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Expression of T-bet, Eomesodermin and GATA-3 in porcine αβ T cells

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ABSTRACT

The transcription factors GATA-3, T-bet and Eomesodermin play important roles in T-cell development, differentiation and memory formation. However, their expression has not been studied in great detail in porcine T cells. We report on protein expression at the single cell-level of these transcription factors in thymocytes and mature αβ T cells. GATA-3 expression was found in $\gamma\delta^-$ thymocytes, with decreasing expression from the $CD4^-CD8\alpha^-$ stage towards single-positive stages. Extra-thymic $CD4^+$ T cells but not $CD8\beta^+$ T cells expressed low levels of GATA-3, which decreased with age. $CD4^+$ and $CD8\beta^+$ T-bet$^+$ cells mainly displayed a $CD8\alpha^+CD27^-$ and perforin$^+CD27^{dim/-}$ phenotype, respectively and had the capacity for IFN-γ production; indicative of an effector/effector memory phenotype. Eomesodermin$^+$ αβ T cells had mixed phenotypes in regard to CD8α, CD27 and perforin expression. In conclusion, our data so far support the hitherto reported roles for GATA-3 in T-cell development and T-bet for Th1 effector-differentiation, but question the role of Eomesodermin for memory formation of porcine T-cells.

1. Introduction

The differentiation of polarized effector $CD4^+$ T cells is controlled by so-called lineage-defining or master-regulatory transcription factors (TFs). Accordingly, the following TFs are considered as the master regulators for commitment to several T helper (Th) subpopulations: T-bet for Th1 differentiation, GATA-3 for Th2 differentiation, ROR-γt for Th17 differentiation and Foxp3 for regulatory T-cell differentiation (Chen et al., 2003; Ivanov et al., 2006; Szabo et al., 2000; Zheng and Flavell, 1997). However, it was also demonstrated in recent years that many of these TFs have functions beyond regulating CD4 T-cell differentiation.

For example, T-bet is expressed also in myeloid dendritic cells, subpopulations of innate lymphoid cells, NK cells and activated γδ T cells (reviewed in Lazarevic et al., 2013). T-bet is also supposed to play a role in the maintenance of memory B cells (Wang et al., 2012) and $CD4^+$ T cells (Marshall et al., 2011). Moreover, together with the TF Eomesodermin (Eomes) it is involved in the regulation of differentiation and long-term survival of $CD8^+$ memory T cells. The available data suggests that high levels of T-bet expression drive $CD8^+$ T cells towards effector function and terminal differentiation; whereas high levels of Eomes expression are involved in long-term survival of memory cells (Banerjee et al., 2010; Intlekofer et al., 2005; Joshi et al., 2007, 2011). Nevertheless, Eomes appears to be also involved in sustaining effector functions in $CD8^+$ T cells in co-operation with T-bet (Pearce et al., 2003).

In addition, in murine $CD4^+$ T cells, Eomes expression is associated with cytolytic function and in human $CD4^+$ T cells also with Th1 differentiation (Eshima et al., 2012; Narayanan et al., 2010; Qui et al., 2011). In a study addressing T-bet and Eomes expression in blood-derived human lymphocytes, the frequency of Eomes$^+$ cells in different subsets of $CD4^+$ T cells from the blood was substantially lower when compared to $CD8^+$ T cells, which already expressed this TF in naïve $CD8^+$ T cells (Knox et al., 2014).

For GATA-3 it has also become clear that this TF has functions beyond regulation of Th2 differentiation. It is involved in T-cell lineage commitment (Ho et al., 1991; Ting et al., 1996) and also later stages of T-cell development in the thymus, in particular for cells of the $CD4^+$ lineage (Pai et al., 2003; Wang et al., 2008). GATA-3 is also expressed in naïve $CD8^+$ T cells in mice, albeit at lower levels than found in $CD4^+$ T cells, where it is involved in maintenance and

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proliferation (Wang et al., 2013). Moreover, data suggest that this TF has a role in NK-cell maturation and function in mice (Samson et al., 2003; Vosshenrich et al., 2006) as well as in the function and maintenance of human and murine type 2 innate lymphoid cells (Hoyler et al., 2012; Mjösberg et al., 2012).

Despite this central role in lymphocyte function, data on the expression of these TFs in cells of the porcine immune system is sparse. One recent report shows an increase in the frequency of T-bet$^+$ and GATA-3$^+$ CD4$^+$ T cells following infection with porcine reproductive and respiratory syndrome virus and *Trichuris suis*, respectively (Ebner et al., 2014). To expand the knowledge on TF expression in porcine αβ T cells, we investigated the expression of T-bet, Eomes and GATA-3 in combination with additional differentiation molecules within CD4$^+$ and CD8β$^+$ T cell subsets from different organs of healthy pigs.

2. Materials and methods

2.1. Animals and cell isolation

Six-month-old fattening pigs and 4- to 5-year-old healthy sows from an abattoir served as donors for blood and organs. Animals were subjected to electric high-voltage anesthesia followed by exsanguination. This procedure is in accordance with the Austrian Animal Welfare Slaughter Regulation. For analyses with peripheral blood mononuclear cells (PBMCs) from aging pigs, blood samples were obtained from the same animals at 2 weeks, 25 weeks and 26 months of age. Repeated blood sampling of these animals was 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). PBMCs were also obtained from the blood of three 5-week-old piglets infected with swine influenza A virus (FLUAVsw). Animals had been infected intratracheally with a H1N2 isolate (FLUAVsw/Kitzen/IDT6142/2007; infection dose: 15 mL of $10^{7.25}$ $TCID_{50}$/mL). Blood samples were taken 12 and 15 days after infection. The FLUAVsw infection experiment was approved by the institutional ethics committee and the national authority according to § 26 of Law for Animal Experiments, Tierversuchsgesetz 2012 – TVG 2012 (reference number BMWF-68.205/0103-II/3b/2013).

PBMCs were isolated by gradient centrifugation using lymphocyte separation medium (density 1.077 g/mL; PAN Biotech, Aidenbach, Germany) as described elsewhere (Saalmüller et al., 1987). Cells from thymus, spleen and mediastinal lymph nodes were isolated as reported previously (Reutner et al., 2012). For the isolation of lymphoid cells from the lung, lung tissue was finely chopped at room temperature using scissors. Subsequently, the tissue was rinsed with phosphate buffered saline (PBS, PAN Biotech) to remove cells of blood-origin. Thereafter, the tissue pieces were subjected to enzymatic digestion in RPMI 1640 with stable L-glutamine supplemented with 2% heat-inactivated fetal calf serum (FCS), 100 IU/mL penicillin, 0.1 mg/mL streptomycin (all from PAN Biotech), 20 mM HEPES (Sigma-Aldrich, Schnelldorf, Germany), 25 U/mL DNase I (Life Technologies, Carlsbad, CA, USA), and 300 U/mL collagenase type I (Life Technologies) for 60 min at 37 °C in a shaking incubator. Dead cells were removed by cotton-wool filtration. This was followed by a gradient centrifugation under the conditions described for the isolation of PBMCs. Isolated cells were either stained and analyzed by flow cytometry (FCM), *in vitro* cultivated (see below for details) or cryopreserved at −150 °C as described elsewhere (Leitner et al., 2012).

2.2. In vitro cultivation

To study the co-expression of T-bet and IFN-γ, PBMCs were defrosted, washed, and re-suspended in pre-warmed (37 °C) culture medium (RPMI 1640 with stable L-glutamine supplemented with 10% (v/v) heat-inactivated FCS, 100 IU/mL penicillin and 0.1 mg/mL streptomycin, all from PAN Biotech). Subsequently, 5×10^5 cells per well were cultivated for 24 h at 37 °C in round-bottomed 96-well plates (Greiner Bio-One, Kremsmünster, Austria) in a total volume of 200 μL. The following stimuli were used: (i) phorbol 12-myristate 13-acetate (PMA, 50 ng/mL, Sigma-Aldrich), (ii) ionomycin (500 ng/mL, Sigma-Aldrich), (iii) PMA and ionomycin (50 ng/mL, 500 ng/mL, respectively), (iv) recombinant porcine (rp) IL-12 and rpIL-18 (25 ng/mL of both cytokines, R&D Systems, Minneapolis, MN, USA), (v) rpIL-2, rpIL-12 and rpIL-18 (20 ng/mL, 25 ng/mL and 25 ng/mL, respectively), (vi) Concanavalin A (ConA) (5 μg/mL, Amersham Biosciences, Uppsala, Sweden), (vii) ConA in combination with rpIL-2, rpIL-12 and rpIL-18 (same concentration of each reagent as mentioned before). Stimuli (i) to (iii) were added for the last 4 h of cultivation, stimuli (iv) to (vii) were present during the entire 24 h of cultivation. Freshly isolated PBMCs obtained from FLUAVsw-infected piglets were *in vitro* re-stimulated with the homologous FLUAVsw strain at a multiplicity of infection of 1 for 24 h. Mock-stimulated microcultures or microcultures with culture medium served as negative controls.

For all mentioned *in vitro* stimulation variants, during the last 4 h, Brefeldin A (1 μg/mL, BD GolgiPlug™, BD Biosciences, San Jose, CA, USA) was added to the microcultures. After 24 h, cells were harvested, washed in PBS (without Ca^{2+}/Mg^{2+}) containing 3% (v/v) of FCS, incubated with the corresponding antibodies listed in Table 1 (see below for staining procedure) and analyzed by FCM.

2.3. Monoclonal antibodies and second-step reagents

Details on monoclonal antibodies (mAbs) and second-step reagents used in this study are summarized in Table 1. Where indicated, fluorochrome conjugation or biotinylation of in-house produced mAbs was performed as reported previously (Talker et al., 2013). Labeling kits used for conjugation are listed in Table 1.

2.4. FCM staining

Incubation steps for all mAbs targeting extracellular antigens took place for 20 min in the fridge. Following each incubation step, the cells were washed twice with 200 μL of a buffer consisting of PBS and 10% (v/v) porcine plasma (in-house preparation). In case of cultivated cells, the buffer consisted of PBS and 3% of FCS. Following surface labeling, all samples were fixed and permeabilized by the Foxp3/Transcription Factor Staining Buffer Set (eBioscience, San Diego, CA, USA) according to manufacturer's instructions. Intracellular staining comprised the use of a single directly conjugated mAb or a mastermix of directly conjugated antibodies for 30 min in the fridge. Cross-reactivity of anti-human/mouse mAbs against porcine T-bet and GATA-3 has been described recently (Ebner et al., 2014) and the same antibody clones were used in this study (Table 1). For Eomes, Clustal X 1.8 multiple amino-acid sequence alignments of human, murine and porcine sequences were performed (Supplementary Fig. 1; Thompson et al., 1997). The calculation of pairwise *p*-distances by using Mega version 6.6 (Tamura et al., 2013) revealed a homology of 96.8% between human and porcine sequences and 92.0% between murine and porcine sequences. On this basis, cross-reactivity of the anti-human mAb WD1928 with porcine Eomes appears very likely.

For all samples where isotype-specific secondary antibodies were used, whole mouse IgG molecules (2 μg per sample;