4A). Stimulation by IL-2, however, caused apparent proliferation (Fig. 4A). The second group is proliferative owing to the apparent proliferation of γδ T cells in RPMI 1640 and under stimulation by PMA or IL-4 (Fig. 4B). As in the case of nonproliferative animals, stimulation by IL-2 caused substantial increase of proliferation (Fig. 4B). Analysis of CD2/CD8 γδ T cell subsets for proliferation capacity showed that IL-2 stimulation caused mainly proliferation of CD2⁺CD8⁻ and CD2⁺CD8⁺ γδ T cells whereas CD2⁺CD8⁺ proliferation was substantially reduced, and this occurs in both nonproliferative (Fig. 4C) and proliferative (Fig. 4D) animals. Notably, the same proportion of cycling cells among CD2/CD8 subsets was also observed during spontaneous proliferation in proliferative animals (data not show).

To further investigate the direct effect of stimulation on individual CD2/CD8 subpopulations and to avoid the effect of CD2/CD8 phenotype alteration during cultivation, we performed flow cytometry sorting (Fig. 4E). Cultivation of sorted subpopulations confirmed data from mixed cell cultures (Fig. 4C, 4D) and showed that sorted CD2⁺CD8⁻ γδ T cells always have the lowest proliferative activity, followed by sorted CD2⁺CD8⁻ and CD2⁺CD8⁺ γδ T cells (Fig. 4E). However, because proliferative and nonproliferative animals were used together, the only significant difference was found for IL-2 stimulation.

Proliferation activity of sorted CD2/CD8 subpopulation as shown in Fig. 4E was always lower than for nonsorted cells where other cell types were present. There was also an apparent lack of activation effect by different activators (namely Con A) on proliferation of sorted γδ T cells when compared with nonsorted cells (compare Fig. 4E and Table I). This observation initiated experiments in which pure γδ T cells and their individual CD2/CD8 subsets were sorted, stained by CFSE, and half of them were cultivated alone whereas the second half were cultivated with an isogenic mixture of unsorted cells (Fig. 4F, 4G). Analysis of spontaneous (Fig. 4F) and IL-2–induced (Fig. 4G) proliferation showed that pure sorted γδ T cells always have a lower proliferation capacity than do sorted γδ T cells cultivated with isogenic unsorted cells (Fig. 4F, 4G). The same applies to CD2/CD8 subpopulations of γδ T cells (Fig. 4F, 4G). However, the capacity of

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**FIGURE 2.** Sorted TCRγδmed and TCRγδhi subsets have different features. Splenocytes from 2- to 8-wk-old piglets were stained for TCRγδ and sorted by flow cytometry according to TCRγδ surface density in TCRγδmed (A) and TCRγδhi (B) subsets. Sorted cells were cultivated for 3 d under different conditions and reanalyzed by four-color flow cytometry after restaining with anti-TCRγδ and anti-CD2, anti-CD8, and anti-CD25.

(C–H) Representative analyses of resulting cells from cultivation with IL-2 are shown and percentages of resulting cells for all culture conditions are stated beside each quadrant: C, control cultivation at 4˚C in medium alone; R, cultivation in RPMI 1640 medium only; P, cultivation with PMA; 2, cultivation with IL-2; 4, cultivation with IL-4. (C and D) Expression of TCRγδ/CD25 on resulting cells from sorted TCRγδmed and TCRγδhi splenocytes, respectively. (E and F) Expression of CD2/CD8 on resulting cells from sorted TCRγδmed and TCRγδhi splenocytes, respectively. (G and H) Expression of CD2/CD8 on resulting cells from sorted TCRγδhi splenocytes gated by region R1 for TCRγδmed and R2 for TCRγδhi, respectively [regions are shown in (D)]. The results are representative of nine independent experiments.
individual CD2/CD8 subpopulations of γδ T cells to proliferate remains unchanged: CD2+CD8+ γδ T cells always have the lowest proliferative activity followed by sorted CD2+CD8− and CD2+CD8+ γδ T cells (Fig. 4F, 4G).

Thymic γδ T cells can be divided into three subsets according to level of TCRγδ and the expression of the CD1 molecule

Expression density of TCRγδ on thymocytes is medium and high (Fig. 5A), resembling the periphery. However, thymic γδ T cells contain developing precursors that can be identified by expression of CD1 (5, 11, 13). Whereas CD1+ γδ thymocytes are strictly TCRγδmed, CD1− γδ thymocytes can be TCRγδmed or TCRγδhi (Fig. 5A). These three subsets differ in expression of CD2 and CD8 (Fig. 5B) and also CD25, SLA-DR (MHC-II), and CD45RC/RA (Fig. 5C). TCRγδmedCD1+ thymocytes are characteristic of the highest frequencies of CD2+CD8− and CD2+CD8+ whereas CD2−CD8− cells are almost absent (Fig. 5B). TCRγδmedCD1+ thymocytes also express low amounts of CD45RC, CD25, and SLA-DR molecules (Fig. 5C). In contrast, TCRγδmedCD1− thymocytes contain all CD2/CD8 subpopulations but most abundant are CD2−CD8− cells (Fig. 5B). TCRγδmedCD1− thymocytes also contain low frequencies of CD45RC+, CD25+, or SLA-DR+ cells
Thymic γδ T cells can be divided into three subsets according to level of TCRγδ and the expression of CD1 molecule. Freshly isolated thymocytes were gated by three regions according to their expression of TCRγδ and CD1 (A). Cells inside these three regions were analyzed for proportion of CD2/CD8 subsets (B) or for expression of CD45RC, CD25, or SLA-DR (C). Bars represent mean values and error bars represent ±SD obtained from at least four animals.

(FIG. 5C). Finally, TCRγδhiCD1+ thymocytes are characteristic by a predominance of CD2+CD8− cells (FIG. 5B), which is similar to blood. This subset has a much higher amount of CD45RC expression than TCRγδlow thymocytes and also higher expression of CD25 and SLA-DR (FIG. 5C).

Sorted γδ thymic subsets can change their phenotype during cultivation

Whereas sorted TCRγδmedCD1+ thymocytes (FIG. 6A) keep the same TCRγδ/CD1 phenotype during cultivation, even with activators (FIG. 6B), sorted TCRγδmedCD1− (FIG. 6C) and TCRγδhi CD1− (FIG. 6E) thymocytes can change their phenotype in vitro (FIG. 6D and 6F, respectively). Similarly to their peripheral counterparts, a portion of TCRγδhiCD1− thymocytes (FIG. 6E) becomes TCRγδmedCD1− (FIG. 6F). This downregulation of TCRγδ occurs quickly (it is visible in a day), spontaneously, and it can be increased by activators, especially using PMA (data not shown). In contrast, a small fraction of sorted TCRγδmedCD1− thymocytes (FIG. 6C) can become TCRγδhiCD1− (FIG. 6D). This upregulation of TCRγδ occurs very slowly (few cells can be detected after 3 d but a significant amount at day 7), only by IL-2 stimulation, and could not be observed in any analyzed tissue except the thymus.

When TCRγδmedCD1− thymocytes were sorted (FIG. 6C) and thereafter cultivated (FIG. 6D), we analyzed resulting cells for CD45RC, CD25, and SLA-DR expression (FIG. 6G–I). The results showed that newly generated TCRγδhiCD1+ cells appear only in IL-2-conditioned cultures (FIG. 6G–I, white dashed bars) with a similar phenotype as freshly isolated TCRγδhiCD1+ thymocytes (FIG. 6G–I, gray bar for 4°C) but are significantly different from original TCRγδmedCD1− cells (FIG. 6G–I, white bar for 4°C). Newly generated TCRγδhiCD1+ cells have much higher expression of CD45RC than do parental TCRγδmedCD1− cells and also higher expression of CD25 and SLA-DR. In contrast, TCRγδmed CD1− cells that keep their phenotype during cultivation (FIG. 6G–I, white bars except for 4°C) do not change their CD45RC, CD25, and SLA-DR expression (FIG. 6G–I, compare the white bars of the other four treatment groups with white bar for 4°C). However, when cultures were prolonged to 7 d, resulting TCRγδmedCD1− cells increased their CD45RC, CD25, and SLA-DR expression (data not shown). This increase occurs spontaneously but to a lesser extent than for newly generated TCRγδhiCD1+ cells.

Analysis for CD2/CD8 subpopulations (J) is shown only for IL-2-conditioned culture because it generates TCRγδhiCD1− from TCRγδmed CD1− γδ T cells. *p < 0.05, **p < 0.01, ***p < 0.001.
When TCRγδCD1− thymocytes were sorted (Fig. 6E) and thereafter cultivated (Fig. 6F), we also analyzed resulting cells for CD45RC, CD25, and SLA-DR expression (Fig. 6G–I). The results show that newly generated TCRγδmedCD1− cells have comparable expression of CD45RC, CD25, and SLA-DR (Fig. 6G–I, compare the gray dashed bars of the other four treatment groups with the gray bar for 4˚C). Again, the only exception is CD25 expression in PMA- and IL-2–stimulated cultures. Differential increase in CD25 expression results in a significant difference of CD25 expression between TCRγδhiCD1− and TCRγδmedCD1− cells that are the product of sorted and cultivated TCRγδCD1− thymocytes and for all culture conditions except PMA (Fig. 6H, compare gray and gray dashed bars).

In agreement with the finding that sorted (Fig. 6A) and thereafter cultivated (Fig. 6B) TCRγδmedCD1+ thymocytes keep the same phenotype during cultivation, we mostly did not observe significant changes in expression of CD45RC, CD25, and SLA-DR (Fig. 6G–I, compare the black bars of the other four treatment groups with the black bar for 4˚C). The only exception was increased expression of CD25 in PMA-conditioned cultures.

The last analysis of sorted and cultivated TCRγδCD1 γδ thymocyte subsets includes an examination of proportional distribution of CD2 and CD8 expression on the resulting cells (Fig. 6J). Results show that whereas TCRγδmedCD1− and TCRγδhi CD1− thymocytes did not change their proportions of CD2/CD8 subsets, TCRγδmedCD1− thymocytes are enriched for the CD2+CD8− subset (compare Fig. 6J with Fig. 5B). Analysis of newly generated TCRγδmedCD1− cells originating from TCRγδhiCD1− cells has a decreased proportion of the CD2−CD8− subset to the detriment of CD2+CD8− and CD2+CD8+ subsets (Fig. 6J).

**Analysis of TCR diversity in sorted subpopulations of peripheral and thymic γδ T cells**

The diversity of the TCRδ repertoire in flow cytometry–sorted subpopulations of γδ T cells was studied by CDR3 spectratyping (Fig. 7). Only the V61 family was studied because it is the most varied family (containing >30 members) whereas the rest of the Vδ families (Vδ2–V65) contains one to two members (19). Analyses of sorted TCRγδmed and TCRγδhi (Fig. 7A, 7B) and CD2/CD8 (Fig. 7C–E) subpopulations of γδ T cells isolated from spleen showed that their V61 repertoire is diverse (polyclonal) in all sorted subpopulations. Note that γδ T cells isolated from the blood gave the same results (data not shown). Similar analysis of CDR3 polymorphism for sorted TCRγδCD1 subpopulations of γδ thymocytes (Fig. 7F–H) showed also polyclonal Vδ1 repertoire.

TCRγδmed cells do not lose TCR completely as evidenced by intracellular staining

In some experiments, TCRγδ downregulation occurs to the level resembling TCRγδ− cells. To exclude the possibility that TCRγδ is completely lost from the surface but cells maintain TCRγδ in cytoplasm, we performed intracellular staining for TCRγδ (Fig. 8). Staining of cells by both mAbs PPT16 and PPT26 (recognizing CD3 molecule expressed specifically on γδ T cells) showed a clear subpopulation of intracellularly positive but extracellularly negative cells for TCRγδ (Fig. 8A and 8B respectively, region R2). Further analysis of this population indicated that these cells resemble αβ (Fig. 8D) rather than γδ T lymphocytes (Fig. 8C). Such an explanation is possible mainly because PPT16 and PPT26 are directed against the CD3-specific form exclusively expressed with TCRγδ. This was confirmed by staining for CD3 (Fig. 8E) where the frequency of CD3+ TCRγδ− αβ T cells (20%) corresponds to the proportion of cells in region R2 (Fig. 8A, 8B). It is likely that fixation during intracellular staining changes the CD3 molecule to a form that can be recognized by mAbs against the CD3-specific forms for TCRγδ, such as PPT16 and PPT26. When we used TCRγδ–specific mAb PGBL22A that recognizes the same cells as PPT16 or PPT26 (Fig. 8F), the artifact of intracellular staining was eliminated (Fig. 8G). Moreover, this staining using PGBL22A mAb (Fig. 8G) proved that TCRγδ molecules are not completely lost from the surface during downregulation and that all γδ T cells are included in the analyses. Evidence that PPT16 and PPT26 mAbs recognize the same γδ T cells as PGBL22A is also shown (compare Fig. 8H with 8F).

**Discussion**

Data reported in this study describe populations of porcine γδ T cells differing in expression of their TCR. Evidence that TCRγδ is expressed differently can be found in other reports (15, 16), but to our knowledge this phenomenon was never studied in detail. Porcine TCRγδmed and TCRγδhi cells are distributed differently among CD2/CD8 subsets, which are known to home differentially into various lymphatic tissues. Whereas CD2+CD8− are preferentially TCRγδhi and are enriched in the blood, CD2−CD8+ and...
CD2⁺CD8⁻ are prevalently TCRγδ⁺ and accumulate in other tissues (8). Such findings are in accordance with reported findings in cows, where CD8⁺ and CD8⁻ γδ T cells also exhibit a defined tissue tropism. This unequal distribution throughout different tissues is probably connected to their differential expression of L-selectin and E-selectin ligand (23). Moreover, another work in cows showed that CD8⁺/− γδ T cell subpopulations differ in expression of many other molecules such as galectin-1, prolactin, IgE-dependent histamine-releasing factor, epidermal growth factor, IL-10 or Gro-γ, IL-1, CD44, CD18, and MHC class I (15). Interestingly, although authors of these findings did not study differential level of TCRγδ expression, they noted that CD8⁺ γδ T cells express lower levels of TCR. These findings collectively indicate that γδ T cells with different level of TCRγδ and CD2/CD8 phenotype express auxiliary surface molecules and produce soluble factors that influence the immune system and explain tissue-specific accumulation of γδ T cell subsets. Similar tissue tropism and functional difference between γδ T cell subpopulations can be found also in humans and mice, although most of these studies discriminate γδ T cell subpopulations on the basis of their TCR usage.

Our studies indicate that CD2⁺ and CD2⁻ γδ T cell subsets represent two independent lineages. This is not only because of differential expression of TCR but mainly because whereas CD2⁺ γδ T cells cannot change their CD2/CD8 expression, CD2⁺ γδ T can modulate CD8 expression. There is also differential susceptibility for proliferation between CD2⁺ and CD2⁻ γδ T cells. Although CD2⁻ CD8⁻ almost do not proliferate, proliferation of CD2⁺CD8⁻ and CD2⁺CD8⁺ is substantial. Lower proliferation of CD2⁻ γδ T cells was also observed in mice (24). A population of CD2⁺ γδ T cells is also absent in CD1⁺ immature thymocytes. Moreover, our earlier finding shows that there is a substantial difference between CD2⁺ and CD2⁻ subsets in expression of CD25, CD11b, SWC1, SWC7, MHC-II, and the family of CD45 molecules (12). The conclusion that CD2⁺ and CD2⁻ γδ T cell are two lineages is also supported by the finding that they differ in expression of TCRγ-chains (8). Furthermore, findings in cows show that these two lineages differ in expression of non–TCRγδ cell Ags WC1 and GD3.5, in expression of cell adhesion molecules, and in ability to infiltrate into inflammatory sites (15, 23). In any case, these two lineages are equally capable of immune responses because analysis of CDR3 length polymorphism showed equal diversification of V61 with no restriction of TCR repertoire. Moreover, existence and behavior of these two lineages are independent of gut colonization because germ-free animals were comparable to conventional ones (data not shown).

Human and mice γδ T cells are generally considered CD2⁺ (25–27), thus resembling the CD2⁺ γδ T cell lineage in swine. This is in sharp contrast with porcine CD2⁻ γδ T cells, which are numerous and preferentially reside in the blood. High occurrence of CD2⁻ γδ T cells is not unique to pigs but also for other members of γδ-high species such as sheep (28, 29), cattle (30), and birds (31). These findings together support the idea that CD2⁻ γδ T cells are a specific lineage, and this lineage is missing in the blood of humans and mice. This may also be the reason why γδ T cells in humans and mice constitute a minority of T cells in the circulation. There are only a few examples in which CD2⁻ γδ T cells were found in humans and mice. Such examples are mouse intestinal (24) or vaginal γδ T cells (27). Other examples in humans are some leukemias (32), autoimmune disorders (33), or some particular individuals (26, 34). Loss of circulating γδ T cells in humans and mice can be connected with nonfunctional orthologs of WC1 genes known in ruminants and pigs (35). In contrast, CD2⁺ γδ T cells in humans and mice can be clearly CD8⁺ or CD8⁻ (26, 36). We show in this study that CD8 expression can be modulated on CD2⁻ γδ T cells in both ways. Moreover, our earlier work indicated that a CD2⁺CD8⁻ subset is more mature than CD2⁺CD8⁺ (12). Such a conclusion agrees with other studies in humans where CD8⁺ γδ T cells were shown to have attributes of regulatory cells (37). Also, some studies indicate that CD2⁺ CD8⁺ γδ T cells can be precursors of CD2⁺CD8⁻ γδ T cells (10, 38). These findings collectively indicate that the CD8 molecule can be modulated on the surface according to actual functional status of CD2⁺ γδ T cells.

Sorting experiments show that CD8 expression can be modulated on CD2⁺ γδ T cells and that TCRγδ can be downregulated, which is in agreement with studies in mice (16). However, analysis of resulting cells from cultures of TCRγδ⁺/TCRγδ⁻ sorted cells does not indicate any significant change in composition of CD2/CD8 subpopulation (Fig. 2). This indicates that downregulation of TCRγδ can clearly occur on CD2⁺CD8⁻ γδ T cells in vitro. In contrast, when freshly isolated cells were analyzed, CD2⁺CD8⁻
γδ T cells are practically absent from the TCRγδmed compartment. Therefore, there has to be some factors that keep expression of TCRγδ on the CD2+CD8− subset in vivo. In this study we were unable to identify those factors, although the potential requirement for these factors was identified by addition of unsorted cells, which reconstitute the proliferation activity of pure γδ T cells. Another possibility can be fast turnover of TCRγδ as shown for TCRαβ (17), which can be constitutive or ligand-induced and is dependent on protein kinase C (39), and is maintained in vivo but not in vitro. Such a conclusion would correspond to our observation that partial loss of TCRγδ expression occurs in hours and this cannot be explained by mRNA modulation and/or proliferation of cells. In vitro experiments exclude the possibility that it is caused by early capping and receptor-mediated endocytosis due to cross-linking of molecules by indirect staining. In any case, more experiments are needed to show what is responsible for the high expression of TCR on CD2−CD8− γδ T cells in vivo. Our work indicates that distribution of CD2/CD8 subsets among TCRγδhi and TCRγδmed cells in vivo is tightly regulated and is already established in the thymus prior to export of γδ T cells to the periphery. In connection with possible different regulation of the TCR level in vitro and in vivo, we also cannot exclude the possibility that CD2+ γδ T cells can acquire and/or that CD2− γδ T cells can lose the CD2 molecule in vivo, which was never observed in vitro. This has to be true at least in thymus where CD2− γδ T cells are generated from CD2+CD11a immature thymocytes (5, 11).

Sorted γδ T lymphocytes are clearly less sensitive to activation of proliferation by different activators than unsorted γδ T cells. Because there is no effect of indirect staining on the proliferative response of γδ T cells, decreased proliferation cannot be explained by cross-linking of signaling molecules. More likely, an effect of activators on γδ T cells is indirect and is mediated by other cell types such as sensitive to Con A and IL-2 activation, and these cells are missing in sorted cells. This would agree with studies showing that CD4+ αβ T cells are involved (40). In this respect, IL-2 is the best candidate for this indirect function but it is probably not the only factor, as evidenced by the inability to fully reconstitute proliferative potential of sorted cells.

Thymic γδ T cells are characterized by the occurrence of CD11a immature cells (13) that are always TCRγδmed. Finding that CD11a+ γδ thymocytes are composed exclusively of CD2−CD8− and CD2+CD8− subsets agrees with previous work showing that the earliest precursors are CD1+CD2−CD8− γδ T cells whereas CD1+CD2+ CD8+ γδ T cells are their progeny (5, 11). Apparently, CD8 molecules can be acquired even in immature stages. Also, CD2−CD8− γδ T cells are missing from the CD11a compartment, which indicates that they arise after CD11 downregulation. With regard to CD11a− γδ thymocytes, distribution of CD2/CD8 subsets among TCRγδhi and TCRγδmed cells resembles the periphery. These TCRγδhiCD11a− and TCRγδmedCD11a− subsets are probably exported from thymus differently; that is, whereas TCRγδhiCD11a+ cells (CD2− lineage) are circulating and remain in the blood, TCRγδmedCD11a− cells (CD2+ lineage) are preferentially homing to tissues. However, there is some difference between CD11a+ TCRγδhi and TCRγδmed thymocytes. It appears that TCRγδhi CD11a− thymocytes are fully mature with many CD45RA/RC- and CD25-expressing cells, which is characteristic of the periphery (12). Similarly to the blood, they can also easily and quickly downregulate TCRγδ. In contrast, TCRγδmedCD11a+ are still in some transitional stage as evidenced by the low proportion of CD45RA/RC+ and CD25+ cells. Moreover, there is still a relatively high proportion of CD2−CD8− γδ T cells that probably further mature into TCRγδhi cells. Finally, at least a part of these cells is capable of upregulation of TCRγδ, which can never be detected in peripheral γδ T cells. Significantly, newly generated TCRγδhi cells show high expression of CD45RA/RC, CD25, and MHC-II. These findings indicate that TCRγδmedCD11a− thymocytes may need further maturation and upregulation of CD45RA/RC, CD25, and MHC-II once they reach their final destination in solid tissues.

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References
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