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# The expression of CD25, CD11b, SWC1, SWC7, MHC-II, and family of CD45 molecules can be used to characterize different stages of γδ T lymphocytes in pigs

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ABSTRACT

The expression of selected molecules was chosen to study porcine γδ lymphocytes and their CD2/CD8 subsets in different lymphoid organs *in vivo* and *in vitro*. Results indicate that many γδ T cells can constitutively express CD25 and MHC-II and that the frequency of γδ T cells positive for CD25, CD11b, SWC1 and SWC7 can be increased by stimulation. A diversified TCRδ repertoire was found inside $CD25^+$, $CD11b^+$, $SWC1^-$ and $CD45RA^-$ cells. Ontogenetic studies revealed various age and/or colonization dependency for expression of all studied molecules except of SWC7. Findings generally indicate that CD25 represent an activation molecule that probably marks a functionally distinct subsets, expression of CD11b is perhaps connected to early functions of naive γδ T cells in the periphery, SWC1 is lineage specific marker, SWC7 may represent an activation molecule with intrinsic or transient expression, and the expression of CD45RA/RC most likely defines naive and terminally differentiated cells.



## 1. Introduction

T lymphocytes of γδ lineage are evolutionary conserved cells which develop in the thymus similarly to αβ T cells (Xiong and Raulet, 2007). However, γδ T cells do not need any selection for pre-antigen receptors (like pre-BCR or pre-TCR αβ) and therefore mature faster than αβ T cells, develop without any $TCR^{low}$ transitional stage and are released much earlier to the periphery (Sinkora et al., 2000a, 2005a, 2007; Xiong and Raulet, 2007). At the effector cell level, γδ T lymphocytes share many features with αβ T cells such as potent cytotoxic activity, regulatory functions including ability to induce maturation of dendritic cell, and the capacity to produce a variety of cytokines (Scotet et al., 2008). They also generate and retain immunologic memory. On the other hand, they respond rapidly to infection (Xiong and Raulet, 2007), are probably involved mainly in mucosal immunity (Hiromatsu et al., 1992; King et al., 1999), can act as potent antigen-presenting cells (Brandes et al., 2005; Takamatsu et al., 2002) and their TCR recognizes a broad spectrum of unprocessed or non-peptide antigens without any requirement for MHC co-signalization (Tanaka et al., 1994). They can also be involved in managing of tumors by recognizing stress-induced conserved antigens (Scotet et al., 2008). Due to their nature, γδ T cells are often categorized into unconventional T cells and probably form a unique link between innate and adaptive immune responses.

Swine together with ruminants and birds belongs to the group of γδ high species in which γδ T cells are not preferentially limited to epithelia and may account for >70% of all T cells (Hein and Dudler, 1993). Traditionally, γδ T-cells in swine are subdivided into three subsets based upon their expression of CD2 and CD8 and include $CD2^-CD8^-$, $CD2^+CD8^-$ and $CD2^+CD8^+$ cells (Sinkora et al., 1998, 2005b, 2007; Yang and Parkhouse, 1996, 1997). These individual subsets differ in their homing characteristic (Saalmüller et al., 1990) and cytotoxic activities (de Bruin et al., 1997; Yang and Parkhouse, 1997). Our previous studies revealed basic distribution of porcine γδ T cells (Sinkora et al., 1998), their ontogeny (Sinkora et al., 1998, 2005a), development in the thymus (Sinkora et al., 2000a, 2005a, 2007) and the repertoire diversification of their TCR (Holtmeier et al., 2004). However, none of these studies focused on a detailed analysis of peripheral γδ T cells, and no other studies have been performed to explain differences in the phenotypic profile of γδ T cells subsets.

In this report, the expression of CD25, CD11b, SWC1, SWC7, MHC class II and family of CD45 was studied. These molecules were chosen because of their differential expression on porcine γδ T cells in our preliminary studies. CD25 is α-chain of IL-2 receptor and it is expressed on activated and regulatory T cells

*Abbreviations:* BAL, bronchoalveolar lavage; BM, bone marrow; GF, germ-free; IL-2, interleukin 2; MHC-II, major histocompatibility complex class II; MLN, mesenteric lymph nodes; PBS, phosphate buffered saline; PMA, phorbol-12-myristate-13-acetate; SWC1, SWC7, swine workshop cluster 1,7.

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and on all B cells (Bailey et al., 1992; Käser et al., 2008). CD25 is generally considered as activation marker because its expression increases after stimulation. CD11b belongs to adhesive molecules of the integrin family. It is α subunit of integrin β2 which clusters with CD18. It is a molecule important for adhesion, chemotaxis and diapedesis and is found not only on monocytes and macrophages, but also on other leukocytes including T cells (McFarland et al., 1992). In humans and mice, CD11b is used to distinguish between naive and memory cytotoxic αβ T cells (McFarland et al., 1992). SWC1 and SWC7 are porcine molecules with unknown function. SWC1 is expressed on a majority of leukocytes but not on B cells (Saalmüller et al., 1987). Previous findings showed the down-regulation of SWC1 on $CD4^+$ and $CD8^+$ T cells after activation (Saalmüller et al., 1987). The SWC7 molecule is expressed on B cells and also on a small fraction of T cells (Denham et al., 1998). Its expression can be induced by mitogens (Bullido et al., 1999). MHC class II (MHC-II, SLA-DR) is associated with antigen presentation and is expressed on cells that have such capabilities like macrophages or B cells. Previous studies indicate an age related correlation between MHC-II and CD8α expression on γδ T cells (Sinkora and Butler, 2009). There are at least CD45RA, CD45RAC, CD45RC and CD45R0 spliced forms of CD45 in swine (Schnitzlein and Zuckermann, 1998). Although precaution must be taken with any inter-species comparison, human and mouse studies show expression of CD45RA on naive lymphocytes while CD45R0 marks memory cells (Bullido et al., 1997; Donovan and Koretzky, 1993).

In the present study, we used germ-free piglets that have a virgin immune system (Sinkora et al., 2011) and compare those with their age-matched conventional mates. This comparison addresses the effect of bacterial colonization and environmental antigens on development of γT cells *in vivo*. In addition, a group of conventional adult pigs was evaluated for effect of age. Analysis was done for all γδ T cells as well as their CD2/CD8 subpopulations. In addition, *in vitro* experiments were performed to explain the effect of different activation stimuli and TCR repertoire studies were conducted to show the extent of diversification. To our knowledge, this is the first extensive study of peripheral γδ T cells and their subsets in swine in which we attempted to identify putative lineages, developmental and activation markers.

## 2. Materials and methods

### 2.1. Experimental animals

Animals used in the study were Minnesota miniature/Vietnam–Asian–Malaysian crossbred piglets bred in Novy Hradek (Sinkora et al., 1998, 2003). All pigs were healthy and normal at slaughter. Germ-free piglets were recovered from gilts by Caesarian section at the 112th day of gestation. Gestation age was calculated from the day of mating. After birth, germ-free piglets were kept in isolator units under germ-free conditions at all times and monitored for the unwanted appearance of bacteria. For the purpose of this report, all analyzed animals were divided into three experimental groups: (1) germ-free piglets of age 2–6 weeks ($n = 21$), (2) young conventional piglets of age 2–6 weeks ($n = 10$) and (3) adult conventional pigs of age 1–2 years ($n = 13$). 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.

### 2.2. Preparation of cell suspensions

Cell suspensions were prepared essentially as previously described (Sinkora et al., 2002a, 2003). Briefly, heparinized (20 U/ml) blood was obtained by intracardial puncture. Cell suspensions from the thymus, lungs, mesenteric lymph nodes (MLN), tonsils and spleen were prepared in cold phosphate-buffered saline (PBS) by carefully teasing the tissues using forceps and then by passage through a 70 μm mesh nylon membrane. Lymphocytes from the bone marrow were isolated by washing femurs with PBS. Lymphocytes from the bronchoalveolar lavage (BAL) were isolated by washing lungs with PBS. Cells from the ileum and jejunum were prepared by cutting corresponding part of gut into pieces and incubation in digestion media (RPMI-1640, 100 U/ml collagenase type IV (Sigma–Aldrich, St. Louis, MO), 2% fetal calf serum) at 37 °C for 1 h. Erythrocytes from all suspensions were removed using hypotonic lysis and washed twice in cold PBS. In the case of the blood, bone marrow and spleen, lymphocyte fractions were purified using a Histopaque-1077 (Sigma–Aldrich, St. Louis, MO) gradient centrifugation. In the case of ileum, jejunum, BAL and lungs, the lymphocyte fraction was separated using a 40–80% Percoll (GE Healthcare, Uppsala, Sweden) gradient centrifugation. In the case of cell cultivation, all suspensions were washed twice in PBS while in the case of flow cytometry all 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 Sigma–Aldrich, St. Louis, MO). All cell suspensions were finally filtered through a 70 μm mesh nylon membranes, and cell numbers were determined by hemacytometer.

### 2.3. Immunoreagents

The following mouse anti-pig monoclonal antibodies (mAbs), whose source and specificity were described earlier (Sinkora et al., 1998, 2005b, 2011), were used as primary immunoreagents: anti-TCR γδ (PPT26, IgG1 or PPT16, IgG2b), anti-CD2 (MSA4, IgG2a or 1038H-5-37, IgM), anti-CD8 (76-2-11, IgG2a or PT36B, IgG1), anti-CD11b (MIL4, IgG1), anti-CD25 (K231-3B2, IgG1), anti-CD45RA (FG2F9, IgG1), anti-CD45RC (MIL5, IgG1), anti-MHC-II (MSA3, IgG2a or 1038H-12-34, IgM), anti-SWC1 (K263.3D7, IgG1 or 76-6-7, IgM) and anti-SWC7 (IAH-CC55, IgG1 or 2F6/8, IgG2a). Goat polyclonal antibodies specific for mouse immunoglobulin subclasses labeled with FITC, PE, PE/Cy5 or APC were used as secondary immunoreagents (Southern Biotechnologies Associates, Inc., Birmingham, AL). All immunoreagents were titrated for optimal signal/noise ratios and isotype-matched mouse anti-rat monoclonal antibodies were used as negative controls. No background staining was observed during any experiments.

### 2.4. Staining of cells

Staining of cells for flow cytometry was performed as described previously (Sinkora et al., 1998, 2005a, 2011) by indirect sub-isotype staining. Briefly, multi-color 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 sub-isotypes. Cells were incubated for 30 min and subsequently washed twice in PBS-GEL. Mixtures of goat secondary polyclonal Abs specific for mouse immunoglobulin subclasses that had been labeled with FITC, PE, PE/Cy5 and APC conjugate 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.

### 2.5. Flow cytometry and cell sorting

Samples were measured or sorted on standard FACSCalibur or FACSAriaIII flow cytometers respectively (BDIS, Mountain View, CA). In each measurement, 300–700 thousand events were collected. Electronic compensation was used to eliminate residual spectral overlaps between individual fluorochromes. The PCLysis