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A novel lineage transcription factor based analysis reveals differences in T helper cell subpopulation development in infected and intrauterine growth restricted (IUGR) piglets

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ABSTRACT

Research in mouse and human clearly identified subsets of T helper (Th) cells based on nuclear expression of specific lineage transcription factors. In swine, however, transcription factor based detection of functional subpopulations of porcine Th cells by flow cytometry is so far limited to regulatory T cells via Foxp3.

T-bet and GATA-3 are the transcription factors that regulate commitment to Th1 or Th2 cells, respectively. In this study we prove GATA-3 and T-bet expression in porcine $CD4^+$ cells polarized *in vitro*. Importantly, GATA-3 and T-bet expressing cells were detectable in pigs infected with pathogens associated with Th2 and Th1 immune responses. Increased frequencies of GATA-3 positive $CD4^+$ cells are found *in vivo* in pigs experimentally infected with the nematode *Trichuris suis*, whereas porcine reproductive and respiratory syndrome virus (PRRSV) infection elicited T-bet positive $CD4^+$ T cells.

Analysing the immune status of pre-weaning piglets with intrauterine growth restriction (IUGR) we found an increased expression of Foxp3, T-bet and GATA-3 in $CD4^+$ and $CD4^+CD8^+$ double-positive T cells in systemic and intestinal compartments of IUGR piglets. Hence, we established the detection of porcine Th1 and Th2 cells via T-bet and GATA-3 and show that the porcine lineage transcription factors are differentially regulated very early in life depending on the developmental status.

1. Introduction

Studying porcine T lymphocytes is of great interest due to their unique characteristics compared to humans and mice (Charerntantanakul and Roth, 2006). The relatively high proportion of TCR-γδ T cells (Yang and Parkhouse, 1996), the high abundance of extrathymic CD4/CD8 double-positive (DP) lymphocytes under non-pathological conditions (Saalmüller et al., 1987; Zuckermann and Gaskins, 1996), and the membrane expression of SLA class II DR on TCR-γδ and TCR-αβ T cells (Gerner et al., 2009) represent major differences with respect to human T cells. Despite the immunological differences between pigs and humans (Bailey et al., 2013) there is growing interest in the pig for comparative studies, as they are similar to humans in size, organ development and physiology (Guilloteau et al., 2010; Lunney, 2007; Sangild et al., 2013).

While numerous porcine T cell subsets have been well-defined based on surface expression of differentiation antigens (Binns et al., 1992; Revilla et al., 2004; Saalmüller et al., 2002), the functional subpopulations of swine $CD4^+$ helper cells (Th1, Th2 and Th17 cells) are still difficult to assess. Cytokine patterns assigning a clear effector function to different $CD4^+$ T cell subsets have so far been generated based on molecular techniques (e.g. real-time PCR) or unsorted lymphocytes (Cano et al., 2013; Mulder et al., 2011; Schmied et al., 2012).

In swine, the Th1/Th2 paradigm has been experimentally addressed by infecting pigs with the intracellular protozoan parasite *Toxoplasma gondii* vs. *Ascaris suum*, an extracellular gastrointestinal nematode (Dawson et al., 2005). By analysing gene expression of Th1/Th2-related cytokines and cytokine receptors the authors reported a prototypical Th1-like cytokine response (IFN-γ/TNF-α/IL-12) for *T. gondii* infection and a Th2-dominated response (IL-13/IL-4/IL-5) after infection with *A. suum* (Dawson et al., 2009, 2005). However, the broad spectrum of commercially available antibodies usable for human and murine studies for flow cytometric analyses in order to support molecular protein data is lacking for swine.

Research in mice and men identified Th lineage-specific transcription factors that are required for differentiation into Th1 and

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Th2 cells. Transcription factors activate or repress their target genes by binding to accessible promoter and enhancer elements (Lazarevic et al., 2013). T-bet (encoded by *Tbx21*) is the master transcription factor expressed in T cells committed to the Th1 lineage and promotes *IFNG* transcription, while at the same time repressing *IL4* (Jenner et al., 2009) and GATA-3 levels (Usui et al., 2006). Conversely, GATA-3 regulates Th2 differentiation leading to secretion of the Th2 signature cytokines IL-4/IL-5/IL-13 and directly represses *IFNG* transcription (Chang and Aune, 2007). To our knowledge protein expression of the transcription factors GATA-3 and T-bet have not been addressed so far in porcine T helper cells. In contrast, the anti-rat/mouse Foxp3 antibody clone FJK-16s has been widely established to identify porcine regulatory T cells based on expression of the Treg-specific transcription factor Foxp3 in $CD4^+$ T cells (Käser et al., 2008). Several studies have recently addressed the importance of tissue-resident or circulating $Foxp3^+$ Treg cells in neonatal pig development or challenge models (Schmied et al., 2012; Wen et al., 2012). Although much effort has been made to dissect the development of adaptive immunity and oral tolerance during the critical neonatal window around weaning (Bailey, 2009; Butler et al., 2009; Inman et al., 2012; Mulder et al., 2011; Rothkötter et al., 1991), the cell types and factors that drive and regulate neonatal immune cell development are only partially described and comprehensive studies in several organs in parallel are lacking. Such insights into immunological processes, however, are critically important in agricultural but also biomedical research.

Among domestic animals, the pig exhibits the highest rate of naturally occurring intrauterine growth restricted neonates (approximately 15–20% of piglets with birth weights below 1.1 kg, (Wu et al., 2006)). Usually, intrauterine growth restriction (IUGR) is defined as impairment of growth and development of the mammalian fetus or its organs, with a fetal weight at or below the 10th percentile for normal gestational age. IUGR is a major problem in human medicine (Pike et al., 2012; Varvarigou, 2010) and domestic animal production (Alvarenga et al., 2013; Nissen and Oksbjerg, 2011) as it reduces neonatal survival and permanently affects postnatal growth performance and long-term health (Foxcroft et al., 2009). For neonates with IUGR increased risks for subsequent intestinal, neurological, respiratory, infectious and circulatory disorders are reported (Wu et al., 2006). IUGR-associated intestinal alterations in neonatal pigs detected by differential gene expression analysis revealed signs of an inflammatory status as well as enhanced regulatory mechanisms presumably in order to control inflammation (D'Inca et al., 2010a,b). However, little is known about the effects of IUGR on neonatal T-cell development, distribution and phenotype.

In this study we describe the detection of the transcription factors T-bet and GATA-3 in porcine $CD4^+$ T cells *in vitro* under Th1 and Th2 polarizing conditions and *in vivo* in *Trichuris suis* and PRRSV infected pigs by taking advantage of anti-human/mouse specific antibodies. To gain further insights into the porcine neonatal immune system, we analysed the expression of T-bet, GATA-3 and Foxp3 in $CD4^+$ and $CD4^+CD8\alpha^+$ double positive (DP) T lymphocytes of different systemic and intestinal compartments before weaning. In particular, we investigated the effect of intrauterine growth restriction on neonatal T lymphocyte subsets.

2. Materials and methods

2.1. Animals

All experiments were performed in accordance with the national authorities and approval of ethical revision at the animal facility of the Institute of Animal Nutrition (Freie Universität Berlin). Purebred Landrace piglets were born and raised at the Institute of Animal Nutrition under standard husbandry conditions under registration A0100/13. Sows received a standard gestational diet according to the recommendations of the Gesellschaft für Ernährungsphysiologie (GfE, 2006) to meet their nutrient requirements. No antibiotics were given to sows and piglets for therapeutic or prophylactic purpose.

For nematode infection studies infective *T. suis* eggs (TSO) were provided by Ovamed GmbH (Barsbüttel, Germany). Piglets ($n = 4$, 6 weeks old) were inoculated with a single dose of 7500 TSO in 2 ml 0.9% $NaCl_2$ solution and euthanized 8 weeks post infection.

For IUGR studies, all piglets were weighed at birth and IUGR piglets had <2 SD below the mean birth weight of piglets from all litters. Normally developed piglets of the Animal Nutrition breeding facility had ~1.4 kg body weight at birth and were within 1 SD of the mean body weight of piglets from all litters. Body weight was recorded at days 1, 3, 7 and 14 of life. At day 14 5 piglets (male and female) from both groups were euthanized for subsequent sampling of different organs.

In addition, we performed analysis on blood PBMCs from PRRSV infected pigs at 35 days p.i. (age: 98 days), which had received a challenge infection with the heterologous European genotype PRRSV strain CReSA 3267 with a titer of 5×10^6 TCID50/ml by intranasal application. The PRRSV study was performed in the BSL-2 experimental facility (Bundesinstitut für Risikobewertung, Berlin, Germany) under the registration number G0116/12.

2.2. Sampling and cell isolation

The piglets were sedated with 20 mg kg^{-1} BW of ketamine hydrochloride (Ursotamin®, Serumwerk Bernburg AG, Germany) and 2 mg kg^{-1} BW of azaperone (Stresnil®, Jansen-Cilag, Neuss, Germany). Blood was taken by heart puncture (7.5 ml/animal) and was collected in heparinized tubes (16 I.E. Heparin/ml blood). The piglets were subsequently euthanized by intracardial injection of 10 mg/kg BW of tetracaine hydrochloride, mebezonium iodide and embutramide (T61, Intervet, Unterschleißheim, Germany). The abdominal cavity was opened and spleen, mesenteric lymph nodes (mLN), the last discrete jejunal Peyer's patches (PP) and colon tissues were obtained. Spleen, mLN and jejunal PP tissues were gently homogenized using the plunger of a 5 ml syringe. Cell suspensions were washed, erythrocytes were lysed and the cell suspensions were filtered through a 70 µm cell strainer to eliminate clumps and debris. Lymphocytes from colon lamina propria (colon LPL) were purified using Collagenase VIII digestion (Sigma–Aldrich, 200 U/ml, 60 min, 37 °C) and Percoll (GE Healthcare) gradient centrifugation (40%/70%, 800g, 30 min).

PBMCs from *T. suis* and PRRSV infected pigs were isolated using density centrifugation via Ficoll gradient (LSM1077, PAA Laboratories) followed by erythrocyte lysis.

2.3. In vitro polarization

Splenocytes purified from 5-weeks aged $n = 4$ piglets were cultivated for 5 days in RPMI-1640 media (Pan™ Biotech) supplemented with 10% FCS (Pan™ Biotech), L-Glutamine (Pan™ Biotech) and 100 IU/ml penicillin and 0.1 mg/ml streptomycin (Pan™ Biotech) in the presence of ConA alone (1 µg/ml, Sigma–Aldrich) or in combination with porcine rIL-4 (10 ng/ml, R&D Systems) or rIL-12 (10 ng/ml, R&D Systems).

2.4. Flow cytometry

Cells were acquired using BD FACSCanto™ II or BD LSR II analyser (BD Biosciences; both with FACSDiva software) and post-acquisition data analysis was carried out using FlowJo™ software