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Phenotypical and functional characterization of porcine Th17 lymphocytes

Bachelor thesis

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1 Introduction

1.1 Cluster of differentiation (CD) 4^+ T-cell biology in mice and humans

T cells expressing a T-cell receptor (TcR) consisting of an α-chain and a β-chain can be separated into $CD4^+$ and $CD8^+$ T cells. $CD4^+$ T cells are also named T-helper (Th) cells and their role is crucial for the adaptive immune system. Th cells have several different functions including the activation of B cells to produce antibodies, the recruitment of granulocytes, macrophages, and other effector cells during an infection, and the production of different cytokines (Zhu and Paul 2008, 2010a). Furthermore, they can also play a role in chronic inflammation and autoimmunity if they respond to autoantigens or induce an excessive immune response (Annunziato et al. 2009; Sallusto and Monticelli 2013). The differentiation of naïve $CD4^+$ T cells into effector cells depends on the antigen-presentation by professional antigen presenting cells via the major histocompatibility complex II (MHC-II) to trigger the TcR, costimulatory signals the T cells receive through CD28 and soluble factors like cytokines (Luckheeram et al. 2012; Zhu 2018). Importantly, these cytokines form a polarizing milieu for the differentiation of the $CD4^+$ T cells into distinct functionally specialized subsets (Figure 1). Furthermore, this polarization is dependent on key transcription factors (TF) regulating subset-specific gene expression (Swain et al. 2012).

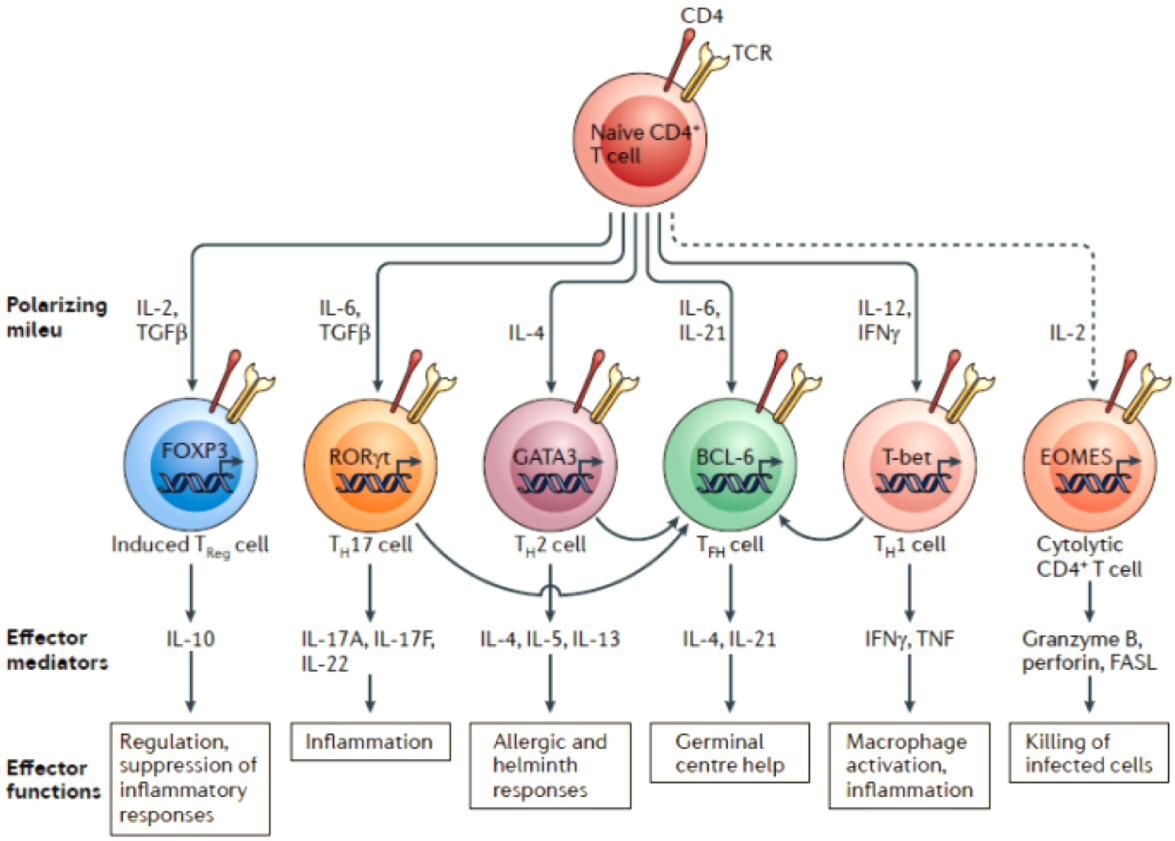

Figure 1: Polarization and functional specialization of CD4⁺ T cells in mice and humans. After activation, naïve $CD4^+$ T cells differentiate into distinct effector subtypes. This functional specialization is dependent on polarizing cytokines and key transcription factors. After specialization, each subset produces a different set of signature cytokines and is involved in distinct immune responses. (Swain et al. 2012)

The first two subtypes of effector cells, namely Th1 and Th2 cells, were first mentioned in 1986. Th1 cells produce interferon (IFN)-γ and tumor necrosis factor (TNF), whereas Th2 cells secrete interleukin (IL)-4, as well as IL-5, IL-9, IL-10, IL-13, IL-25 and amphiregulin (Mosmann et al. 1986; Zhu and Paul 2008). Th1 differentiation is regulated by the TF T-bet (T-box protein expressed in T cells) that also controls the expression of the Th1 cytokine IFN-γ (Szabo et al. 2000). Through IFN-γ Th1 cells are able to activate macrophages which then show enhanced antimicrobial activity (Nathan et al. 1983; Paul and Seder 1994). Th1 cells play an important role in the immune response against intracellular pathogens but have also been associated with organ-specific autoimmunity (Murphy and Reiner 2002). The master TF identified for Th2 cells is GATA-3 (Zheng and Flavell 1997). Th2 cells act in response to

extracellular parasites like helminths but are also involved in the development of allergies and asthma (Cortés et al. 2017; Nakayama et al. 2017).

Another effector T-cell subset, the Th17 subset, has only been discovered recently. Th17 cells are essential in protection against extracellular bacteria and fungi and also play a role in inflammatory responses (Korn et al. 2007). This subset will be discussed in more detail in chapter 1.1.1.

Regulatory T cells (Treg) can either develop in the thymus (natural Treg, nTreg) or be induced in the periphery (induced Treg, iTreg) when they encounter an antigen in a specific cytokine milieu. For both Treg lineages the expression of the key TF Forkhead-Box-Protein P3 (Foxp3) is crucial (Chen et al. 2003). Tregs are important for immunological tolerance and express IL-10, transforming growth factor (TGF) β, and IL-35 (Chen et al. 2003; Wei et al. 2017). Follicular B-helper T cells (Tfh) represent another effector subset. Tfh cells provide costimulatory signals that are necessary for the survival and maturation of B cells (Breitfeld et al. 2000). The B-cell lymphoma 6 (Bcl6) is the master TF for Tfh cells (Nurieva et al. 2009). For their development they need the co-stimulatory signals from IL-21 and IL-6 (Nurieva et al. 2008; Nurieva et al. 2009). The cytokines they produce include IL-21 and IL-4 (Crotty 2011). Furthermore, a perforin expressing subset of $CD4^+$ T cells with cytotoxic potential was reported that may play a role in viral infections (Appay et al. 2002).

1.1.1 Th17 cells

Th17 cells were first identified as a distinct lineage different from Th1 and Th2 cells due to their differentiation requirements that are independent from the stimuli that are necessary for the development of the other effector-cell subsets. It was shown that cytokines like IFN-γ and IL-4 needed for Th1 and Th2 differentiation inhibit the development of IL-17 producing cells (Park et al. 2005; Harrington et al. 2005). The breakthrough of discovering this new effector subset came with studies showing that IL-23 is involved in the survival and expansion of Th17 cells in autoimmune models in mice (Langrish et al. 2005). However, IL-23 cannot induce the differentiation into Th17 effector cells and the pro-inflammatory cytokines IL-6 and TGF-β are needed for the development of Th17 cells in mice (Bettelli et al. 2006). The role of Th17 cells is mainly associated with autoimmunity and inflammation (Yasuda et al. 2019). However, they are not only harmful but are important for clearance of extracellular pathogens like bacteria as

was shown in *Klebsiella* and *Pneumococcus* infections (Amezcua Vesely et al. 2019; Crome et al. 2010; Wang et al. 2019; Wright et al. 2013). The cytokines that play a role in this immune response include IL-17A, IL-17F, IL-21 and IL-22 (Korn et al. 2009).

1.1.1.1 RORγt

Besides the cytokine milieu, the differentiation into an effector subset is also regulated by transcription factors. The master TF that leads to the differentiation of Th17 cells is the retinoic acid–related orphan receptor (ROR) γt (Ivanov et al. 2006). RORα, another member of this transcription factor family likewise is involved in the development of Th17 cells and the expression of cytokines associated with this T-cell effector lineage like IL-17 (Yang et al. 2008). Both, RORγt and RORα are induced by TGF-β and IL-6 in a signal transducer and activator of transcription 3 (STAT3) dependent manner (Yang et al. 2008; Yang et al. 2007). It was shown that Th17 cells are absent in RORγt deficient mice and that these animals are less susceptible to autoimmunity as shown in studies on experimental autoimmune encephalomyelitis (EAE) (Ivanov et al. 2006).

1.1.1.2 IL-17

IL-17 was first cloned and described in 1993 by Rouvier et al., named Cytotoxic T-lymphocyte-associated antigen 8 (CTLA8) and only later renamed to IL-17A (Rouvier et al. 1993). There are five more members of the IL-17 family, named IL-17B to IL-17F (Korn et al. 2009; Kolls and Lindén 2004). Th17 cells express both, IL-17A and IL-17F (Korn et al. 2009). These two cytokines are the most similar within the IL-17 family although they are only 50% identical to each other. They share a chromosomal location and bind to the same receptor (Hymowitz et al. 2001; Kuestner et al. 2007). Both, IL-17A and IL-17F, can be secreted as homodimers as well as heterodimers. Because those two cytokines share a receptor complex, they also induce a similar response in the cells that express their receptor (McGeachy et al. 2019). Activation leads to the production of different pro-inflammatory cytokines, chemokines as well as antimicrobial peptides by different cell types, such as epithelial cells or fibroblasts (Moseley et al. 2003; Iwakura et al. 2011). Thereby they induce inflammation and have the capability to recruit neutrophils to sites of infection (Iwakura et al. 2011; Zhu and Paul 2008; Ye et al. 2001). Despite the similar roles of IL-17A and IL-17F, studies showed that IL-17A homodimers have

the capability to induce a stronger signal in comparison to IL-17F homodimers (Wright et al. 2007).

1.1.1.3 Chemokine receptor (CCR) 6

Chemokine receptors can also be differentially expressed on the different $CD4^+$ T cell types. The chemokine receptor CCR6 has been identified to be expressed by human Th17 cells (Acosta-Rodriguez et al. 2007). The expression of CCR6 is important for inflammatory and immunological responses as well as for the recruitment of lymphocytes (Baba et al. 1997). CCR6 also plays a role in several autoimmune diseases. In EAE in mice models it was shown that CCR6 deteriorates the disease through activation of myelin oligodendrocyte glycoprotein-specific T cells (Moriguchi et al. 2013). There is only one ligand that interacts with CCR6, namely liver and activation-regulated chemokine, which is also known as C-C Chemokine Ligand (CCL) 20 (Baba et al. 1997). Studies showed that CCL20 is upregulated by IL-17 (Kao et al. 2005). Therefore it could be possible that the expression of CCR6 by Th17 cells leads to a positive feedback loop that attracts more Th17 cells (Acosta-Rodriguez et al. 2007).

1.2 $CD4^+$ T cells in swine

Likewise to humans and mice, $CD4^+$ T cells also exist in the pig. A peculiarity of these cells is that extrathymic T-cells exist that express both CD4 and CD8α (Saalmüller and Bryant 1994). The expression of CD8α on $CD4^+$ T cells is believed to be a sign of antigen contact and those cells represent memory-T cells (Zuckermann and Husmann 1996, Gerner et al. 2015). In addition to CD8α CD27 can also be used to discriminate naïve $CD4^+$ T cells ($CD8\alpha^-CD27^+$) as well as central memory ($CD8\alpha^+CD27^+$) and effector memory cells ($CD8\alpha^+CD27^-$) in the pig (Reutner et al. 2013). Naïve $CD4^+$ T cells are known to differentiate upon antigen contact in a stimulating cytokine milieu that leads to different effector cell types. However, the different effector subsets in swine are not as thoroughly studied as in humans and mice (Gerner et al. 2015). Nevertheless, recent studies could demonstrate the role of key TFs for $CD4^+$ T-cell functional specialization also in the pig. Anti-human/mouse monoclonal antibodies (mAbs) directed against Foxp3, T-bet and GATA-3 were shown to identify porcine Treg, Th1 and Th2 cells (Bolzer et al. 2009; Rodríguez-Gómez et al. 2016). $T\text{-bet}^+$ cells were shown to produce IFN-γ after *in vitro* stimulation (Rodríguez-Gómez et al. 2016). Furthermore, T-bet and GATA-

3 were inducible *in vitro* after stimulation with Th1 or Th2-driving cytokines, respectively (Ebner et al. 2014). A final proof of the Th1 and Th2 specialization in the context of TF expression was given by the induction of T-bet in $CD4^+$ T cells during experimental viral infection and GATA-3 in helminth infection experiments (Ebner et al. 2014). Up to date, less in-depth studies are available on porcine Th17 cells.

1.2.1 Porcine Th17 cells

It has been shown that $CD4^+$ T cells capable of producing IL-17 exist in the pig (Stepanova et al. 2012). *In vitro*, porcine recombinant IL-17 showed similar functions as human or mice IL-17 (Katoh et al. 2004). Studies showed that the *in vitro* development of porcine Th17 cells is driven by the presence of TGF-β and IL-6, like it is the case in mice and humans (Kiros et al. 2011; Bettelli et al. 2006). TGF-β and IL-6 stimulation leads to an increase in IL-17 and IL-21 production as well as a higher expression of the transcription factor RORγt on the mRNA level. Additionally, TGF-β and IL-6 stimulation leads to a downregulation of IFN-γ, which suggests the suppression of Th1 cells (Kiros et al. 2011). In an experimental infection study, IL-17A production was induced in $CD4^+$ T cells in pigs infected with *Actinobacillus pleuropneumoniae* (Sassu et al. 2017). These findings lead to the assumption that IL-17 producing $CD4^+$ T cells could represent the porcine Th17 cells. A first study on the key TF RORγt for Th17 cells also exist in the pig. Increased numbers of $CD4^+ROR\gamma t^+$ cells were found in pigs that were infected with *Helicobacter pylori* (Kronsteiner et al. 2013). In the same infection study also increased IL-17A production could be observed, suggesting a correlation of $CD4^+ROR\gamma t^+$ and IL-17A and co-expression of the Th17 key TF and effector cytokine. Nevertheless, in this study no co-expression of RORγt and IL-17A was shown. Furthermore, up to date no data exist on the expression of CCR6 on the putative porcine Th17 cells.

1.3 Cross-reactivity testing of monoclonal antibodies (mAbs)

For the characterization of human and mouse immune cells a broad range of species-specific mAbs is available to detect different CD molecules, cytokines, and TFs. For other species, including pigs, the selection is limited. It is however possible to test mAbs specific for human/mouse molecules for cross-reactivity on orthologous proteins in other species, like the pig (Cossarizza et al. 2019). A high amino acid homology of the antigens between the species

will increase the likelihood of cross-reactivity. Furthermore, a positive staining with the mAb candidate in flow cytometry (FCM) leads to the assumption that the antibody is cross-reactive. However, final proof will only be obtained when, in our case, the porcine-specific protein is cloned, expressed in a cell line and then analyzed with the potential cross-reactive mAb using immunofluorescence staining in FCM or microscopy (Cossarizza et al. 2019). This was already successfully tested for cross-reactive mAbs against the TFs Helios und Foxp3 in the pig (Bolzer et al. 2009; Käser et al. 2015).

2 Aims of the study

Th17 cells can be identified by the expression of IL-17A, RORγt and CCR6 in humans and mice. Very little is known about the Th17 lineage in pigs. However, for the identification of porcine IL-17A producing cells, anti-human/mouse mAbs with proven cross-reactivity are available and $CD4^+$ cells that are capable of IL-17A production have already been identified in pigs. Nevertheless, a distinct characterization of porcine Th17 cells was hampered so far due to the lack of species-specific or cross-reactive mAbs against porcine RORγt and CCR6 molecules. Therefore, the aim of this study was to identify further tools for a detailed characterization of porcine Th17 cells.

In a first step, anti-human/mouse mAbs specific for RORγt and CCR6 were tested for potential cross-reactivity on porcine lymphocytes in multi-color FCM stainings in combination with CD4 and IL-17A. In a second step, cross-reactivity of two mAbs against RORγt and CCR6 had to be proven on their porcine orthologues. This was achieved by the cloning and expression of recombinant porcine RORγt and CCR6 in a mammalian cell line. Cross-reactivity had to be shown by a positive signal in FCM with the mAb candidates on the recombinant porcine molecules.

3 Material and Methods

3.1 Buffers and solutions

Table 1: Buffers and solutions that were used in the study.

Reagent/Solution	Company
Porcine plasma buffer: PBS (without Ca^{2+}, Mg^{2+}) 10% (v/v) porcine plasma	 PAN-Biotech (Aidenbach, Germany) in-house production
FCS buffer: PBS (without Ca^{2+}, Mg^{2+}) 3% (v/v) FCS	 PAN-Biotech Sigma-Aldrich (Vienna, Austria)
Cell culture medium PBMC: RPMI 1640 with stable glutamine 100 IU/ml penicillin 0.1 mg/ml streptomycin 10% (v/v) heat-inactivated FCS	 PAN-Biotech PAN-Biotech PAN-Biotech Sigma-Aldrich
Freezing medium: 50% (v/v) RPMI 1640 with stable glutamine 40% (v/v) heat-inactivated FCS 10% (v/v) DMSO	 PAN-Biotech Sigma-Aldrich Sigma-Aldrich
Washing medium: RPMI 1640 with stable glutamine 100 IU/ml penicillin 0.1 mg/ml streptomycin 5% (v/v) heat-inactivated FCS	 PAN-Biotech PAN-Biotech PAN-Biotech Sigma-Aldrich
Cell culture medium HEK293T cells: DMEM 1 mM Na-Pyruvate 10% (v/v) heat-inactivated FCS 100 IU/ml penicillin 0.1 mg/ml streptomycin	 PAN-Biotech PAN-Biotech Sigma-Aldrich PAN-Biotech PAN-Biotech
50 x TAE (Tris/Acetate/EDTA) electrophoresis buffer: 242 g Tris base 57.1 ml glacial acetic acid 37.2 g Na_2EDTA (ethylenediamine tetraacetic acid) in 1 liter water for a 1x TAE dilution of the 50x stock in water	 Carl Roth, Karlsruhe, Germany Carl Roth Carl Roth
LB (Luria or Lenox broth) medium (pH 7.0): 10 g tryptone 5 g yeast extract 5 g NaCl in 1 liter water adjust pH to 7.0 with NaOH 100 µg/ml Ampicillin	 Carl Roth Scharlau Merck, Darmstadt, Germany Serva, Heidelberg, Germany

OR 50 µg/ml Kanamycin	 Serva
LB plates with Ampicillin: 15 g agar in 1 l of LB medium 100 µg/ml Ampicillin OR 50 µg/ml Kanamycin	 Carl Roth Serva Serva

3.2 Cells and cell culture

3.2.1 Isolation of porcine peripheral blood mononuclear cells (PBMC)

Porcine blood was obtained from six- to seven-month-old healthy pigs from a slaughterhouse in Lower Austria. Animals underwent electric high-voltage anesthesia and subsequent exsanguination. This procedure is in accordance with the Austrian Animal Welfare Slaughter Regulation. For the isolation of PBMC, heparinized blood was mixed with equal amount of PBS (without Ca^{2+}, Mg^{2+}, PAN-Biotech) at room temperature (RT) and layered over 15 ml lymphocyte separation medium (Pancoll human, density 1.077 g/ml, PAN-Biotech) in sterile 50 ml tubes. The tubes were then centrifuged for 30 minutes at 920 x g at RT, with an acceleration of 4 and a deceleration of 1. Thereafter, the monolayer containing the PBMC was collected and transferred to new 50 ml tubes on ice. The tubes were filled with cold PBS (without Ca^{2+}, Mg^{2+}, PAN-Biotech) and centrifuged for 10 minutes at 470 x g at 4 °C. The supernatant was then discarded and the cells were resuspended in residual PBS. After pooling cells from the same animal into one tube, the washing step was repeated. Then, a final washing step was performed with cold washing medium (Table 1) and centrifuged as described above. After discarding the supernatant, the cells were resuspended in cold cell culture medium for PBMC (Table 1). Cells were either used directly for FCM staining and cell culture or frozen for future tests.

3.2.2. Human embryonic kidney cells (HEK293T)

Cells were cultured in cell culture medium for HEK293T cells (Table 1) in T25 (growth area 25 cm² for adherent cells) tissue flasks and incubated at 37°C with 5% CO_2. For splitting of the adherent cells, cell culture medium was removed and cells were washed twice with PBS (without Ca^{2+}, Mg^{2+}, PAN-Biotech). To detach cells, 2.5 ml pre-warmed Trypsin-EDTA (PAN-Biotech) was added to the cells and the flasks were incubated at 37°C with 5% CO_2 for 4

minutes. After gently patting the flasks to make sure that the cells were detached, 5 ml of the HEK293T cell culture medium (Table 1) were added and the cells transferred into 50 ml tubes. The cells were then centrifuged for 8 minutes at 350 x g for 6 minutes.

3.2.2 Freezing and defrosting of cells

3.2.2.1 Freezing of cells

For freezing, cells were centrifuged for 8 min at 350 x g at 4°C. The supernatant was discarded and the cell pellet was resuspended in freezing medium (Table 1). Between 3 x 10^7 (in 1 ml freezing medium) and 6 x 10^7 cells (in 1.5 ml freezing medium) were placed per cryo-vial. The cryo-vials were then placed at -80°C. After 24-72 h they were transferred into a -150°C freezer.

3.2.2.2 Defrosting of cells

Dependent on the number of cryo-vials that were to be defrosted, either a 50 ml tube (two vials) or a 15 ml tube (one vial) was chosen. The tube was filled with either cell culture medium or FCM buffer if the cells were put in culture or stained directly. 20 ml or 10 ml of medium/buffer were used for the 50 ml tube or the 15 ml tube respectively and pre-warmed in a water bath at 37°C. The cryo-vials were likewise put into the water bath at 37°C for defrosting. When only a small rest of ice remained in the vial, 1 ml of warm medium/buffer was added slowly to the cells, which were then transferred into the prepared tubes. The cells were centrifuged for 8 min at 350 x g at RT. After that, the supernatant was removed, and the cells were resuspended in medium/buffer for further use.

3.2.3 Cell counting

Cell counting for blood and PBMC was performed using a Sysmex XP-300 automated hematology analyzer (Sysmex, Vienna, Austria). For counting of the HEK293T cells the CASY Model TT (OLS OMNI Life Science, Bremen, Germany) was used.

3.2.4 PMA/Ionomycin stimulation of PBMC

For the PMA/Ionomycin stimulation, PBMC were seeded into 96-well round-bottom plates with 2 x 10^5 cells in 200 µl per well in cell culture medium. The cells were incubated at 37°C and 5% CO_2 overnight. 20 µl cell culture medium per well were added the next morning with stimuli and a Golgi Block to reach a final concentration of 50 ng/ml Phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich), 500 ng/ml Ionomycin (Sigma-Aldrich), and 1 µg/ml Brefeldin

A (BD Biosciences, San Jose, CA, USA). After incubation at 37°C and 5% CO_2 for additional four hours, the cells were harvested and analyzed for cytokine production by FCM.

3.3 Flow cytometry (FCM)

FCM analyses were performed using a FACS Canto II (BD Biosciences), which was equipped with three lasers (red, violet, and blue). At least 4 x 10^5 cells were recorded per sample. For the analysis of the data the FACSDiva software (version 8.0, BD Biosciences) and FlowJo software (version 10.6, Tree Star, Ashland, OR, USA) were used.

3.3.1 Staining of PBMC

PBMC were stained in 96-well round-bottom plates with 2 x 10^6 cells per well. All the staining steps were performed at 4°C for 20 minutes in the dark. The primary antibodies and secondary reagents (Table 2) for the FCM analyses were used in different combinations in the experiments. For a discrimination of living and dead cells, Viability Dye eFluor780 (Thermo Fisher Scientific, Vienna, Austria) was used according to manufacturer's instructions with 0.025 µl of the dye per sample. Before applying the viability dye, cells were washed twice in pure PBS (without Ca^{2+}, Mg^{2+}, PAN-Biotech), as proteins interfere with the staining. After each staining step, the cells were washed twice in their respective staining buffer. Therefore, 200 µl staining buffer per well were added and the plates were centrifuged at 470 x g for 4 minutes at 4°C. The supernatant was discarded and the plates were pulse-shaken on a plate-shaker. These steps were performed a second time before the next staining step.

For *ex vivo* analyses of cells, the porcine plasma buffer was used (Table 1). For *in vitro* activated cells, the FCS buffer was used (Table 1). For the detection of intracellular antigens, the cells were fixed and permeabilized using either the Foxp3 staining buffer set (eBioscience, San Diego, CA, USA) or the BD Cytofix/Cytoperm™ Fixation/Permeabilization Solution Kit (BD Biosciences) according to manufacturer's instruction. After fixation, the appropriate permeabilization buffer was used for all following washing steps.

Table 2: Primary antibodies and secondary reagents used for FCM analyses of porcine PBMC.

Antigen	Clone	Isotype	Fluorochrome	Labeling Strategy	Source of primary Ab
Ex vivo RORγt mAb test					
CD3	PPT3	mouse IgG1	Alexa488	secondary antibody[a)]	in-house
CD4	74-12-4	mouse IgG2b	BV421	Biotin[b)]-Streptavidin[c)]	in-house
RORγt	AFKJS-9	rat IgG2a	APC	directly conjugated	Thermo Fisher Scientific
Ex vivo CCR6 mAb test					
CD3	PPT3	mouse IgG1	Alexa488	secondary antibody[a)]	in-house
CD4	74-12-4	mouse IgG2b	BV421	Biotin[b)]-Streptavidin[c)]	in-house
CCR6	G034E3	mouse IgG2b	PE	directly conjugated	BioLegend
IL-17A and RORγt co-expression					
CD4	74-12-4	mouse IgG2b	PE	secondary antibody[d)]	in-house
IL-17A	MT504	mouse IgG1	FITC	directly conjugated	Mabtech[e)]
RORγt	AFKJS-9	rat IgG2a	APC	directly conjugated	Thermo Fisher Scientific
IL-17A and CCR6 co-expression					
CD4	74-12-4	mouse IgG2b	Alexa488	Biotin[b)]-Streptavidin[f)]	in-house
IL-17A	SCPL1362	mouse IgG1	Alexa647	directly conjugated	BD Biosciences
CCR6	G034E3	mouse IgG2b	PE	directly conjugated	BioLegend

a) goat anti-mouse anti-IgG1-Alexa488, Thermo Fisher Scientific
b) EZ-Link™ Sulfo-NHS-LC-Biotin, Thermo Fisher Scientific
c) Streptavidin-Brilliant Violet 421, BioLegend, San Jose, CA, USA
d) goat anti-mouse anti-IgG2b-PE, Southern Biotech, Birmingham, AL, USA
e) Mabtech, Nacka Strand, Sweden
f) Streptavidin-Alexa488, Thermo Fisher Scientific

3.3.2 Gating strategy to analyze porcine lymphocytes

For the analyses of $CD4^+$ T-helper cells, the same gating strategy was used for all samples (Figure 2). Lymphocytes were gated based on their forward and side scatter properties. The gate was slightly enlarged to also include activated cells that might show increased size. To exclude doublet cells from the analyses a FSC-H/FSC-A gate was introduced. For a

discrimination of live/dead cells the fixable Viability Dye eFluor780 (eBioscience) was used and a gate was placed on the living cell population (negative fixable Viability Dye eFluor780).

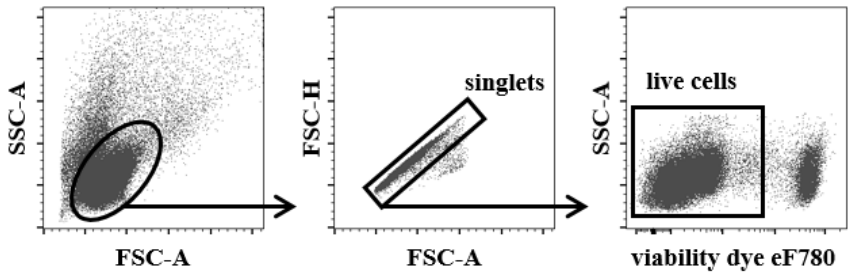

Figure 2: General gating hierarchy for porcine PBMC.
Lymphocytes were gated based on their forward and side scatter properties. The gate was slightly enlarged to also include activated cells that might show increased size. To exclude doublet cells from the analyses a FSC-H/FSC-A gate was introduced. A gate was placed on the living cell population (negative fixable Viability Dye eFluor780).

3.4 Cloning of porcine RORγt and CCR6

3.4.1 Polymerase chain reaction (PCR)

The cDNA to amplify porcine RORγt (using isolated RNA of porcine lymphocytes isolated from spleen) and CCR6 (using isolated RNA of porcine PBMC) in the PCR reaction was kindly provided by Mahsa Adib-Razavi (Institute of Immunology, University of Veterinary Medicine Vienna). A separate PCR reaction was run for each of the two targets. The mastermixes for both targets consisted of 4 µl 5x HF buffer for a final 1x concentration (Biozym Scientific, Hessisch Oldendorf, Germany), 0.4 µl of a 10 mM deoxyribonucleoside triphosphate mix for a final 200 µM concentration (dNTP, Biozym Scientific), 12.4 µl RNAse free water and 0.2 µl of a 2.0 U/µl S7 Fusion Polymerase for a final concentration of 0.02 U/µl (Biozym Scientific). Additionally, 1 µl of the specific forward and reverse primers (Table 3) for a final concentration of 500 nM (Eurofins Genomics, Ebersberg, Germany) and 1 µl of the respective porcine cDNA was added in a final reaction volume of 20 µl.

Table 3: Primers used for PCR.

Primer specifications, NCBI accession number of mRNA sequence as well as expected product length in base pairs (bp) are indicated. The restriction sites are underlined in the primer sequence, start/stop codons are shown in bold letters. For RORγt the start codon is part of the forward primer and no stop codon is present in the reverse primer as this will be provided within the final mammalian expression vector. For CCR6 the opposite situation is given.

Target	forward/ reverse	Position on mRNA sequence	Sequence (5'-3') and restriction site	Product length (bp)
RORγt	forward	236 NCBI: XM_003355171.4	GCGGCCGCRCC**ATG**AGAACACAAATTGAAGTGATCCC NotI	1504 bp
	reverse	1725 NCBI: XM_003355171.4	GAATTCTCGGACAGCCCATCAGATGAC EcoRI	
CCR6	forward	521 NCBI: XM_021086056.1	GATATCCGGACGTGTACCTCCTGAAC EcoRV	905 bp
	reverse	1413 NCBI: XM_021086056.1	TCTAGACCGCCG**TCA**CATGGTGAAG XbaI	

The PCR reactions were run on a MultiGene™ OptiMax Thermal Cycler (Labnet International, Cary, NC, USA) according to the temperature profile in Table 4 for RORγt and Table 5 for CCR6, respectively. To determine at which temperature the primers work best, six different annealing temperatures were tested in one PCR setup from 59°C to 64°C.

Table 4: RORγt temperature profile for PCR.

Step	Time	Temperature	Number of cycles
step 1	2 min	95°C	1 x
step 2	30 sec	95°C	35 x
step 3	30 sec	gradient from 59 – 64°C	
step 4	1 min and 31 sec	72°C	
step 5	5 min	72°C	1 x

Table 5: CCR6 temperature profile for PCR.

Step	Time	Temperature	Number of cycles
step 1	2 min	95°C	1 x
step 2	30 sec	95°C	35 x
step 3	30 sec	gradient from 59 – 64 °C	

step 4	30 sec	72°C	
step 5	5 min	72°C	1 x

3.4.2 Gel electrophoresis

After the PCR was completed, the products were analyzed by agarose gel electrophoresis. A 1% (w/v) agarose gel (LE Agarose, Biozym Scientific) in 1x TAE electrophoresis buffer (Table 1) was prepared. After cooling of the melted agarose, GelStar Nucleic Acid Gel Stain (Cambrex, Rockland, USA) for a final concentration of 0.05 x was added for the visualization of the PCR product by UV light. For loading of samples the DNA Gel Loading Dye (6x) (Thermo Fisher Scientific) was mixed with the PCR products for a final 1x concentration and loaded onto the gel. 2 µl of Gene Ruler Express DNA Ladder (Thermo Fisher Scientific) were used as standard for estimating the sizes of the PCR products. The gel was run at 100 V for a minimum of 30 minutes. After completion of the gel electrophoresis, the gel was visualized using the G:Box gel doc system (Syngene, Synoptics Ltd, Cambridge, England). All further upcoming gel electrophoresis analyses were performed in the same way.

3.4.3 Recovery of the DNA from agarose gel

The DNA from the agarose gel was extracted using the QIAquick Gel Extraction Kit (all kit components Qiagen, Hilden, Germany) according to the manufacturer's protocol. All the centrifugation steps were carried out at 17000 x g at RT. DNA fragments at the expected band length in agarose gel electrophoresis were cut out with a sharp scalpel under UV light. The extracted gel was weighed in a 1.5 ml tube and 3x volumes of the QG buffer (w/v) were added to the gel. The tube was incubated at 50°C until the gel was completely dissolved. One gel volume of isopropanol was added to the sample. Then a QIAquick spin column was placed into a 2 ml collection tube, the sample was added to the column and the assembly was centrifuged for one minute. The flow-through was discarded and 500 µl of the QG buffer were added to the column. After an one-minute incubation step the sample was centrifuged for one minute to get rid of residual buffer. After discarding the flow-through, 750 µl of PE buffer were added to the column. After five minutes of incubation at RT, the sample was centrifuged for one minute. The flow-through was discarded again and the column was centrifuged for 5 minutes for drying. For the elution of the DNA, the column was put into a new 1.5 ml tube and 20 µl of DNAse

and RNAse free water were added. An incubation step at RT for two minutes and a centrifugation step for one minute followed. The last two steps for the elution were repeated with additional 15 µl of DNAse and RNAse free water. After purification of the DNA, the concentration was measured using the NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific).

3.4.4 Blunt-end cloning

In the blunt-end cloning the gene-specific product obtained by PCR was introduced into a blunt-end vector, the bacterial vector was amplified in bacteria and vector DNA was isolated for further processing.

3.4.4.1 Blunt-end ligation reaction

For the ligation of the purified DNA into the blunt-end vector system, the Clone JET1.2 PCR Cloning Kit (Thermo Fisher Scientific) was used for both, the RORγt and the CCR6 product. The ligation was done according to manufacturer's instructions. The ligation reactions were set up in a 1.5 ml reaction tube for a molecular ratio of 1:3 between the backbone and the insert. For the ligation of RORγt, 4.3 µl of the purified PCR product (17.45 ng/µl) were added to 10 µl of a 2 x reaction buffer, 2.7 µl of nuclease free water, 2 µl of T4 DNA ligase and 1 µl of the pJET 1.2/blunt cloning vector (50 ng/µl) for a total of 20 µl reaction mix. For CCR6 the same ligation reaction mix was used, but upscaled to 24 µl total volume due to the low concentration of the CCR6 DNA. Therefore, 8.3 µl of the purified PCR product (5.5 ng/µl) were added to 12 µl of a 2 x reaction buffer, 0.7 µl of nuclease free water, 2 µl of T4 DNA ligase and 1 µl of the pJET 1.2/blunt cloning vector (50 ng/µl). The ligation reactions where then incubated at RT for one hour and 30 minutes.

3.4.4.2 Transformation of *Escherichia coli (E.coli)*

After the ligation reaction was completed, the ligated products were introduced into competent *E. coli* cells (*E. coli* JM109, Promega Corporation). For the transformation 5 µl of the ligation reaction were added to 50 µl of the competent cells that were thawed on ice. The tubes containing the cells were gently flicked to mix the cells with the DNA and then incubated on ice for 20 minutes. After that, the reaction was heat-shocked for one minute in a water bath at 42°C and then placed on ice for two minutes. Next, 500 µl of pre-warmed SOC medium (Sigma-Aldrich) was added to the cells and the solution was incubated for 1.5 hours at 37°C on

a heat-shaker at 250 rpm. The bacteria were then plated onto LB plates with Ampicillin in two steps. In each step, 150 µl of the transformation reaction were added to the plates and thoroughly spread. Finally, the plates were put into an incubator overnight at 37°C. Only successfully transformed bacteria grew on the agar plates due to the Ampicillin resistance gene in the vector sequence. Furthermore, the pJET 1.2/blunt vector contains the lethal gene eco47IR which is activated if the vector circularizes and does not include an insert. Therefore, only bacteria with an insert survived and grew.

3.4.4.3 Colony PCR

To control whether the transformation was successful, a colony PCR was run for both RORγt and CCR6. Therefore, single colonies were picked from each plate with sterile toothpicks and were put into 0.5 ml reaction tubes containing 100 µl of sterile water with Ampicillin (100 µg/ml). The tubes were then put on a shaker to disperse the cells for 5 minutes. The mastermix for the colony PCRs of both reactions consisted of 6.25 µl GoTaq® G2 Green mastermix (Promega Corporation, Madison, WI, USA) resulting in a 1 x concentration, as well as 1.25 µl RNAse free water and 0.25 µl of the forward and reverse primers (Table 6) for a final concentration of 200 nM (Eurofins Genomics). To this mastermix 4.5 µl of the bacteria/water mix was added for a final reaction volume of 12.5 µl. The PCR reactions were run on a MultiGene^{TM} OptiMax Thermal Cycler (Labnet International) according to the temperature profile in Table 7.

Table 6: Primers used for pJET 1.2/blunt vector colony PCR.
Primer specifications and expected product length in base pairs (bp) are indicated.

Target	forward/ reverse	Sequence (5'-3')	Product length (bp)
pJET1,2/blunt vector	forward	CGACTCACTATAGGGAGAGCGGC	RORγt insert: 1626 bp CCR6 insert: 1024 bp empty: 119 bp
	reverse	AAGAACATCGATTTTCCATGGCAG	

Table 7: Temperature profile for pJET 1.2/blunt vector colony PCR.

Step	Time	Temperature	Cycle
step 1	2 min	95°C	1 x
step 2	30 sec	95°C	35 x
step 3	30 sec	60°C	
step 4	1 min and 37 sec	72°C	
step 5	5 min	72°C	1 x

The PCR products were analyzed by gel electrophoresis as described in chapter 3.4.2. The GoTaq® G2 Green mastermix already contained a loading dye.

3.4.4.4 Plasmid Miniprep

For the plasmid miniprep, 4 ml of LB medium (Table 1) with 100 µg/ml Ampicillin were inoculated with 10 µl of the bacteria/water mix obtained in 3.4.4.3 and incubated at 37°C overnight on a shaker at 250 rpm. The plasmid miniprep was done using the ZR Plasmid Miniprep – Classic kit (all kit components Zymo Research, Irvine, CA, USA) according to manufacturer's instructions. After the overnight incubation, the bacterial culture was centrifuged 5 minutes at 1200 x g at RT and the supernatant was discarded. All further centrifugation steps were done at 17000 x g at RT. 200 µl of the P1 buffer were added to the bacterial cells and the pellet was resuspended completely. Thereafter, 200 µl of the P2 buffer were added and the tubes were inverted four times for thoroughly mixing. A one-minute incubation step at RT followed. Next, 400 µl of the P3 buffer were added and the mixture was mixed gently and incubated for 2 minutes at RT until the sample turned yellow. Then the samples were centrifuged for 4 minutes. After centrifugation, a Zymo-spin IIN column was placed into a collection tube and the supernatant was transferred onto the column. The assembly was then centrifuged for 30 seconds and the flow-through was discarded. For the next step, 200 µl Endo-Wash Buffer were added to the column and the samples were centrifuged for additional 30 seconds. After adding 400 µl of Plasmid Wash Buffer a further one-minute centrifugation step followed. The flow-through was discarded thereafter and the column/collection tube assembly was centrifuged for another 3 minutes for drying. The column was then placed into a new 1.5 ml tube, 20 µl of water were added to the column. After incubation for one minute at RT a one-minute centrifugation step followed to elute the DNA. The last two steps were

repeated with additional 20 µl of water. Concentration of the purified DNA was measured using the NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific).

3.4.5 Sticky-end cloning

To clone the RORγt and CCR6 sequences into mammalian expression vectors, sticky-end cloning was performed using the restriction sites that were introduced by the PCR primers (3.4.1). For the two targets different mammalian expression vectors were selected that are shown in Figure 3. Both vectors contain a FLAG-sequence, leading to the expression of a FLAG-tag fused to the introduced gene. Commercially available anti-FLAG mAbs recognize the FLAG peptide sequence DYKDDDDK and can be used as control for the correct expression of the recombinant fusion protein. The pSF-CMV-Puro-COOH-TEV-FLAG vector (Sigma-Aldrich) was used for the RORγt cloning. Here, the RORγt sequence will be cloned upstream of the FLAG sequence, resulting in a C-terminal FLAG-tag in the protein. The protein will stay intracellular, as no signal sequence is present. The pSF-CMV-NH2-PPT-3xFLAG vector (Sigma-Aldrich) was used for the CCR6 cloning. Here, the CCR6 sequence was cloned downstream of the FLAG sequence, resulting in a N-terminal FLAG tag in the protein. Before the FLAG sequence a signal sequence was present in the vector leading to extracellular expression of the fusion protein.

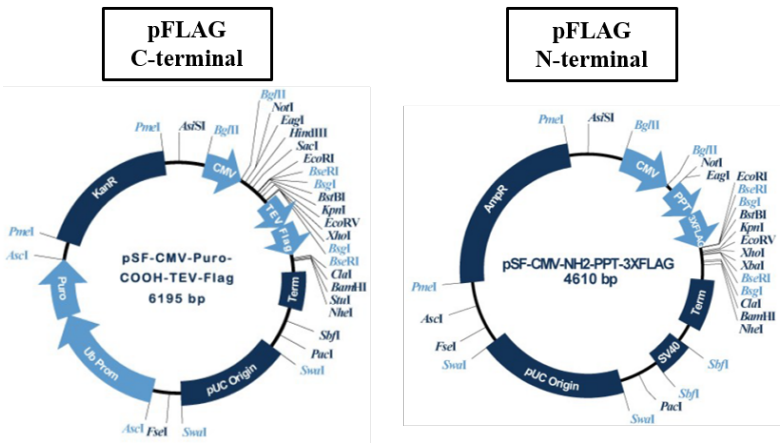

Figure 3: Mammalian expression vectors used in the study.
The pSF-CMV-Puro-COOH-TEV-FLAG vector has the multiple cloning site before the FLAG sequence and contains a Kanamycin resistance gene for selection in bacterial cells. The pSF-CMV-NH2-PPT-3xFLAG vector has the multiple cloning site after a signal sequence and the FLAG sequence and contains an Ampicillin resistance gene for selection in bacterial cells. https://www.sigmaaldrich.com/austria.html

3.4.5.1 Restriction digest and phosphatase treatment

Restriction digest reactions were set up for the RORγt-pJET 1.2/blunt vector and the pSF-CMV-Puro-COOH-TEV-FLAG vector as well as for the CCR6-pJET 1.2/blunt vector and the pSF-CMV-NH2-PPT-3xFLAG vector. For RORγt, the reaction was set up in a 1.5 ml tube for a total 25 µl reaction volume with 2.5 µl O buffer (Thermo Fisher Scientific), 16.3 µl of nuclease free water, 2.9 µl of RORγt-pJET 1.2/blunt vector DNA (228.35 ng/µl), 2 µl of EcoR1 and 2 µl of Not1 (both enzymes Thermo Fisher Scientific). Parallel to this, a digestion of 2.5 µl of O Buffer (Thermo Fisher Scientific), 16.2 µl of nuclease free water, 2.3 µl of pSF-CMV-Puro-COOH-TEV-FLAG vector DNA (218 ng/µl) as well as 2 µl of each EcoRI and NotI (both Thermo Fisher Scientific) was set up. For CCR6, the reaction was set up in a 1.5 ml tube for a total 25 µl reaction volume with 5 µl Tango buffer (Thermo Fisher Scientific), 11.8 µl of nuclease free water, 2.3 µl of the CCR6-pJET 1.2/blunt vector DNA (205.95 ng/µl), 2 µl of the enzyme EcoR1 and 4 µl of the enzyme Xba1 (both Thermo Fisher Scientific). Parallel to this, a digestion reaction of 5 µl Tango buffer (Thermo Fisher Scientific), 11.6 µl nuclease free water, 2.4 µl of pSF-CMV-NH2-PPT-3xFLAG vector DNA (209.4 ng/µl) as well as 2 µl of EcoRV and 4 µl of XbaI (both Thermo Fisher Scientific) was set up. The tubes containing the restriction digest reactions were mixed gently and incubated for one hour at 37°C. After the restriction digest, the samples were incubated at 80°C for 20 minutes for heat inactivation of the enzymes.

To verify that the restriction digest worked properly, a gel electrophoresis of the samples was performed as described in chapter 3.4.2. For this, 5 µl of the digestion samples was used. The expected DNA fragments are outlined in Table 8.

Table 8: Expected lengths of the DNA fragments after restriction digest.
The lengths of the DNA fragments were predicted using the free online tool NEBcutter V2.0 (New England BioLabs, http://nc2.neb.com/NEBcutter2/).

Vector	Restriction enzymes	Fragment length in base pairs (bp)	Fragment description
RORγt-pJET 1.2/blunt	NotI and EcoRI	2937 bp 1500 bp	backbone insert to be cloned
pSF-CMV-Puro-COOH-TEV-FLAG	NotI and EcoRI	6153 bp 44 bp	backbone to be cloned insert
CCR6- pJET 1.2/blunt	EcorV and XbaI	2971 bp 897 bp	backbone insert to be cloned
pSF-CMV-NH2-PPT-3xFLAG	EcorV and XbaI	4594 bp 16 bp	backbone to be cloned insert

Thereafter a phosphatase treatment of the reaction mixtures with the pJET 1.2/blunt vector for both, RORγt and CCR6 followed to avoid re-ligation of the insert into the vector backbone. Therefore, 1 µl (1 U) of a calf intestinal alkaline phosphatase (CIAP, Thermo Fisher Scientific) was added directly to the digestion mix and incubated for 30 minutes at 37°C.

For the purification of DNA fragments of the restriction reactions the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's protocol was used as described in 3.4.3 without the agarose gel melting step. After this procedure, the samples of the pJET 1.2/blunt vector for RORγt or CCR6 should contain the backbone as well as the insert (re-ligation not possible due to the CIAP treatment). The samples of the two pFLAG vectors should only contain the backbone for further cloning, as the small DNA fragments of 44 bp and 16 bp should be removed during the washing steps as the column membrane has a range >70 bp.

3.4.5.2 Sticky-end ligation, amplification, and purification of vector DNA

For the ligation a molecular ratio of 1:6 for the pFLAG backbones and the RORγt or CCR6 DNA was selected. The ligation reactions were set up in a 1.5 ml reaction tubes. For the ligation of RORγt, 6.5 µl of the purified DNA fragments (11.85 ng/µl) were added to 4 µl of the 5 x Rapid Ligase buffer (Thermo Fisher Scientific), 4.5 µl of nuclease free water, 2 µl of T4 DNA ligase (Thermo Fisher Scientific) and 3.0 µl of the pSF-CMV-Puro-COOH-TEV-FLAG DNA (16.16 ng/µl) for a total of 20 µl. For CCR6 the same ligation reaction mix was used with 2.3 µl of the purified DNA fragments (19.4 ng/µl), 4 µl of the 5 x Rapid Ligase buffer (Thermo Fisher Scientific), 9.4 µl of nuclease free water, 2 µl of T4 DNA ligase (Thermo Fisher Scientific) and 2.4 µl of the pSF-CMV-NH2-PPT-3xFLAG DNA (21.3 ng/µl) for a total of 20 µl. The ligation reactions where then incubated at RT for one hour and 30 minutes.

After the ligation reaction was completed, the ligated products were introduced into competent *E. coli* cells as described in 3.4.4.2. For RORγt, LB agar plates with Kanamycin (Table 1) were used, while LB agar plates with Ampicillin (Table 1) were used for CCR6.

To control whether the transformation was successful, colony PCRs were run as described in 3.4.4.3. The same PCR mastermix was used, but with different forward and reverse primers specific for the pFLAG vectors (Table 9). The PCR reactions were run on a MultiGene™ OptiMax Thermal Cycler (Labnet International) according to the temperature profiles in Table 10.

Table 9: Primers used for pFLAG vector colony PCR.
Primer specifications and expected product length in base pairs (bp) are indicated.

Target	forward/ reverse	Sequence (5'-3')	Product length (bp)
pSF-CMV-Puro-COOH-TEV-FLAG vector	forward	TGTCGTAACAACTCCGCCC	RORγt insert: 1877 bp empty: 419 bp
	reverse	ACTGCATTCTAGTTGTGGTTTGT	
pSF-CMV-NH2-PPT-3xFLAG vector	forward	TGTCGTAACAACTCCGCCC	CCR6 insert: 1307 bp empty: 426 bp
	reverse	TGTGAGCTGAAGGTACGCTG	

Table 10: Temperature profile for pFLAG vector colony PCR.

Step	Time	Temperature	Cycle
step 1	2 min	95°C	1 x
step 2	30 sec	95°C	35 x
step 3	30 sec	60°C	
step 4	1 min and 53 sec for RORγt 1 min and 19 sec for CCR6	72°C	
step 5	5 min	72°C	1 x

The PCR products were analyzed by gel electrophoresis as described in chapter 3.4.2. The GoTaq® G2 Green mastermix already contained a loading dye.

Finally, vector DNA was further amplified and purified from bacteria by plasmid minipreps as described in 3.4.4.4. Due to the different antibiotic resistances, LB medium with 100 µg/ml Ampicillin was used for CCR6, while LB medium with 50 µg/ml Kanamycin was used for RORγt (Table 1).

3.5 Test on cross-reactivity of the human/mouse RORγt and CCR6 mAbs

3.5.1 Transfection of HEK293T cells

For the transfection of HEK293T cells, cells were defrosted and cultured according to chapters 3.2.2 and 3.2.3.2. To reach 70-80% confluency of cells, 1.6 x 10^6 cells were seeded two days before transfection in T25 cell culture flasks in 5 ml HEK293T cell culture medium (Table 1). In a first step for the transfection, cell culture medium was removed and the cells were washed two times with PBS (without Ca^{2+}, Mg^{2+}, PAN-Biotech). Thereafter, 2 ml of the cell culture medium for HEK293T cells (Table 1) without antibiotics were added to the cells. For the

transfection 5 µg of DNA of either the RORγt-pFLAG or CCR6-pFLAG vector were diluted in 100 µl pure DMEM without any additives (PAN-Biotech). 50 µl of PolyFect transfection reagent (Qiagen) were added to the DNA and incubated for 10 minutes at RT to allow complex formation. After that, 0.5 ml cell culture medium for HEK293T cells (Table 1) without antibiotics were added to the complex and the whole solution was added to the cells. The flasks were incubated at 37°C with 5% CO_2 for 3 hours before adding another 2 ml of medium to the cells and finally incubating them for further 16 hours. In parallel, HEK293T cells were treated the same way without vector transfection and served as negative untransfected controls.

3.5.2 Cross-reactivity testing by FCM

After the incubation of the transfected cells, cells were detached as described in 3.2.2. and forwarded to FCM analyses. FCM analyses were performed as described in 3.3.1 including all washing steps and the viability dye. The respective primary mAbs and secondary reagents used are listed in Table 11. For RORγt, intracellular staining was performed to detect both, RORγt and the FLAG-tag. For this approach, the Foxp3 staining buffer set (eBioscience) was used. For CCR6 the FCS buffer was used (Table 1) and no fixation/permeabilization was applied to these cells. For analyses, cells were gated on single live cells.

Table 11: Primary antibodies and secondary reagents used for FCM analyses of HEK293T.

Antigen	Clone	Isotype	Fluorochrome	Labeling Strategy	Source of primary Ab
RORγt mAb test					
FLAG	M2	mouse IgG1	BV421	secondary antibody[a)]	Sigma-Aldrich
RORγt	AFKJS-9	rat IgG2a	APC	directly conjugated	Thermo Fisher Scientific
CCR6 mAb test					
FLAG	M2	mouse IgG1	BV421	secondary antibody[a)]	Sigma-Aldrich
CCR6	G034E3	mouse IgG2b	PE	directly conjugated	BioLegend

a) rat anti-mouse anti-IgG1-BV421, clone RMG1-1, BioLegend

3.6 Sequencing

Sanger sequencing of the RORγt-pFLAG and CCR6-pFLAG vectors used for the transfection was performed on purified vector DNA (Eurofins Genomics). Sequences were kindly analyzed and interpreted by Mahsa Adib-Razavi (Institute of Immunology, University of Veterinary Medicine Vienna).

4 Results

4.1 Test of potentially cross-reactive mAbs against RORγt and CCR6 on porcine lymphocytes

Potentially cross-reactive mAbs against RORγt and CCR6 were tested in multicolor FCM to characterize the expression pattern of these markers on porcine $CD4^+$ T cells. For the experiments, a general gating strategy was used as described in chapter 3.3.2 to exclude non-lymphocytes, doublet cells and dead cells.

4.1.1 Potential RORγt$^+$ and CCR6$^+$ porcine lymphocytes

As reported by Kronsteiner et al., $CD4^+$ cells expressing RORγt have been identified in the pig by the use of the anti-human/mouse mAb clone AFKJS-9 (Kronsteiner et al. 2013). However, in this study no original FCM data on the RORγt expression was shown and no proof of real cross-reactivity of the mAb exists. To assess the cross-reactivity of the RORγt antibody (clone AFKJS-9) a multicolor FCM experiment was performed with porcine lymphocytes. For this experiment, PBMC were fixed using the Foxp3 staining buffer set (eBioscience). As shown in Figure 4A, a small but distinct population of RORγt$^+$ cells of approximately 1.5% was visible. The majority of those cells were found amongst $CD3^+$ cells and most of them were $CD4^+$.

The antibody specific for human/mouse CCR6 (clone G034E3) has also been tested using multicolor FCM. For the detection of CCR6 no fixation was necessary as the chemokine receptor is expressed on the cell surface (Figure 4B). In this first experiment, cells were gated on single lymphocytes without live/dead discrimination, as this is not routinely done on unfixed cells for *ex vivo* analyses. A small population of CCR6$^+$ cells of under 0.5% could be detected and parts of them were $CD3^+$ as well as $CD3^-$. Similar results were obtained when the expression of CCR6 was compared to the expression of CD4. Nevertheless, when including the viability dye eFluor780 in the staining panel an unexpected result was observed. The last graph of Figure 4B shows the expression of CCR6 against the viability dye eFluor780 and the red arrow indicates cells that are double positive. Therefore, most of the CCR6 positive cells are amongst the dead cells. In Figure 4C, the gate was set on living, single lymphocytes, and the percentage of CCR6$^+$ dropped to about 0.1% and most of the CCR6$^+$ cells were $CD3^-$ as well as $CD4^-$.

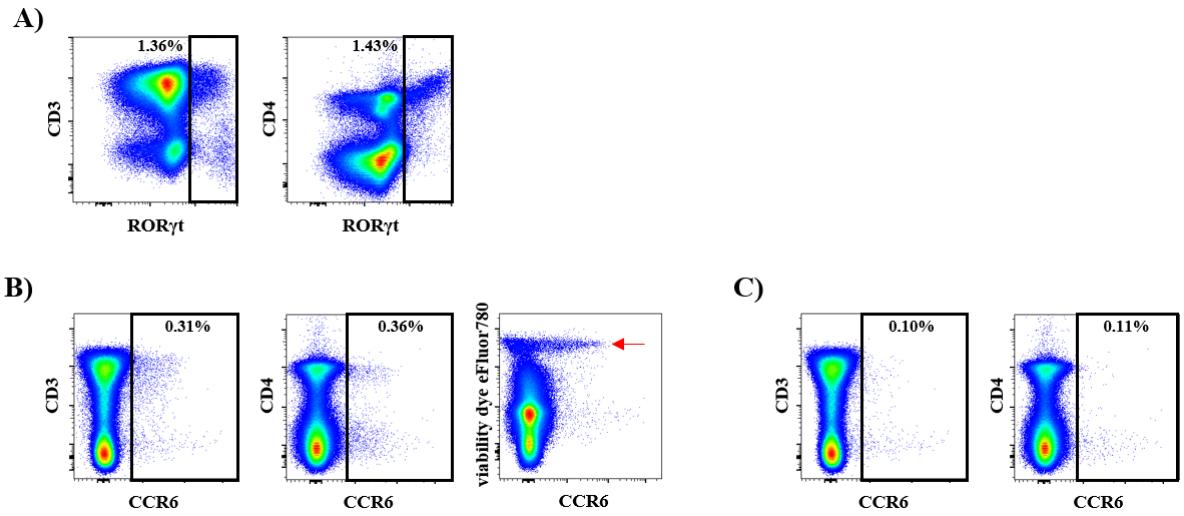

Figure 4: Expression of RORγt and CCR6 on porcine lymphocytes.
Porcine lymphocytes that express RORγt and CCR6 were detected by multicolor FCM using a RORγt antibody (clone AFKJS-9) and a CCR6 antibody (clone G034E3) in combination with mAbs against CD3 and CD4. **(A)** Cells fixed by Foxp3 staining buffer set (eBioscience) were analyzed for RORγt expression. The percentages of RORγt$^+$ cells are indicated in the graphs. Cells were gated on live, single lymphocytes as shown in Figure 2. **(B)** Unfixed cells with a gate on single lymphocytes were analyzed for the expression of CCR6. The red arrow indicates CCR6$^+$ cells amongst a population of dead cells. **(C)** Unfixed cells with a gate on live, single lymphocytes were analyzed for the expression of CCR6. The percentage of CCR6$^+$ cells is indicated in the graphs.

4.1.2 Co-expression of IL-17A with RORγt and CCR6 on porcine lymphocytes

IL-17A is the main effector cytokine produced by Th17 cells (Korn et al. 2009). In the pig, the use of cross-reactive anti-human mAbs against IL-17A are already described to identify IL-17A producing cells. A co-expression of IL-17A with either RORγt or CCR6 after cell stimulation would further strengthen the potential cross-reactivity of the two tested mAbs.

For the staining of intracellular markers, the cells that are to be analyzed need to be fixed and permeabilized. The BD Cytofix/Cytoperm Fixation/Permeabilization Solution Kit (BD Biosciences) is recommended for the detection of cytokines but might be too weak to make the detection of transcription factors possible. The Foxp3 staining buffer set (eBioscience) on the other hand is recommended for the staining of intracellular markers like transcription factors but might lead to weaker signals of cytokine stainings (in-house observations). Since the co-expression of both IL-17A and RORγt was to be studied, both permeabilization kits were tested (Figure 5) on porcine lymphocytes activated with PMA/Ionomycin for 4 hours. No obvious difference in the percentages of IL-17A positive cells between the two permeabilization kits

were seen (0.26% and 0.23%, respectively). Nevertheless, the median fluorescence intensity (MFI) of IL-17A using the BD Biosciences kit was slightly higher with 3518 (Figure 5A) compared to the MFI of 3273 using the eBioscience kit (Figure 5B). The majority of IL-17A producing cells co-expressed CD4. There was a clear population of RORγt^{+} cells, both amongst CD4^{+} as well as CD4^{-} cells with a total of approximately 1.0% (Figure 5A and B). In a next step, the cells were further gated on CD4 and CD4^{+} cells and were analyzed for a co-production of IL-17A and RORγt (graphs on the right in Figure5A and B). A clear single positive population for RORγt was visible as well as an IL-17A^{+}RORγt^{dim} population for both fixation methods (0.60% and 0.58%).

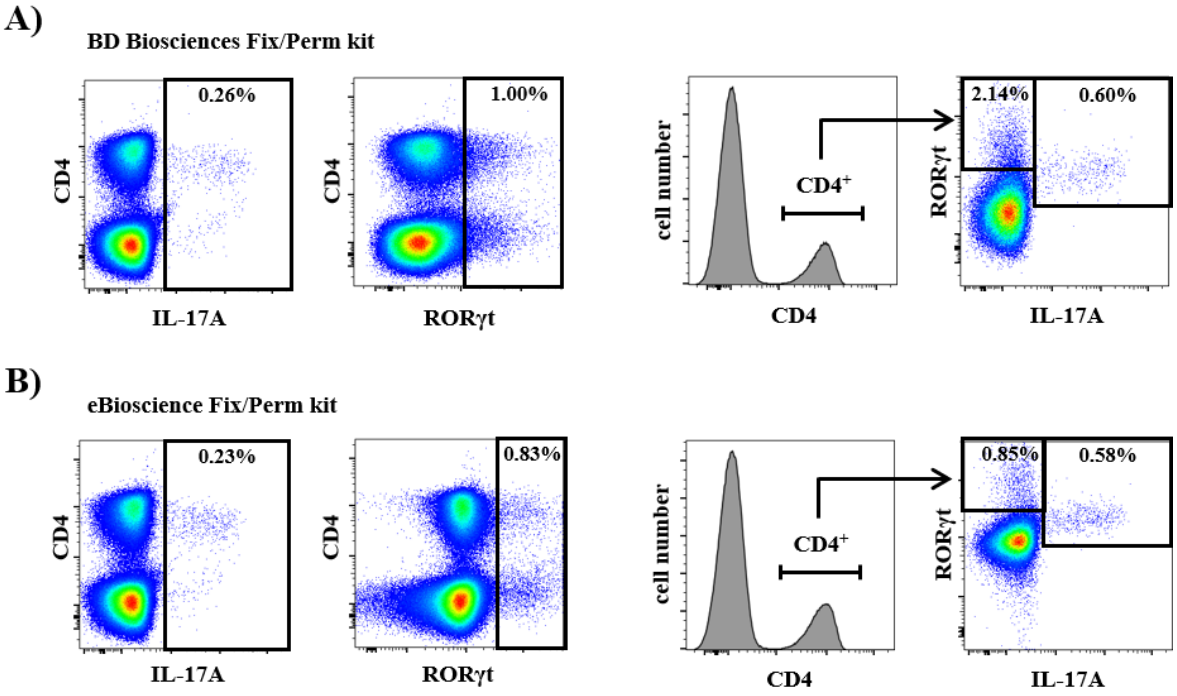

Figure 5:Co-expression of IL-17A and RORγt on activated porcine lymphocytes using two different fixation/permeabilization kits.
For the detection of co-expression of IL-17A and RORγt two multicolor FCM experiments were performed, one using the BD Cytofix/Cytoperm Fixation/Permeabilization Solution Kit (BD Biosciences) **(A)** and the other using the Foxp3 staining buffer set (eBiosciences) **(B)**. Cells were gated on live, single lymphocytes as shown in Figure 2. The two graphs on the left show the IL-17A and RORγt co-expression with CD4. The two graphs on the right show further gating on CD4^{+} cells and IL-17A co-expression with RORγt. The percentages of IL-17A^{+} and RORγt^{+} cells are indicated in the gates.

The detection of CCR6 should be possible without fixation and permeabilisation of the cells. Nevertheless, to detect co-expression with IL-17A this treatment was necessary. The BD

Cytofix/Cytoperm Fixation/Permeabilization Solution Kit (BD Biosciences) was chosen for this approach for an optimized IL-17A expression (Figure 6). After activation of cells with PMA/Ionomycin for 4 hours, IL-17A production could be observed. Likewise, a small population of $CCR6^+$ cells could be detected, however most of the positive cells were $CD4^-$ and showed a $CCR6^{dim}$ phenotype. When the cells were further gated for $CD4^+$ cells, no IL-17A and CCR6 double positive cells were visible (Figure 6, graphs on the right).

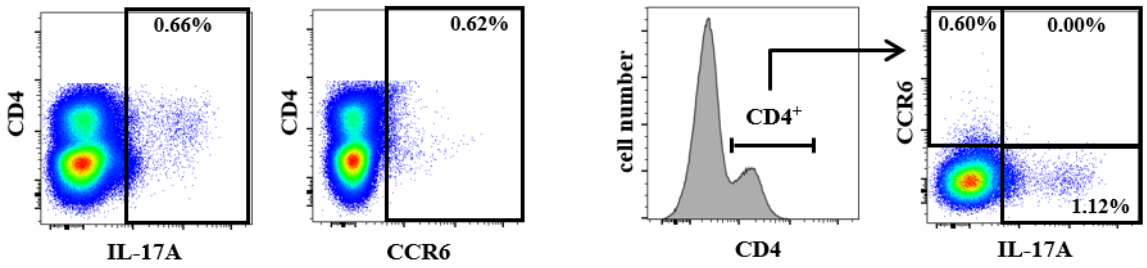

Figure 6: Co-expression of IL-17A and CCR6 on activated porcine lymphocytes.
The co-expression of IL-17A and CCR6 was studied by multicolor FCM. The cells were fixed and permeabilized using the BD Cytofix/Cytoperm Fixation/Permeabilization Solution Kit (BD Biosciences) for the detection of IL-17A. Cells were gated on live, single lymphocytes as shown in Figure 2. The two graphs on the left show the IL-17A and CCR6 co-expression with CD4. The two graphs on the right show further gating on $CD4^+$ cells and IL-17A co-expression with CCR6. The percentages of $IL\text{-}17A^+$ and $CCR6^+$ cells are indicated in the gates.

4.2 Molecular proof of cross-reactivity

To get the final proof that the mAbs against RORγt and CCR6 are cross-reactive to the porcine proteins, the porcine molecules were cloned into expression vectors, expressed in a mammalian cell line, and finally detected by multicolor FCM using the antibodies in question.

4.2.1 Cloning of porcine RORγt and CCR6

At first, the porcine-specific sequences for both RORγt and CCR6 were amplified by PCR using gene-specific primers with restriction overhangs. For RORγt several unspecific bands were detected by gel electrophoresis after PCR. Nevertheless, also a band for the PCR product of the expected length of 1504 bp was found (Figure 7A). The expected length for the PCR product for CCR6 was 905 bp, and there were no other unspecific bands visible (Figure 8A).

Using the CloneJET PCR Cloning Kit (Thermo Fisher Scientific), the sequences were ligated into the pJET 1.2/blunt cloning vector and competent *E. coli* were transformed. Thereafter, 20 single colonies were picked, and presence of the insert was analyzed by PCR and gel electrophoresis. If the ligation worked, a band at 1626 bp was expected for RORγt and a band of 1024 bp for CCR6. If the vector ligated without the insert a band of 119 bp would be visible. 4 out of 20 colonies were positive for RORγt blunt-end cloning (Figure 7B). One sample, number 10, showed a band slightly higher than expected. The no template control (NTC) showed the same band, which suggests a contamination in the mastermix. For CCR6 all 20 colonies that were picked showed a band at the expected length (Figure 8B).

Positive clones for both targets where then selected to be expressed in a pFLAG vector. Here the gene of interest was cloned in-frame with a FLAG-tag resulting in a fusion protein that could be expressed in mammalian cells. Sticky-end cloning by digestion with restriction enzymes was used for this approach. For the RORγt blunt-end vector three positive clones were selected and a restriction digest with the enzymes NotI and EcoRI was performed for the samples as well as for a C-terminal pFLAG vector. To make sure, that the restriction digest worked properly a gel electrophoresis was run. The expected lengths for the blunt-end vector RORγt insert were 1500 bp and 2937 bp for the plasmid backbone. Only one of the three clones that were digested showed bands at the expected length (Figure 7C). For the C-terminal pFLAG vector a band at 6153 bp was expected. A small sequence of 44 bp, that was cut out between the restriction sites was also produced but was too small to be visible in the gel. For CCR6 the restriction digest was done for one positive clone as well as for a N-terminal pFLAG vector with EcoRV and XbaI. For CCR6 the expected length of the CCR6 insert was 897 bp and 2971 bp for the backbone of the blunt-end plasmid vector (Figure 8C) and was visible for the digested clone. The expected band for the N-terminal pFLAG vector was at 4594 bp. The sequence cut out in between the restriction sites consisted of 16 bp and was too small to be visible in the gel.

After a successful restriction digest, the gene-specific sequences were ligated into the opened pFLAG vectors and competent *E. coli* were transformed. A colony PCR was run to screen for positively ligated bacterial clones. For RORγt six colonies out of 20 were positive and showed a band at 1877 bp (Figure 7D). For the colony PCR for CCR6 19 colonies were tested and 9

showed a band at the expected length of 1307 bp (Figure 8D). Lower bands of 419 bp (Figure 7D) and 426 bp (Figure 8D) indicate colonies with un-ligated pFLAG vectors.

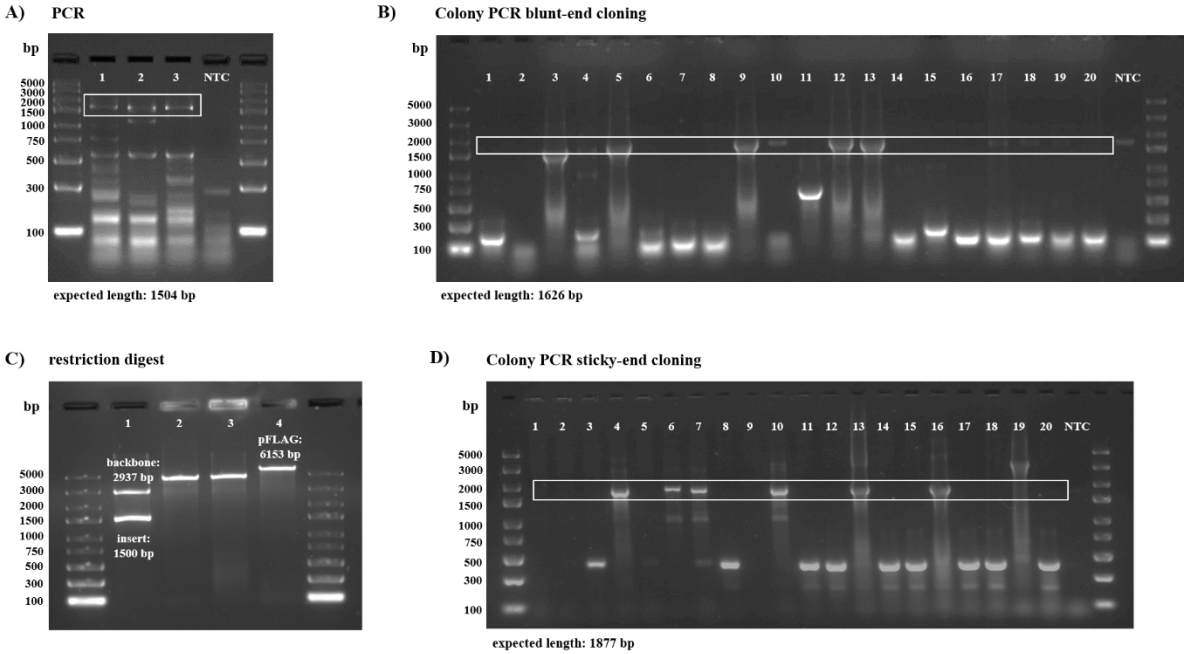

Figure 7: Blunt-end and sticky-end cloning of porcine RORγt.
(A) The coding sequence of porcine RORγt was amplified by PCR and amplification was confirmed with three replicates by gel electrophoresis (lanes 1-3). **(B)** After transformation of *E. coli* a colony PCR of 20 colonies was performed (lanes 1-20). **(C)** After restriction digest of the RORγt blunt-end vector and the pFLAG vector with NotI and EcoRI fragments were analyzed by gel elecrophoresis. **(D)** After ligation of the RORγt sequence into the pFLAG vector and the transformation of competent *E. coli* a colony PCR was performed for 20 colonies (lanes 1-20). For all gels a 1% (v/v) agarose gel was used and the GeneRuler express DNA ladder (Thermo Fisher Scientific) was applied to the first and last lane of the gel as size standard. The expected lengths of the products are indicated and highlighted within the white boxes for PCR reactions at the expected band length. NTC = no template control

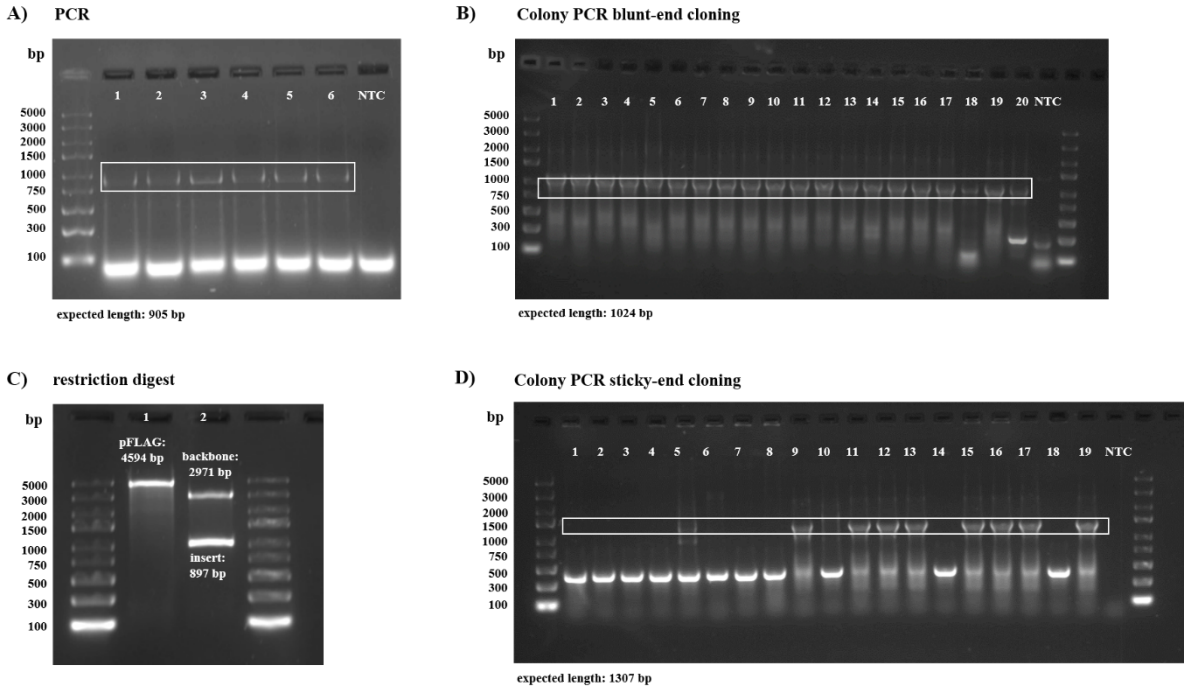

Figure 8: Blunt-end and sticky-end cloning of porcine CCR6.
(A) The coding sequence of porcine CCR6 was amplified by PCR. The temperature gradient for the annealing temperature of the primers is shown (59 - 64°C, lanes 1-6). **(B)** After transformation of *E. coli* a colony PCR of 20 colonies was performed (lanes 1-20). **(C)** After restriction digest of the CCR6 blunt-end vector and the pFLAG vector with EcoRV and XbaI fragments were analyszed by gel elecrophoresis. **(D)** After ligation of the CCR6 sequence into the pFLAG vector and the transformation of competent *E. coli* a colony PCR was performed for 19 colonies (lanes 1-19). For all gels a 1% (v/v) agarose gel was used and the GeneRuler express DNA ladder (Thermo Fisher Scientific) was applied to the first and last lane of the gel as size standard. The expected lengths of the products are indicated and highlighted within the white boxes for PCR reactions at the expected band length. NTC = no template control

4.2.2 Analysis of transfected HEK293T cells with recombinant porcine RORγt and CCR6

After transiently transfecting HEK293T cells with the pFLAG plasmid vector containing either the porcine RORγt or the CCR6 sequence, the transfected cells were analyzed by FCM. In parallel, untransfected HEK293T cells were used as control. Cells were stained with the cross-reactive mAb candidates. In parallel a commercially availbale mAb directed aginst the FLAG-tag was used as control for correct expression of the recombiant protein.

For RORγt two different clones were used for the transfections and compared to the controls (Figure 9). The cells that were untransfected showed no signal with the specific mAbs,

comparable to the unstained control (no mAb, empty). Transfected cells for one clone did not show signals for the RORγt nor the FLAG staining (clone 1). The other RORγt transfectant showed a clear signal for RORγt, but no signal for the FLAG tag (clone 2).

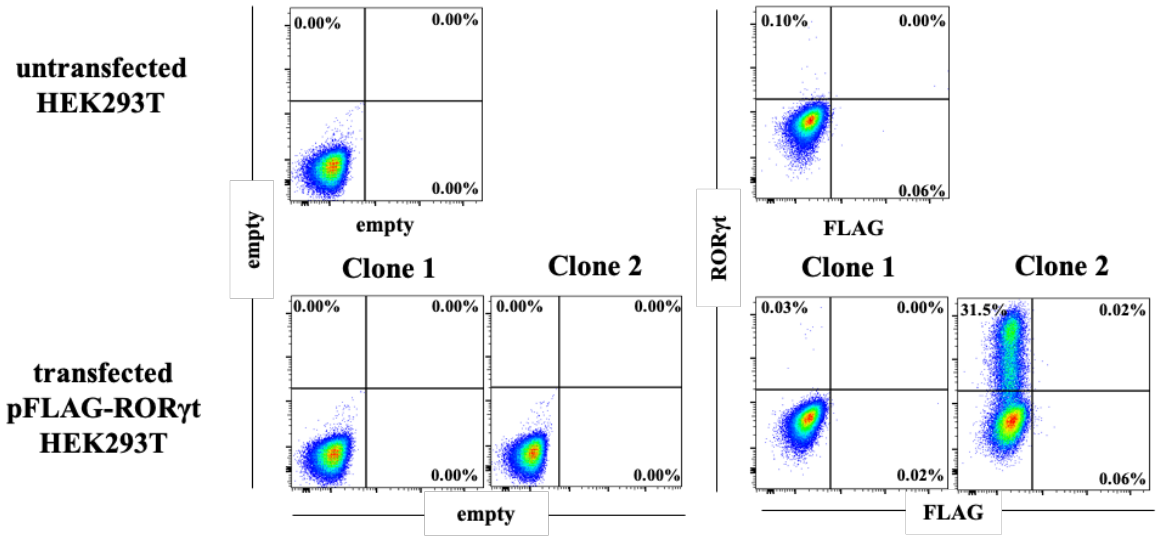

Figure 9: FCM analysis of HEK293T cells transfected with a pFLAG vector containing the porcine RORγt sequence.
The transfected HEK293T cells with two different RORγt-pFLAG clones were analyzed using multicolor FCM and compared to untransfected HEK293T cells. Cells were either not stained with mAbs (empty, graphs on the left) or with the specific anti-RORγt and anti-FLAG mAbs (graphs on the right). The percentages of positive signals are indicated in the quadrants.

For CCR6 one vector clone was used for the transfection (Figure 10). No clear staining with the anti-CCR6 mAb was observed in the transfected cells. Interestingly, in the untransfected cells a shift in the population was seen when using the anti-CCR6 mAb that cannot be explained at the moment. The untransfected as well as the transfected cells showed weak, non-specific stainings with the anti-FLAG mAb. Therefore, also the correct expression of the recombinant fusion protein could not be proven. The repetition of this experiment will be necessary in the future.

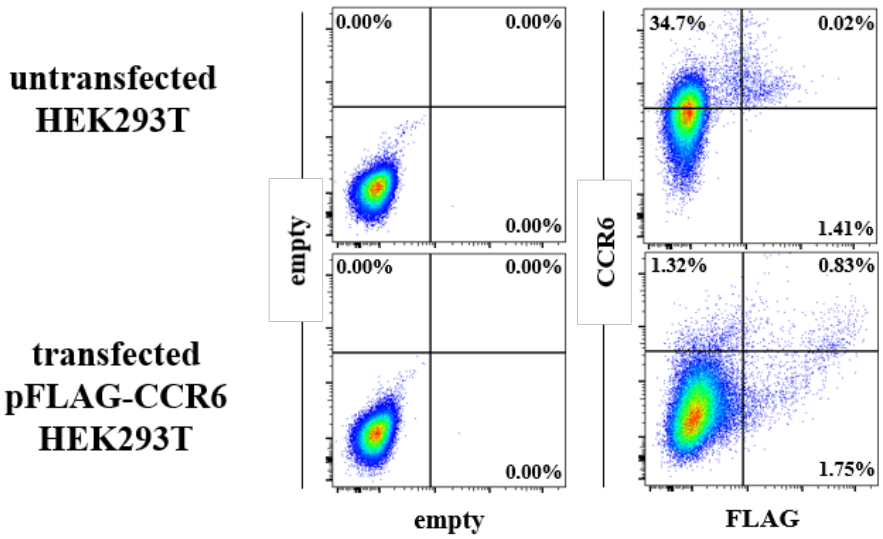

Figure 10: FCM analysis of HEK293T cells transfected with a pFLAG vector containing the porcine CCR6 sequence.

The transfected HEK293T cells with the CCR6-pFLAG clone were analyzed using multicolor FCM and compared to untransfected HEK293T cells. Cells were either not stained with mAbs (empty, graphs on the left) or with the specific anti-CCR6 and anti-FLAG mAbs (graphs on the right). The percentages of positive signals are indicated in the quadrants.

5 Discussion and Outlook

Th17 cells play important roles in the defense against extracellular bacteria but also in autoimmunity and inflammation (Crome et al. 2010; Yasuda et al. 2019). The Th17 subset is already well characterized in humans and mice. Beside the production of high levels of IL-17A after stimulation, Th17 cells are also characterized by the expression of the master TF RORγt and the chemokine receptor CCR6 in these species (Ivanov et al. 2006; Korn et al. 2009; Singh et al. 2008). In swine however, the Th17 cells are less characterized due to the lack of species-specific markers for a detailed identification and characterization. Therefore, a good approach is the use of cross-reactive anti-human/mouse mAbs. $CD4^+$ cells that are capable to produce IL-17A have been already successfully identified with the help of cross-reactive anti-IL17A mAbs (Stepanova et al. 2012). This work focused on the testing of two further mAb candidates for potential cross-reactivity in the pig – the anti-RORγt mAb clone AFKJS-9 and the anti-CCR6 mAb clone G034E3.

5.1 Discussion of results

In a first step, both mAbs were tested on porcine lymphocytes. In a second step, cross-reactivity should be proven by recombinant porcine RORγt and CCR6 proteins transiently expressed by a human cell line. The anti-RORγt mAb clone AFKJS-9 was already reported to stain a subset of porcine $CD4^+$ lymphocytes. Nevertheless, in this publication no original FCM data was shown (Kronsteiner et al. 2013). Consistent with Kronsteiner et al. (Kronsteiner et al. 2013) a $ROR\gamma t^+$ population amongst $CD4^+$ cells could be identified using the anti-human/mouse mAb clone AFKJS-9. Further indications on cross-reactivity would be a co-expression with IL-17A after activation of cells. To obtain optimal staining results for cytokine and TF labelling, two different fixation/permeabilization kits were tested for studying the co-expression of IL-17A and RORγt after stimulation of PBMC with PMA/Ionomycin. There were no obvious differences between the two fixation/permeabilization kits. Although a clear RORγt single positive population could be observed, the $IL\text{-}17A^+$ cells only showed a very dim RORγt expression. This might make data interpretation difficult in regard to defining them as real double positive cells. The next step was therefore to prove the cross-reactivity of the mAb by expression of the porcine recombinant protein in HEK293T cells and analysis by FCM using

the potentially cross-reactive mAb. The recombinant protein additionally had a FLAG-tag for the use of a commercially available anti-FLAG mAb as positive control for correct expression of the protein. From the two clones tested, transfectants with one clone neither showed a signal for RORγt, nor a signal for the FLAG-tag after FCM staining. Sequencing of the vector revealed several single nucleotide insertions compared to the original sequence, probably leading to a shift in the reading frame. Cells transfected with the other vector clone showed a clear RORγt signal after staining with the mAb, but no signal for the FLAG-tag. Sequencing for that clone revealed a deletion of over 100 base pairs that might represent an alternative splice variant. This deletion was leading to a frame shift causing an early internal stop-codon upstream of the FLAG sequence. The missing FLAG signal of this clone can therefore be explained by the stop codon. As a clear RORγt signal compared to the untransfected cells could be observed, this strongly indicates cross-reactivity of the mAb.

The FCM experiments with the mAb against CCR6 (clone G034E3) on porcine lymphocytes showed a very small positive population as well. Nevertheless, after comparing the findings to literature in humans where CCR6 expression is described on naïve and memory B cells (Krzysiek et al. 2000) as well as on a subset of CTL memory cells (Kondo et al. 2007) in peripheral blood we would expect much higher numbers of $CCR6^+$ cells within porcine lymphocytes. Additionally, most of the $CCR6^+$ cells were found within dead cells in our experiments. Thus, the question arises if the observed staining with the mAb are rather unspecific. Also, no co-expression of IL-17A and CCR6 was seen on PMA/Ionomycin stimulated porcine cells. This is in vast contrast to literature, as it was shown that all IL-17A producing $CD4^+$ cells express CCR6 at high levels (Singh et al. 2008). Further tests were conducted to validate the cross-reactivity of the CCR6 mAb. The analysis of HEK293T cells transfected with a plasmid vector containing the porcine CCR6 sequence with a FLAG-tag by FCM unfortunately showed no interpretable results. No specific stainings could be detected with the anti-CCR6 mAb, pointing to non-cross reactivity. Nevertheless, also no positive staining for the FLAG-tag control was observed. The sequence of this clone revealed no mutations that could have led to a frameshift or stop-codon preventing the correct expression of the protein. Therefore, no statement about the cross-reactivity can be made at this time point, and the cloning experiment needs to be repeated.

5.2 Outlook on future experiments

Although cross-reactivity for the anti-RORγt mAb was proven by the transfectants, further clones of the expression vector should be sequenced to identify clones without the internal stop codon. With this vector the transfection of the HEK293T cells should be repeated to obtain a co-staining of anti-RORγt and anti-FLAG mAbs. As a rather dim RORγt expression was observed on IL-17A cells, an unspecific isotype control antibody should be included in further FCM stainings for a better separation of positive and negative cells. Additionally, the anti-RORγt mAb should be tested in cells derived from lymphatic organs. In lymphocytes isolated from the intestine of mice, cells with a higher RORγt expression on non-$CD4^+$ T cells was shown compared to a likewise dim RORγt expression on $CD4^+$ T cells (Ivanov et al. 2006). Also, investigating RORγt expression in the thymus might be of interest, as is was shown that RORγt-deficient mice show a high reduction of $CD4^+CD8^+$ double positive cells in the thymus, indicating that these cells might express higher levels of the TF (Sun et al. 2000). In a next step, further multi-color staining panels should be tested in FCM on porcine lymphocytes, including the Th1 and Th2 specific TFs T-bet and GATA3 to confirm that there is no co-expression with RORγt. Further steps for the characterization of porcine Th17 cells will include the polyclonal stimulation and expansion of Th17 cells, as demonstrated by Cunha et al. for bovine Th17 cells (Cunha et al. 2019). By i*n vitro* stimulation with antibodies against CD3 and CD28 as well as the cytokines TGF-β, IL-6 and IL-2, expansion of the $ROR\gamma t^+$ population in porcine lymphocytes should be achieved.

Results on CCR6 obtained so far indicate no cross-reactivity of this mAb. Nevertheless, a repetition of the transfection or even the cloning is necessary for a final proof to obtain at least the FLAG signal to verify the correct expression of the recombinant fusion protein. A next step will be the testing of other mAbs apart from clone G034E3 for staining on porcine lymphocytes. A CCR6-sprecific mAb would be important for Th17 analyses in the pig as so far, only intracellular markers like IL-17A and now RORγt are available. This would always require fixation/permeabilization of cells and therefore killing of the cells. Having reliable extracellular markers beside CD4 will facilitate sorting of porcine Th17 cells for further functional assays. If no cross-reactive antibodies are available, the production of a porcine-specific CCR6 mAb by mouse immunization should be considered for further research regarding Th17 cells in the pig.

6 Summary

CD4$^+$ T-helper cells play an important role in the adaptive immune response. Naïve CD4$^+$ cells can differentiate into differently specialized effector subsets upon activation. One of those subsets are the Th17 cells that are involved in the protection against extracellular bacteria and fungi and play a role in inflammatory responses. They are characterized by the production of IL-17A, the expression of the transcription factor RORγt and the chemokine receptor CCR6. In pigs, IL-17A producing CD4$^+$ T cells were identified. However, no further markers are available for a more detailed characterization of porcine Th17 cells.

Aim of this study was therefore the validation of markers that can be used for a better characterization of porcine Th17 cells. As currently no species-specific mAbs against RORγt and CCR6 are available, potential cross-reactive anti-human/mouse mAbs directed against the two markers were tested in FCM on porcine PBMC. The anti-RORγt mAb clone AFKJS-9 showed a distinct staining on porcine lymphocytes *ex vivo* that also correlated with CD4 expression. Furthermore, a CD4$^+$IL-17A$^+$RORγt^{dim} population was observed after stimulation of cells with PMA/Ionomycin. The anti-CCR6 mAb clone G034E3 was likewise tested on porcine PBMC *ex vivo* and after stimulation. Only a weak staining could be seen with this antibody without clear co-expression of CD4 and IL-17A.

Recombinant porcine proteins of both RORγt and CCR6 were generated for cross-reactivity proof of the mAb candidates. Working steps for this test included amplifying RORγt and CCR6 by PCR with gene-specific primers and cloning them into separate mammalian expression vectors containing a FLAG sequence. In a last step, HEK293T cells were transiently transfected with the expression vectors and the transfectants were analyzed in FCM with the potential cross-reactive mAbs and a commercially available mAb against the FLAG-tag as control. For RORγt a clear signal was visible, indicating that the mAb is indeed cross reactive. A missing signal for the FLAG-tag was explained by an internal stop-codon upstream of the FLAG sequence. For the CCR6 transfectants on the other hand, no specific stainings for the cross-reactivity candidate as well as the FLAG control were observed. As sequencing of the tested expression vector did not reveal any mutations the failed expression of the recombinant protein cannot be explained at the moment. Therefore, no final statement about the CCR6 cross-reactivity can be made at this time point, and the experiments need to be repeated.

7 Zusammenfassung

$CD4^+$ T-Helferzellen spielen eine wichtige Rolle für das adaptive Immunsystem. Naive $CD4^+$ Zellen können nach Aktivierung in verschiedene spezialisierte Effektor-Zelltypen differenzieren. Eine dieser Effektor Subpopulationen sind die Th17 Zellen. Sie spielen eine wichtige Rolle bei der Immunantwort gegen extrazelluläre Bakterien und sind in Entzündungsreaktionen involviert. Th17 Zellen sind charakterisiert durch die Produktion von IL-17A und die Expression des Transkriptionsfaktors RORγt und des Chemokinrezeptors CCR6. Bei Schweinen ist jedoch nur wenig über Th17 Zellen bekannt. Es wurden IL-17A produzierende $CD4^+$ Zellen im Schwein gefunden, jedoch fehlen bis heute zusätzliche Marker für eine bessere Charakterisierung der porzinen Th17 Zellen.

Das Ziel dieser Arbeit war es daher, zusätzliche Marker zur Th17 Charakterisierung zu testen. Da es bis jetzt keine spezies-spezifischen monoklonalen Antikörper (mAk) für RORγt und CCR6 im Schwein gibt, wurden potenziell kreuzreaktive anti-Human/-Maus Antikörper getestet. Dies wurde mittels Mehrfarben-Durchflusszytometrie auf porzinen mononukleären Blutzellen getestet. Mit dem anti-RORγt mAk Klon AFKJS-9 konnte ein eindeutiges Signal auf porzinen Lymphozyten gezeigt werden und eine Koexpression mit CD4. Zusätzlich konnte eine Population von $CD4^+$IL-$17A^+$RORγt^{dim} Zellen nach PMA/Ionomycin Stimulation identifiziert werden. Der anti-CCR6 mAk Klon G034E3 zeigte aber nur ein sehr schwaches Signal auf porzinen Zellen und es konnte auch keine Koexpression mit CD4 und IL-17A gezeigt werden.

Für den Beweis der Kreuzreaktivität der Antikörper wurden rekombinante porzine Proteine von RORγt und CCR6 erstellt. Dazu wurden die mRNA Sequenzen mittels PCR mit gen-spezifischen Primern amplifiziert und in Expressionsvektoren für Säugetierzellen kloniert. Diese Vektoren enthielten zusätzlich eine FLAG Sequenz. HEK293T Zellen wurden mit den Vektorkonstrukten transient transfiziert und die Zellen in der Durchflusszytometrie analysiert. Hierfür wurde der zu testende mAk in Kombination mit einem FLAG-spezifischem mAk als Kontrollmarkierung verwendet. Für RORγt war ein klares positives Signal sichtbar, was für die Kreuzreaktivität des Antikörpers spricht. Das fehlende FLAG Signal konnte nach der Sequenzierung auf ein internes Stop-Codon vor der FLAG Sequenz zurückgeführt werden, wodurch es zum verfrühten Abbruch der Translation kam. Bei den CCR6 Transfektanten konnten nur unspezifische Signale mit dem anti-CCR6 mAk, aber auch der FLAG-Kontrolle gesehen werden. Die Sequenzierung des Expressionsvektors konnte keine Erklärung liefern,

wieso der FLAG-tag nicht exprimiert wurde, da keine Veränderungen in der Sequenz gefunden wurden. Daher können zum jetzigen Zeitpunkt keine finalen Aussagen zu der CCR6 Kreuzreaktivität gemacht werden und eine Wiederholung der Experimente ist als nächster Schritt geplant.

8 Abbreviations

Table 12: List of abbreviations.

Bcl6	B-cell lymphoma 6
bp	base pairs
CD	cluster of differentiation
CCL	C-C Chemokine Ligand
CCR	chemokine receptor
CTLA	cytotoxic T-lymphocyte-associated antigen
CIAP	calf intestinal alkaline phosphatase
EAE	experimental autoimmune encephalomyelitis
E.coli	Escherichia coli
EDTA	ethylenediaminetetraacetic acid
FCM	flow cytometry
Foxp3	Forkhead-Box-Protein P3
FSC	forward scatter
HEK293T	human embryonic kidney 293T cells
IFN-γ	Interferon-γ
IL	Interleukin
mAb	monoclonal antibody
MFI	median fluorescence intensity
MHC	major histocompatibility complex
NTC	no template control
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PMA	phorbol 12-myristate 13-acetate
ROR	retinoic acid-related orphan receptor
RT	room temperature
TAE	Tris/Acetate/EDTA
T-bet	T-box protein expressed in T cells
TcR	T-cell receptor
TF	transcription factor
Tfh	follicular B helper T cells
TGF	transforming growth factor
Th	T-helper cell
TNF	tumor necrosis factor

Treg	regulatory T cells
STAT	signal transducer and activator of transcription
SSC	sideward scatter
v/v	volume per volume
w/v	weight per volume

9 References

Acosta-Rodriguez, Eva V.; Rivino, Laura; Geginat, Jens; Jarrossay, David; Gattorno, Marco; Lanzavecchia, Antonio et al. (2007): Surface phenotype and antigenic specificity of human interleukin 17-producing T helper memory cells. In *Nature Immunology* 8 (6), pp. 639–646. DOI: 10.1038/ni1467.

Amezcua Vesely, Maria Carolina; Pallis, Paris; Bielecki, Piotr; Low, Jun Siong; Zhao, Jun; Harman, Christian C. D. et al. (2019): Effector TH17 Cells Give Rise to Long-Lived TRM Cells that Are Essential for an Immediate Response against Bacterial Infection. In *Cell* 178 (5), 1176-1188.e15. DOI: 10.1016/j.cell.2019.07.032.

Annunziato, Francesco; Cosmi, Lorenzo; Liotta, Francesco; Maggi, Enrico; Romagnani, Sergio (2009): Type 17 T helper cells-origins, features and possible roles in rheumatic disease. In *Nature Reviews. Rheumatology* 5 (6), pp. 325–331.

Appay, Victor; Zaunders, John J.; Papagno, Laura; Sutton, Julian; Jaramillo, Angel; Waters, Anele et al. (2002): Characterization of CD4(+) CTLs ex vivo. In *Journal of immunology (Baltimore, Md. : 1950)* 168 (11), pp. 5954–5958. DOI: 10.4049/jimmunol.168.11.5954.

Baba, M.; Imai, T.; Nishimura, M.; Kakizaki, M.; Takagi, S.; Hieshima, K. et al. (1997): Identification of CCR6, the specific receptor for a novel lymphocyte-directed CC chemokine LARC. In *The Journal of Biological Chemistry* 272 (23), pp. 14893–14898. DOI: 10.1074/jbc.272.23.14893.

Bettelli, Estelle; Carrier, Yijun; Gao, Wenda; Korn, Thomas; Strom, Terry B.; Oukka, Mohamed et al. (2006): Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. In *Nature* 441 (7090), pp. 235–238. DOI: 10.1038/nature04753.

Bolzer, Kerstin; Käser, Tobias; Saalmüller, Armin; Hammer, Sabine E. (2009): Molecular characterisation of porcine Forkhead-box p3 (Foxp3). In *Veterinary Immunology and Immunopathology* 132 (2-4), pp. 275–281. DOI: 10.1016/j.vetimm.2009.05.014.

Breitfeld, D.; Ohl, L.; Kremmer, E.; Ellwart, J.; Sallusto, F.; Lipp, M.; Förster, R. (2000): Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and

support immunoglobulin production. In *The Journal of Experimental Medicine* 192 (11), pp. 1545–1552. DOI: 10.1084/jem.192.11.1545.

Chen, WanJun; Jin, Wenwen; Hardegen, Neil; Lei, Ke-Jian; Li, Li; Marinos, Nancy et al. (2003): Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. In *The Journal of Experimental Medicine* 198 (12), pp. 1875–1886. DOI: 10.1084/jem.20030152.

Cortés, Alba; Muñoz-Antoli, Carla; Esteban, J. Guillermo; Toledo, Rafael (2017): Th2 and Th1 Responses: Clear and Hidden Sides of Immunity Against Intestinal Helminths. In *Trends in Parasitology* 33 (9), pp. 678–693. DOI: 10.1016/j.pt.2017.05.004.

Cossarizza, Andrea; Chang, Hyun-Dong; Radbruch, Andreas; Acs, Andreas; Adam, Dieter; Adam-Klages, Sabine et al. (2019): Guidelines for the use of flow cytometry and cell sorting in immunological studies (second edition). In *European Journal of Immunology* 49 (10), pp. 1457–1973. DOI: 10.1002/eji.201970107.

Crome, S. Q.; Wang, A. Y.; Levings, M. K. (2010): Translational mini-review series on Th17 cells: function and regulation of human T helper 17 cells in health and disease. In *Clinical and Experimental Immunology* 159 (2), pp. 109–119. DOI: 10.1111/j.1365-2249.2009.04037.x.

Crotty, Shane (2011): Follicular helper CD4 T cells (TFH). In *Annual Review of Immunology* 29, pp. 621–663. DOI: 10.1146/annurev-immunol-031210-101400.

Cunha, Patricia; Le Vern, Yves; Gitton, Christophe; Germon, Pierre; Foucras, Gilles; Rainard, Pascal (2019): Expansion, isolation and first characterization of bovine Th17 lymphocytes. In *Scientific reports* 9 (1), p. 16115. DOI: 10.1038/s41598-019-52562-2.

Ebner, F.; Rausch, S.; Scharek-Tedin, L.; Pieper, R.; Burwinkel, M.; Zentek, J.; Hartmann, S. (2014): A novel lineage transcription factor based analysis reveals differences in T helper cell subpopulation development in infected and intrauterine growth restricted (IUGR) piglets. In *Developmental and Comparative Immunology* 46 (2), pp. 333–340. DOI: 10.1016/j.dci.2014.05.005.

Gerner, Wilhelm; Talker, Stephanie C.; Koinig, Hanna C.; Sedlak, Corinna; Mair, Kerstin H.; Saalmüller, Armin (2015): Phenotypic and functional differentiation of porcine αβ T cells:

current knowledge and available tools. In *Molecular Immunology* 66 (1), pp. 3–13. DOI: 10.1016/j.molimm.2014.10.025.

Harrington, Laurie E.; Hatton, Robin D.; Mangan, Paul R.; Turner, Henrietta; Murphy, Theresa L.; Murphy, Kenneth M.; Weaver, Casey T. (2005): Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. In *Nature Immunology* 6 (11), pp. 1123–1132. DOI: 10.1038/ni1254.

Hymowitz, S. G.; Filvaroff, E. H.; Yin, J. P.; Lee, J.; Cai, L.; Risser, P. et al. (2001): IL-17s adopt a cystine knot fold: structure and activity of a novel cytokine, IL-17F, and implications for receptor binding. In *The EMBO Journal* 20 (19), pp. 5332–5341. DOI: 10.1093/emboj/20.19.5332.

Ivanov, Ivaylo I.; McKenzie, Brent S.; Zhou, Liang; Tadokoro, Carlos E.; Lepelley, Alice; Lafaille, Juan J. et al. (2006): The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. In *Cell* 126 (6), pp. 1121–1133. DOI: 10.1016/j.cell.2006.07.035.

Iwakura, Yoichiro; Ishigame, Harumichi; Saijo, Shinobu; Nakae, Susumu (2011): Functional specialization of interleukin-17 family members. In *Immunity* 34 (2), pp. 149–162. DOI: 10.1016/j.immuni.2011.02.012.

Kao, Cheng-Yuan; Huang, Fei; Chen, Yin; Thai, Philip; Wachi, Shinichiro; Kim, Christy et al. (2005): Up-regulation of CC chemokine ligand 20 expression in human airway epithelium by IL-17 through a JAK-independent but MEK/NF-kappaB-dependent signaling pathway. In *Journal of Immunology (Baltimore, Md. : 1950)* 175 (10), pp. 6676–6685. DOI: 10.4049/jimmunol.175.10.6676.

Käser, Tobias; Mair, Kerstin H.; Hammer, Sabine E.; Gerner, Wilhelm; Saalmüller, Armin (2015): Natural and inducible Tregs in swine: Helios expression and functional properties. In *Developmental and Comparative Immunology* 49 (2), pp. 323–331. DOI: 10.1016/j.dci.2014.12.005.

Katoh, Shinichiro; Kitazawa, Haruki; Shimosato, Takeshi; Tohno, Masanori; Kawai, Yasushi; Saito, Tadao (2004): Cloning and characterization of Swine interleukin-17, preferentially expressed in the intestines. In *Journal of Interferon & Cytokine Research : the official*

Journal of the International Society for Interferon and Cytokine Research 24 (9), pp. 553–559. DOI: 10.1089/jir.2004.24.553.

Kiros, Tadele G.; van Kessel, Jill; Babiuk, Lorne A.; Gerdts, Volker (2011): Induction, regulation and physiological role of IL-17 secreting helper T-cells isolated from PBMC, thymus, and lung lymphocytes of young pigs. In *Veterinary Immunology and Immunopathology* 144 (3-4), pp. 448–454. DOI: 10.1016/j.vetimm.2011.08.021.

Kolls, Jay K.; Lindén, Anders (2004): Interleukin-17 family members and inflammation. In *Immunity* 21 (4), pp. 467–476. DOI: 10.1016/j.immuni.2004.08.018.

Kondo, Takaaki; Takata, Hiroshi; Takiguchi, Masafumi (2007): Functional expression of chemokine receptor CCR6 on human effector memory CD8+ T cells. In *European Journal of Immunology* 37 (1), pp. 54–65. DOI: 10.1002/eji.200636251.

Korn, Thomas; Bettelli, Estelle; Oukka, Mohamed; Kuchroo, Vijay K. (2009): IL-17 and Th17 Cells. In *Annual Review of Immunology* 27, pp. 485–517. DOI: 10.1146/annurev.immunol.021908.132710.

Korn, Thomas; Oukka, Mohamed; Kuchroo, Vijay; Bettelli, Estelle (2007): Th17 cells: effector T cells with inflammatory properties. In *Seminars in Immunology* 19 (6), pp. 362–371. DOI: 10.1016/j.smim.2007.10.007.

Kronsteiner, Barbara; Bassaganya-Riera, Josep; Philipson, Casandra; Viladomiu, Monica; Carbo, Adria; Pedragosa, Mireia et al. (2013): Helicobacter pylori infection in a pig model is dominated by Th1 and cytotoxic CD8+ T cell responses. In *Infection and Immunity* 81 (10), pp. 3803–3813. DOI: 10.1128/IAI.00660-13.

Krzysiek, R.; Lefevre, E. A.; Bernard, J.; Foussat, A.; Galanaud, P.; Louache, F.; Richard, Y. (2000): Regulation of CCR6 chemokine receptor expression and responsiveness to macrophage inflammatory protein-3alpha/CCL20 in human B cells. In *Blood* 96 (7), pp. 2338–2345.

Kuestner, Rolf E.; Taft, David W.; Haran, Aaron; Brandt, Cameron S.; Brender, Ty; Lum, Karen et al. (2007): Identification of the IL-17 receptor related molecule IL-17RC as the receptor for IL-17F. In *Journal of Immunology (Baltimore, Md. : 1950)* 179 (8), pp. 5462–5473. DOI: 10.4049/jimmunol.179.8.5462.

Langrish, Claire L.; Chen, Yi; Blumenschein, Wendy M.; Mattson, Jeanine; Basham, Beth; Sedgwick, Jonathan D. et al. (2005): IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. In *The Journal of Experimental Medicine* 201 (2), pp. 233–240. DOI: 10.1084/jem.20041257.

Luckheeram, Rishi Vishal; Zhou, Rui; Verma, Asha Devi; Xia, Bing (2012): $CD4^+$T cells: differentiation and functions. In *Clinical & Developmental Immunology* 2012, p. 925135. DOI: 10.1155/2012/925135.

McGeachy, Mandy J.; Cua, Daniel J.; Gaffen, Sarah L. (2019): The IL-17 Family of Cytokines in Health and Disease. In *Immunity* 50 (4), pp. 892–906. DOI: 10.1016/j.immuni.2019.03.021.

Moriguchi, Kota; Miyamoto, Katsuichi; Tanaka, Noriko; Yoshie, Osamu; Kusunoki, Susumu (2013): The importance of CCR4 and CCR6 in experimental autoimmune encephalomyelitis. In *Journal of Neuroimmunology* 257 (1-2), pp. 53–58. DOI: 10.1016/j.jneuroim.2013.02.002.

Moseley, T. A.; Haudenschild, D. R.; Rose, L.; Reddi, A. H. (2003): Interleukin-17 family and IL-17 receptors. In *Cytokine & Growth Factor Reviews* 14 (2), pp. 155–174. DOI: 10.1016/S1359-6101(03)00002-9.

Mosmann, Timothy R.; Cherwinski, Holly; Bond, Martha W.; Giedlin, Martin A.; Coffman, Robert (1986): Two types of murine helper t cell clone. In *The Journal of Immunology* 136 (7), pp. 2348–2357.

Murphy, Kenneth M.; Reiner, Steven L. (2002): The lineage decisions of helper T cells. In *Nature reviews. Immunology* 2 (12), pp. 933–944. DOI: 10.1038/nri954.

Nakayama, Toshinori; Hirahara, Kiyoshi; Onodera, Atsushi; Endo, Yusuke; Hosokawa, Hiroyuki; Shinoda, Kenta et al. (2017): Th2 Cells in Health and Disease. In *Annual Review of Immunology* 35, pp. 53–84. DOI: 10.1146/annurev-immunol-051116-052350.

Nathan, C. F.; Murray, H. W.; Wiebe, M. E.; Rubin, B. Y. (1983): Identification of interferon-gamma as the lymphokine that activates human macrophage oxidative metabolism and antimicrobial activity. In *The Journal of Experimental Medicine* 158 (3), pp. 670–689. DOI: 10.1084/jem.158.3.670.

Nurieva, Roza I.; Chung, Yeonseok; Hwang, Daehee; Yang, Xuexian O.; Kang, Hong Soon; Ma, Li et al. (2008): Generation of T follicular helper cells is mediated by interleukin-21 but independent of T helper 1, 2, or 17 cell lineages. In *Immunity* 29 (1), pp. 138–149. DOI: 10.1016/j.immuni.2008.05.009.

Nurieva, Roza I.; Chung, Yeonseok; Martinez, Gustavo J.; Yang, Xuexian O.; Tanaka, Shinya; Matskevitch, Tatyana D. et al. (2009): Bcl6 mediates the development of T follicular helper cells. In *Science (New York, N.Y.)* 325 (5943), pp. 1001–1005. DOI: 10.1126/science.1176676.

Park, Heon; Li, Zhaoxia; Yang, Xuexian O.; Chang, Seon Hee; Nurieva, Roza; Wang, Yi-Hong et al. (2005): A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. In *Nature Immunology* 6 (11), pp. 1133–1141. DOI: 10.1038/ni1261.

Paul, William E.; Seder, Robert A. (1994): Lymphocyte responses and cytokines. In *Cell* 76 (2), pp. 241–251.

Reutner, Katharina; Leitner, Judith; Müllebner, Andrea; Ladinig, Andrea; Essler, Sabine E.; Duvigneau, J. Catharina et al. (2013): CD27 expression discriminates porcine T helper cells with functionally distinct properties. In *Veterinary Research* 44, p. 18. DOI: 10.1186/1297-9716-44-18.

Rodríguez-Gómez, Irene M.; Talker, Stephanie C.; Käser, Tobias; Stadler, Maria; Hammer, Sabine E.; Saalmüller, Armin; Gerner, Wilhelm (2016): Expression of T-bet, Eomesodermin and GATA-3 in porcine αβ T cells. In *Developmental and Comparative Immunology* 60, pp. 115–126. DOI: 10.1016/j.dci.2016.02.022.

Rouvier, E.; Luciani, M. F.; Mattéi, M. G.; Denizot, F.; Golstein, P. (1993): CTLA-8, cloned from an activated T cell, bearing AU-rich messenger RNA instability sequences, and homologous to a herpesvirus saimiri gene. In *Journal of Immunology (Baltimore, Md. : 1950)* 150 (12), pp. 5445–5456.

Saalmüller, Armin; Bryant, Jane (1994): Characteristics of porcine T lymphocytes and T-cell lines. In *Veterinary Immunology and Immunopathology* 43 (1-3), pp. 45–52. DOI: 10.1016/0165-2427(94)90119-8.

Sallusto, Federica; Monticelli, Silvia (2013): The many faces of CD4 T cells: roles in immunity and disease. In *Seminars in Immunology* 25, pp. 249–251.

Sassu, Elena L.; Ladinig, Andrea; Talker, Stephanie C.; Stadler, Maria; Knecht, Christian; Stein, Heiko et al. (2017): Frequency of Th17 cells correlates with the presence of lung lesions in pigs chronically infected with Actinobacillus pleuropneumoniae. In *Veterinary Research* 48 (1), p. 4. DOI: 10.1186/s13567-017-0411-z.

Singh, Satya P.; Zhang, Hongwei H.; Foley, John F.; Hedrick, Michael N.; Farber, Joshua M. (2008): Human T cells that are able to produce IL-17 express the chemokine receptor CCR6. In *Journal of Immunology (Baltimore, Md. : 1950)* 180 (1), pp. 214–221. DOI: 10.4049/jimmunol.180.1.214.

Stepanova, Hana; Mensikova, Marketa; Chlebova, Katarina; Faldyna, Martin (2012): CD4+ and γδTCR+ T lymphocytes are sources of interleukin-17 in swine. In *Cytokine* 58 (2), pp. 152–157. DOI: 10.1016/j.cyto.2012.01.004.

Sun, Z.; Unutmaz, D.; Zou, Y. R.; Sunshine, M. J.; Pierani, A.; Brenner-Morton, S. et al. (2000): Requirement for RORgamma in thymocyte survival and lymphoid organ development. In *Science (New York, N.Y.)* 288 (5475), pp. 2369–2373. DOI: 10.1126/science.288.5475.2369.

Swain, Susan L.; McKinstry, K. Kai; Strutt, Tara M. (2012): Expanding roles for $CD4^+$ T cells in immunity to viruses. In *Nature Reviews in Immunology* 12 (2), pp. 136–148. DOI: 10.1038/nri3152.

Szabo, Susanne J.; Kim, Sean T.; Costa, Gina L.; Zhang, Xiankui; Fathman, C.Garrison; Glimcher, Laurie H. (2000): A Novel Transcription Factor, T-bet, Directs Th1 Lineage Commitment. In *Cell* 100 (6), pp. 655–669. DOI: 10.1016/S0092-8674(00)80702-3.

Wang, Yanling; Yin, Yeshi; Chen, Xin; Zhao, Yongjia; Wu, Yichen; Li, Yifei et al. (2019): Induction of Intestinal Th17 Cells by Flagellins From Segmented Filamentous Bacteria. In *Frontiers in Immunology* 10, p. 2750. DOI: 10.3389/fimmu.2019.02750.

Wei, Xundong; Zhang, Jianhua; Gu, Qianchong; Huang, Man; Zhang, Wei; Guo, Jie; Zhou, Xuyu (2017): Reciprocal Expression of IL-35 and IL-10 Defines Two Distinct Effector Treg

Subsets that Are Required for Maintenance of Immune Tolerance. In *Cell Reports* 21 (7), pp. 1853–1869. DOI: 10.1016/j.celrep.2017.10.090.

Wright, Adam K. A.; Bangert, Mathieu; Gritzfeld, Jenna F.; Ferreira, Daniela M.; Jambo, Kondwani C.; Wright, Angela D. et al. (2013): Experimental human pneumococcal carriage augments IL-17A-dependent T-cell defence of the lung. In *PLoS Pathogens* 9 (3), e1003274. DOI: 10.1371/journal.ppat.1003274.

Wright, Jill F.; Guo, Yongjing; Quazi, Amira; Luxenberg, Deborah P.; Bennett, Frann; Ross, John F. et al. (2007): Identification of an interleukin 17F/17A heterodimer in activated human CD4+ T cells. In *The Journal of Biological Chemistry* 282 (18), pp. 13447–13455. DOI: 10.1074/jbc.M700499200.

Yang, Xuexian O.; Panopoulos, Athanasia D.; Nurieva, Roza; Chang, Seon Hee; Wang, Demin; Watowich, Stephanie S.; Dong, Chen (2007): STAT3 regulates cytokine-mediated generation of inflammatory helper T cells. In *The Journal of Biological Chemistry* 282 (13), pp. 9358–9363. DOI: 10.1074/jbc.C600321200.

Yang, Xuexian O.; Pappu, Bhanu P.; Nurieva, Roza; Akimzhanov, Askar; Kang, Hong Soon; Chung, Yeonseok et al. (2008): T helper 17 lineage differentiation is programmed by orphan nuclear receptors ROR alpha and ROR gamma. In *Immunity* 28 (1), pp. 29–39. DOI: 10.1016/j.immuni.2007.11.016.

Yasuda, Keiko; Takeuchi, Yusuke; Hirota, Keiji (2019): The pathogenicity of Th17 cells in autoimmune diseases. In *Seminars in Immunopathology* 41 (3), pp. 283–297. DOI: 10.1007/s00281-019-00733-8.

Ye, P.; Rodriguez, F. H.; Kanaly, S.; Stocking, K. L.; Schurr, J.; Schwarzenberger, P. et al. (2001): Requirement of interleukin 17 receptor signaling for lung CXC chemokine and granulocyte colony-stimulating factor expression, neutrophil recruitment, and host defense. In *The Journal of Experimental Medicine* 194 (4), pp. 519–527. DOI: 10.1084/jem.194.4.519.

Zheng, Wei-ping; Flavell, Richard A. (1997): The Transcription Factor GATA-3 Is Necessary and Sufficient for Th2 Cytokine Gene Expression in CD4 T Cells. In *Cell* 89 (4), pp. 587–596. DOI: 10.1016/S0092-8674(00)80240-8.

Zhu, Jinfang (2018): T Helper Cell Differentiation, Heterogeneity, and Plasticity. In *Cold Spring Harbor Perspectives in Biology* 10 (10). DOI: 10.1101/cshperspect.a030338.

Zhu, Jinfang; Paul, William E. (2008): CD4 T cells: fates, functions, and faults. In *Blood* 112 (5), pp. 1557–1569.

Zhu, Jinfang; Paul, William E. (2010a): Heterogeneity and plasticity of T helper cells. In *Cell research* 20 (1), pp. 4–12.

Zuckermann, F. A.; Husmann, R.)J. (1996): Functional and phenotypic analysis of porcine peripheral blood CD4/CD8 double-positive T cells. In *Immunology (United Kingdom)*.

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