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Phenotypic maturation of porcine NK- and T-cell subsets

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ABSTRACT

Detailed information concerning the development of the immune system in young pigs is still rudimental. In the present study, we analyzed changes in phenotype and absolute numbers of natural killer cells, γδ T cells, T helper cells, regulatory T cells and cytolytic T cells in the blood of pigs from birth to six months of age. For each lymphocyte subpopulation, a combination of lineage and differentiation markers was investigated by six-color flow cytometry. Major findings were: (i) absolute numbers of γδ T cells strongly increased from birth until 19–25 weeks of age, indicating an important role for these cells during adolescence; (ii) phenotype of T helper cells changed over time from $CD8\alpha^{-}$SLA-$DR^{-}CD27^{+}$ towards $CD8\alpha^{+}$SLA-$DR^{+}CD27^{-}$ but $CD45RC^{-}$ T helper cells were found immediately after birth, therefore questioning the role of this marker for the identification of T-helper memory cells; (iii) for cytolytic T cells, putative phenotypes for early effector ($CD3^{+}CD8\alpha\beta^{+}$perforin$^{+}CD27^{dim}$) and late effector or memory cells ($CD3^{+}CD8\alpha\beta^{+}$perforin$^{+}CD27^{-}$) could be identified.

1. Introduction

As soon as a fetus leaves the uterus, its immune system is confronted with a multitude of antigens. To alleviate this sudden change, the concept of maternal immunity evolved, where antibodies and maternal immune cells are transferred via milk from the mother to the newborn (Labbok et al., 2004; Ma et al., 2008). Nevertheless, although still in its development, the immune system of neonates can mount first immune responses (Marchant and Goldman, 2005). On the cellular level of the immune system, this leads to activation and, at least for T and B cells, to proliferation and differentiation of hitherto naïve cells. This process is accompanied by an up- or down-regulation of certain molecules, which can be used to identify cell subpopulations in particular developmental stages. For example, in human immunology, a combination of the markers CD27, CD28, CD45RA and CCR7 can be used to discriminate various developmental stages of T helper cells and CTLs from naïve to terminally differentiated (Appay et al., 2008).

Despite the importance of swine as a livestock species and its role as a model for human infectious diseases (Meurens et al., 2012), knowledge about the ontogeny of the cellular immune system in this species is still sparse. Development of αβ T cells in the thymus follows the model of T-cell maturation established in mice and humans (Sinkora et al., 2000). For γδ T cells, which are a prominent T-cell subset in swine, various thymic differentiation pathways have been described (Sinkora et al., 2005). However, in regard to phenotypic changes in the periphery, which are related to differentiation following antigen contact during the first months of life, the knowledge is rather fragmentary. A limited number of studies have analyzed T- and/or B-cell differentiation, but only in combination with one or two differentiation markers (Grierson et al., 2007; Juul-Madsen et al., 2010; Solano-Aguilar et al., 2001; Stepanova et al., 2007; Wen et al., 2011). Also, none of these studies addressed all major T-cell subpopulations, i.e. γδ T cells, T helper cells and CTLs, at the same time. To expand this knowledge, in the present study, we investigated phenotypic changes of NK cells, γδ T cells, T helper cells, regulatory T cells (Tregs) and CTLs in the blood of pigs from the day of birth until six months of age. For each lymphocyte subset, a marker panel for six antigens was designed that consisted of a combination of lineage markers as well as established or putative differentiation markers. Each panel was then applied in six-color flow cytometry (FCM).

For porcine NK cells, an established lineage marker is not yet available. Therefore, NK cells were identified by the phenotype $CD2^{+}CD3^{-}CD8\alpha^{+}$. As putative differentiation or activation markers,

Abbreviations: CD, cluster of differentiation; FCM, flow cytometry; mAb, monoclonal antibody; PBMCs, peripheral blood mononuclear cells; PE, phycoerythrin; Treg, regulatory T cell; SWC, swine workshop cluster.

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perforin, swine leukocyte antigen-DR (SLA-DR) and NKp46 (CD335) were analyzed. Although the latter is considered as a lineage marker for NK cells across mammalian species (Walzer et al., 2007), work from our laboratory recently indicated that not all porcine $CD3^{-}CD8\alpha^{+}$ lymphocytes with functional characteristics of NK cells express NKp46 (Mair et al., 2012).

γδ T cells were identified by a monoclonal antibody (mAb) specific for a CD3 molecule only expressed by porcine γδ T cells (clone PPT16; Yang et al., 2005). As potential differentiation markers, we analyzed CD2 and CD8α expression, since both markers have been used in numerous studies in the past to delineate γδ T cells (summarized in Takamatsu et al., 2006). Moreover, we analyzed expression of swine workshop cluster 5 (SWC5). SWC5 has a molecular weight of 180 kDa and is expressed only on a subset of porcine γδ T cells (Lunney et al., 1994). The orthologous human CD molecule is currently unknown. As further putative differentiation markers, SLA-DR and CD27 were analyzed. For the latter, we could recently show that the mAb clone b30c7, which had been clustered within SWC2, binds to porcine CD27 (Reutner et al., 2012). In humans, CD27 is expressed on all major T-cell subsets, including γδ T cells (deBarros et al., 2011; Nolte et al., 2009). The study by deBarros et al. (2011) could show that human Vγ9Vδ2 T cells partially loose CD27 expression following stimulation with hydroxy-2-methyl-2-buten-4-yl 4-diphosphate (HDMAPP) and IL-2. Moreover, for T helper cells and CTLs it is well established that CD27 expression is lost during differentiation into effector memory T cells (Nolte et al., 2009). Therefore, we analyzed CD27 expression not only for porcine γδ T cells, but also for T helper cells and CTLs (see below).

T helper cells were identified by expression of the lineage markers CD3 and CD4. For potential differentiation, expression of CD8α, SLA-DR, CD45RC and CD27 was analyzed. In the past, a number of studies could show that CD8α and SLA-DR are up-regulated following antigen contact and that expression may persist on T helper cells with memory function (Blanco et al., 2000; Gerner et al., 2006; Revilla et al., 2005; Saalmüller et al., 1987a, 2002). On the opposite, for CD45RC, a partial down-regulation following polyclonal stimulation was described. Also, antigen-specific *in vitro* recall assays indicated that porcine T-helper memory cells do not express this molecule anymore (Saalmüller et al., 2002).

Tregs were identified by a co-expression of CD25 and Foxp3, according to previous studies showing that cells with this phenotype have regulatory properties in swine (Käser et al., 2008b, 2011). For information on lineage commitment, CD4 expression was studied. In regard to potential differentiation, CD8α, SLA-DR and CD45RC expression was analyzed, although precise knowledge on the role of these markers for Treg differentiation is currently lacking (Käser et al., 2012).

Lastly, CTLs were addressed by a combination of the lineage markers CD3, CD8α and CD8β. Knowledge about marker expression in regard to differentiation of CTLs in the pig following antigen contact is rather sparse. Early work indicated SLA-DR expression on CTLs, but the functional role of this expression is not known (Saalmüller et al., 1991; Saalmüller and Maurer, 1994). Perforin expression in porcine CTLs was first described by Denyer et al. (2006), showing an increase in the frequency of $perforin^{+}$ CTLs with age. Therefore, CTLs were investigated for the putative differentiation markers CD27, SLA-DR and perforin.

2. Materials and methods

2.1. Animals

Twelve crossbred (sow: Landrace × Large White; boar: Pietrain) and conventionally reared piglets were used for this study, randomly selected at the day of birth. Until weaning, the piglets stayed at their place of birth – a breeding farm in Lower Austria – where they received vaccinations against *Mycoplasma hyopneumoniae* (MycoFLEX©, Boehringer Ingelheim Vetmedica GmbH, Germany) and porcine circovirus type 2 (PCV2) (CircoFLEX©, Boehringer Ingelheim Vetmedica) at day 14 of age. Male piglets were castrated at the third day after birth. After weaning on day 21, the piglets were transferred to the University of Veterinary Medicine Vienna, where they were kept under ordinary husbandry conditions. The animals were clinically monitored, and weighed five times during the investigated time period. No signs of disease were noticed during the entire time of the study. Details on the animals are summarized in Supplementary Table 1. One animal (#9) died in the course of the study (week 11). Experimental procedures with the piglets were approved by the institutional ethics committee, the Advisory Committee for Animal Experiments (§12 of Law for Animal Experiments, Tierversuchsgesetz – TVG) and the Federal Ministry for Science and Research (reference number BMWF-68.205/0021-II/3b/2011).

2.2. Sample collection

Blood was taken by puncturing the anterior *vena cava* or the jugular vein. The first blood samples were taken before colostrum intake right after birth. Until weaning in week 3, samples were taken weekly, followed by sampling every two weeks until week 13 and sampling every three weeks until the end of the study in week 25. Leukograms (total white blood cell count and differential) were generated by using the hematology system ADVIA2120i (Siemens Healthcare Diagnostics, Eschborn, Germany).

2.3. Isolation of PBMCs

Peripheral blood mononuclear cells (PBMCs) were isolated by gradient centrifugation using lymphocyte separation medium (PAA, Pasching, Austria). In order to improve separation of erythrocytes from PBMCs in young animals, the commercially available separation medium was diluted with PBS (PAA) to a density of 1.075 g/ml during the first seven weeks of life. Thereafter, the conventional density of 1.077 g/ml was used. The isolation procedure was performed as described elsewhere (Saalmüller et al., 1987b). Isolated PBMCs were counted and 6×10^5 PBMCs per sample were transferred into U-bottomed 96-well microtiterplates (Greiner Bio One, Frickenhausen, Germany) for FCM staining.

2.4. Monoclonal antibodies and second-step reagents

Details on mAbs and second-step reagents used in this study are summarized in Table 1. Where indicated, for direct fluorochrome labeling or biotinylation, in-house produced mAbs from hybridoma supernatants were purified using HiTrap™ Protein G HP affinity columns (GE Healthcare, Little Chalfont, UK) according to manufacturer's protocol. Purified IgGs were quantified by the NanoDrop™ 2000c spectrophotometer (Thermo Scientific Inc., Waltham, MA). Antibody concentrations were adjusted by centrifugation using Vivaspin 20 columns (Molecular Weight Cut Off: 30,000 Dalton, Sartorius, Göttingen, Germany) at 4000 g and 4 °C. Biotinylation was performed using Sulfo-NHS134LC-Biotin (Thermo Scientific, Pierce, Rockford, IL) following manufacturer's instructions. Direct conjugation to fluorochromes was performed by commercially available labeling kits according to manufacturer's instructions. Labeling kits used for conjugation are listed in Table 1.

2.5. FCM staining of PBMCs

Incubation steps for all antibodies took place for 20 min in the fridge. Staining of cell surface markers was performed in two steps: