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Natural and inducible Tregs in swine: Helios expression and functional properties

Tobias Käser *, Kerstin H. Mair, Sabine E. Hammer, Wilhelm Gerner, Armin Saalmüller

Institute of Immunology, Department of Pathobiology, University of Veterinary Medicine Vienna, Veterinärplatz 1, 1210 Vienna, Austria

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ABSTRACT

Within the population of regulatory T cells (Tregs) natural Tregs (nTregs) and inducible Tregs (iTregs) can be distinguished. Although information about Tregs in swine exists, porcine iTregs were not under investigation yet. In this study, Foxp3+ iTregs were generated from CD4+Foxp3− T cells by *in vitro* stimulation in the presence of IL-2 and TGF-β. In comparison to *ex vivo* Tregs these iTregs had a similar suppressive capacity on the proliferation of CD3-stimulated PBMC, caused higher levels of IL-10 in PBMC/Treg co-cultures, but did not suppress IFN-γ levels. The Ikaros family member Helios is currently discussed to distinguish iTregs and nTregs or to serve as an activation marker of Tregs. In this study, we demonstrate the cross-reactivity of an anti-mouse/human Helios mAb with porcine Helios. Flow cytometric analyses with this antibody showed that porcine iTregs do not express Helios after *in vitro* iTreg induction. Nevertheless, thymic Foxp3+ T cells, which arise at the CD4/CD8α single-positive stage of T-cell development and are defined as nTregs, entirely expressed Helios. Although this might suggest the suitability of Helios as an nTreg–iTreg differentiation marker we also found that Helios− Tregs displayed a phenotype of naive CD4+ T cells *in vivo*. Since iTregs are by definition activated/differentiated Tregs, this finding precludes that all Helios− Tregs are iTregs and thus also the use of Helios as a selection marker for porcine nTregs. Furthermore, Helios+ Tregs displayed a more differentiated phenotype indicating that Helios might rather serve as a Treg activation/differentiation marker.

1. Introduction

Regulatory T cells (Tregs) are an important lymphocyte subset involved in the fine tuning of immune responses and the maintenance of self-tolerance. For the latter, an essential role of thymic-derived CD4+Foxp3+ Tregs, designated as "naturally occurring" Tregs (nTregs), has been demonstrated in various species. Besides these nTregs, other T cells with suppressive capabilities have been described. In addition to γδ T cells, such cells could be found in CD4+ and CD8+ T-cell subsets after subimmunogenic stimulation, e.g. in the presence of immunosuppressive cytokines as IL-10 or TGF-β and therefore were termed inducible Tregs (iTregs) (Mills, 2004). These iTregs are considered to be mainly responsible for a controlled decline of an immune response against pathogens (Povoleri et al., 2013) and suppress anti-tumour immune responses, thereby promoting tumour growth (Whiteside et al., 2012). Due to these different roles in the immune system much effort has been put into the phenotypical discrimination of iTregs and nTregs. A first approach in mice demonstrated a different methylation status of nTregs and *in vitro* induced iTregs (Floess et al., 2007) but this difference was not present *in vivo* (Polansky et al., 2008). Another promising approach was the differential expression of the transcription factor Helios. Thornton et al. (2010) showed that Helios was expressed in all thymic Tregs but neither in *in vitro* nor in *in vivo* induced iTregs (Thornton et al., 2010). However, in more recent studies Helios expression could be induced in *in vitro*- as well as *in vivo* generated iTregs, thereby questioning the use of Helios as a marker for nTregs (Gottschalk et al., 2012). Additionally, Akimova et al. suggested that Helios could be used as a marker for Treg activation and experience in mice and humans (Akimova et al., 2011).

In swine, Tregs could also be defined as immunosuppressive CD4+Foxp3+ T cells (Käser et al., 2008a, 2008b). Thus far, a suppressive effect of porcine Tregs on the proliferation of T-helper cells, cytotoxic T lymphocytes and TCR-γδ T cells could be demonstrated. Suppression can be mediated either via cell–cell contact, soluble components and/or the competition for growth factors (Käser et al., 2011b, 2012). In addition to the described CD4 expression of Foxp3+ Tregs also a substantial subset of CD8α+Foxp3+ Tregs either CD4+ or CD4− could be identified in swine (Käser et al., 2008a; Talker et al., 2013). With the information that surface expression of CD8α

Abbreviations: APC, antigen presenting cells; FCM, flow cytometry; HEK293T cells, human epithelial 293 kidney cells; iTregs, inducible Tregs; nTregs, naturally occurring Tregs; mAb, monoclonal antibody; rh, recombinant human; Tregs, regulatory T cells.

* Corresponding author. University of Saskatchewan, VIDO-Intervac, 120 Veterinary Road, S7N 5E3 Saskatoon, Canada. Tel.: +1 306 966 1510; fax: 01-306-966-7478.
E-mail address: tobias.kaeser@usask.ca (T. Käser).

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is up-regulated on porcine T cells upon activation (Saalmüller et al., 2002) these CD8α+ Tregs might include activated as well as differentiated iTregs. However, basic research on iTregs in swine is missing. Therefore, this study investigated the induction, phenotype and functions of porcine iTregs and compared them with *in vivo* occurring Tregs. Additionally, we investigated the possible use of Helios either as an nTreg/iTreg discrimination marker or as an activation marker for porcine Tregs.

2. Materials and methods

2.1. Cell isolation

For the isolation of PBMC blood of 6-month-old pigs was obtained from an abattoir which slaughters animals from different conventional finishing farms. The general health status of all animals was controlled before transportation and after arrival at the slaughter plant and all animals appeared clinically healthy. Animals were subjected to electric high voltage anaesthesia followed by exsanguination. This procedure is in accordance to the Austrian Animal Welfare Slaughter Regulation. Blood was collected during exsanguination into heparinised sample tubes. PBMC were isolated by gradient centrifugation using lymphocyte separation medium (PAA, Pasching, Austria) as described elsewhere (Saalmüller et al., 1987). For the isolation of antigen presenting cells (APCs), up to 7×10^7 PBMC were incubated for 90 min at 37 °C in a 10 cm Petri dish. Thereafter, non-adherent cells were taken off. Adherent APCs were detached using a cell scraper and used for iTreg induction studies. Thymocytes were gained by sieving small pieces of thymus through steel meshes. Dead cells were separated from cell suspensions by cotton–wool filtration.

2.2. Sorting of T-cell subsets

CD4+ lymphocytes were at first sorted from PBMC by MACS (Quadro-MACS system, Miltenyi Biotec, Bergisch Gladbach, Germany) using monoclonal antibodies (mAbs) against porcine CD4 (clone 74-12-4, mouse IgG2b, (Pescovitz et al., 1984)) and anti-mouse IgG2a+b microbeads (Miltenyi Biotec). Afterwards, CD4+ T cells were sorted according to their CD25 expression (CD25− and CD25high) by FACS (FACSAria, BD Biosciences, San Jose, CA) using mAbs against porcine CD25 (clone 3B2, mouse IgG1, (Bailey et al., 1992)) and isotype-specific fluorescence-labelled secondary antibodies (see Table 1). All sorted cell subsets had a purity of >97%.

2.3. Phenotypic characterisation of Tregs by flow cytometry (FCM)

Cells derived from different isolation procedures or *in vitro* cultivation regimens were labelled with mAbs and second step reagents listed in Table 1. Where indicated, in-house produced mAbs were purified and either covalently conjugated to fluorochromes or biotin by commercially available kits according to manufacturer's instructions as described elsewhere (Talker et al., 2013). Cells were first stained for surface marker expression by incubation for 10 minutes at room temperature with the indicated antibodies and reagents. Between incubations, cells were washed twice with PBS. During the last step of surface staining, a live/dead discrimination dye (LIVE/DEAD® Fixable Aqua or Near IR Dead Cell Stain Kits, Life Technologies) was included in the staining solution. After surface staining, cells were washed again twice in PBS, and fixed and permeabilised using the "Foxp3/Transcription Factor Staining Buffer Set" (eBioscience, San Diego, CA) according to the manufacturer's instructions. Afterwards, cells were stained for intracellular Foxp3 and Helios expression for 10 minutes at room temperature using an Armenian hamster anti-mouse/human Helios-Alexa647 mAb (clone 22F6, BioLegend, San Diego, CA) and an anti-Foxp3-PE mAb (clone FJK-16s, eBioscience). FCM analyses of stained cells were performed using a FACSCantoII (BD Biosciences, San Jose, CA) flow cytometer equipped with three lasers (405, 488 and 633 nm) and a high throughput sampler (HTS). Data analyses of flow cytometric raw data were performed by FACSDiva 6.1.3 Software (BD Biosciences) and by FlowJo version 7.6 (Tree Star, Ashland, OR).

2.4. iTreg induction

PBMC and sorted cell subsets were cultivated at 2×10^5 cells per well in cell culture medium consisting of RPMI 1640 with stable glutamine (PAA, Pasching, Austria) supplemented with 10% FCS (PAA), 100 IU/ml penicillin and 0.1 mg/ml streptomycin (PAA). Where indicated, cells were stimulated by plate-bound anti-CD3 mAbs (clone PPT7, mouse IgG1, 1.5 μg/ml (Yang et al., 1996), 10 IU/ml recombinant human (rh)IL-2 (Roche, Mannheim, Germany) or 4 μg/ml rhTGF-β (R&D Systems, Minneapolis, MN)).

Table 1
Antibodies used for surface staining in FCM analyses.

Antigen	Clone	Isotype	Fluorochrome	Labeling strategy	Primary ab source
Figure 1 (sorted CD4+CD25− cells from PBMC*)					
CD4	74-12-4	IgG2b	Alexa488	Secondary ab[a]	In house
CD25	3B2	IgG1	Alexa647	Directly conjugated	In house
Figure 4a (*ex vivo* thymocytes*)					
CD3	PPT3	IgG1	eFluor450	Directly conjugated	In house[f]
CD4	74-12-4	IgG2b	Alexa488	Secondary ab[a]	In house
CD8α	11/295/33	IgG2a	Qdot605	Biotin–streptavidin[b]	In house
Figure 4b (sorted CD4+CD25− cells from PBMC*)					
CD4	74-12-4	IgG2b	BrilliantViolet421	Biotin–streptavidin[c]	In house
CD25	3B2	IgG1	Alexa488	Directly conjugated	In house
Figure 5 (*ex vivo* PBMC*)					
CD4	74-12-4	IgG2b	FITC	Directly conjugated	BD Biosciences
CD8α	11/295/33	IgG2a	PE-Cy7	Secondary ab[d]	In house
CD27	b30c7	IgG1	BrilliantViolet421	Biotin–streptavidin[e]	In house

* Cells used for staining.
[a] Goat anti-mouse IgG2b-Alexa488, Life Technologies.
[b] Goat anti-mouse IgG2a-biotin, Southern Biotech (Birmingham, AL) + streptavidin-Qdot605, Life Technologies.
[c] Goat anti-mouse IgG2b-biotin, Southern Biotech (Birmingham, AL) + streptavidin-BrilliantViolet421, BioLegend.
[d] Goat anti-mouse IgG2a-PE-Cy7, Southern Biotech.
[e] Biotinylated primary antibody + streptavidin-BrilliantViolet421, BioLegend.
[f] Custom conjugation by eBioscience.