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In-depth characterization of porcine naive and memory CD4$^+$ T cells

Bachelor thesis

University of Veterinary Medicine Vienna

Submitted by
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Table of content

1. Introduction

1.1. The biology of T lymphocytes

T lymphocytes[1] (or T cells) are a type of white blood cells and a crucial component of the adaptive immune system. They carry a T-cell receptor (TCR), consisting either of αβ- or γδ-chains[2] on their cell surface, linked to five CD3 subunits for signal transduction. Expression of these markers distinguishes them from other lymphocytes. T-cell precursors develop from hematopoietic stem cells in the bone marrow and migrate then to the thymus to mature into cluster of differentiation (CD) 8^+ cytotoxic and $CD4^+$ "helper" T cells. These T cells migrate then through various tissues to get in contact with antigens[3]. Here, an important role especially play secondary lymphoid organs (SLOs) like lymph nodes that can be regarded as a crossroad of antigen-presenting cells (APCs) like B cells, macrophages, dendritic cells with T cells. Focusing on $CD4^+$ T cells, the activation happens through three signals. First, recognition of foreign antigen presented by major histocompatibility complex II (MHC-II) of APCs by TCR along with binding of the coreceptor CD4 on T cells. The signal can be further extended by T-cell's CD28 costimulatory molecule[4]. A third signal is provided by cytokines produced by APCs. This leads to full activation, differentiation, and expansion of $CD4^+$ T cells in functionally distinct subsets, like Th1, Th2, Th17 as well as regulatory and follicular T-helper cells[5,6]. The activated $CD4^+$ T cell can enhance the overall immune response by secreting various cytokines that help in activation and differentiation of other immunological components or can even suppress immune reaction. Such examples are the recruitment of macrophages, granulocytes and other effector cells or activation of B cells for production of antibodies against certain antigens or even inflammation of tissue[7]. After successful elimination of the pathogen, the effector $CD4^+$ T cells can either undergo apoptosis or further differentiate and remain as memory cells to help the host abruptly in case of re-infection. Although being an important component, there is a seamy side to $CD4^+$ T lymphocytes. If they remain responsible to autoantigens and are not in time eliminated or are excessively activated, it can lead to dangerous cytokine storms, tissue damage, organ failure and death, therefore a subsequential need of drugs and therapy development is needed[8,9].

1.2. The maturation and differentiation of $CD4^+$ T lymphocytes in human and mice

The studies of maturation and differentiation of $CD4^+$ T cells from naïve (TN) to effector and memory date back to the last century. The research was mainly based on former observations

in $CD8^+$ T cells that have always been the major area of research interest due to their cytotoxicity and with that linked possible immunotherapy, battling the cancer/viruses and vaccine development[10,11]. Nevertheless, over the last 25 years studies showed $CD4^+$ T-cell immunogenicity to be of an equal importance as $CD8^+$ T cells, especially due to their manifold functions in immune responses. Therefore, the memory capacity of $CD4^+$ T cells has extensively been studied.

According to our knowledge, naïve $CD4^+$ T cells develop into cells with effector function upon antigen priming (activation) that is accompanied with clonal expansion and differentiation as mentioned above (expansion phase). However, after successful pathogen elimination some cells are destroyed (regarded as effector cells, contraction phase) and some persist in the host and can provide long-lasting immunity against re-infection (memory cells)[12]. The discrimination between effector and memory cells is still unclear, for this reason they are often regarded as one. In 1999 one of the first studies addressing in-depth characterization of human memory $CD4^+$ T cells was published introducing two functionally distinct memory subsets – the central memory (TCM) and effector memory (TEM) T lymphocytes[13] (gating strategy alongside with used markers further discussed in 1.2.1 and 1.2.2.). TCM, while expressing lymph-node-homing receptors and lacking immediate effector function in contrast to TEM, play a role in dendritic-cell stimulation and are able to differentiate into TEM *in vitro* (not vice versa). Also, TCM having longer telomeres than TEM suggests them being an earlier differentiation stage[13].

1.2.1. CD45R and its isoforms

CD45R is a receptor-linked protein tyrosine phosphatase also known as leukocyte common antigen expressed on all hematopoietic cells, except mature erythrocytes and platelets[14–17]. Its role in T-cell mediated immunity is challenging to summarize simply as CD45R was reported having various functions like activation of T cells via TCR, modulation of cytokine and chemokine production and signaling and many more[18,19]. The mammalian CD45 gene comprises up to 35 exons (depends on the species) which provide the cell high number of splicing possibilities. There have been six human CD45R isoforms observed[20], from which three variants are mostly used for describing T-cell maturation and differentiation stages. CD45RA together with its other isoforms CD45R0 and CD45RC sheds light in basic T-cell research as these particular isoforms can only be expressed on certain T-cell populations. The shortest isoform CD45R0, lacking all three exons and so having the lowest molecular weight, is regarded as a memory T-cell marker because of its broad distribution in adults[21]. CD45RA is a variant of CD45R with high relative molecular mass in which the exon A is expressed. At

birth, most cells express CD45RA suggesting CD45RA as a naïve T-cell marker[21]. However, that contradicts the findings of memory $CD4^+$ T-cells subset expressing CD45RA[22]. The exon C expressing variant CD45RC was also reported to be differentially expressed between functionally distinct T-cell subsets as the $CD45RC^+$ subset contained naïve T cells whereas central and effector memory T cells were $CD45RC^-$ suggesting it as a substitute of CD45RA[23] in human and mouse research.

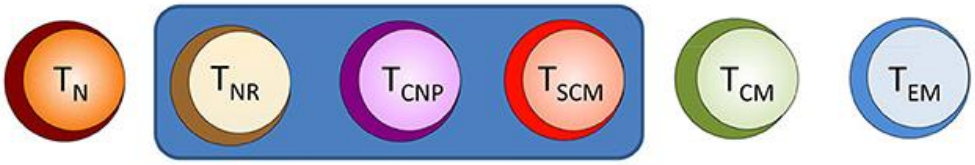
1.2.2. Chemokine receptor type 7 (CCR7)

One of the crucial receptors for leukocyte's homing to SLOs is CCR7. Adhesion and diapedesis of lymphocytes and dendritic cells into SLOs starts with the binding of leukocyte's CD62L to its ligand on high endothelial venules[24]. Next, CCR7 interacts with CCL19 and CCL21 ligands and the leukocyte transmigrates into the SLOs where it can crosstalk[25]. Based on the coexpression of CD62L and CCR7, there were two memory ($CCR7^+CD45RA^-$ TCM and $CCR7^-CD45RA^-$ TEM) and one naïve ($CCR7^+CD45RA^+$) $CD4^+$ T-cell subsets revealed by investigating the co-expression of CCR7 and the naïve marker CD45RA[13], making CCR7 and CD45RA the fundamental marker combination for discrimination of naïve and memory T-cell subsets in humans and mice.

1.2.3. Further markers used to define effector/memory cells in humans and mice

Following Sallusto et al.[26], other research groups focused on the in-depth characterization of human and mice $CD4^+$ T cells with the markers CD45R0, CD62L, CD27, CD28 and others alongside CCR7 and CD45RA. There was a heterogeneity of marker expression observed in most subsets. The constant development of new assays, like applying complex multicolor flow cytometry (FCM), gave scientists a better overview due to easier and precise analysis of complex staining panels[11,27,28]. In Figure 1 is the lymphocyte heterogeneity depicted. The $CD4^+$ T-cell differentiation process undergoes many stages starting with TN, followed by atypical naïve-like T-cell subsets observed by Caccamo et al. Both had a naïve $CCR7^+CD27^+CD28^+CD45R0^-CD45RA^+CD62L^+$ phenotype although only naïve-like T cells displayed effector function and a low expression of CXCR3[29], a receptor important for migration to inflamed tissues[30]. TCM populations showed a very stable $CCR7^+CD27^+CD28^+CD45R0^+CD45RA^-CD62L^+CXCR3^+$ phenotype with possible variations in the CD27 expression[28], still being able to home to SLOs and generate new effector/memory T cells. The expansion potential and telomere length decreased with further differentiation

towards CCR7^{-}CD45R0^{+}CD45RA^{-}CXCR3^{+} TEM. This population displayed a very heterogenous and individual phenotype as the expression of CD27, CD28 and CD62L differed across studies[26,28,29]. Therefore, Mahnke et al. introduced a population of transitional memory T cells (TTM) found within TEM that expressed CD28 suggesting it as transitional stage from TCM to TEM[27]. On top of this, there were CD45RA re-expressing TEM (TEMRA) reported[13,26] with great degree of variation in their frequency between individuals. TEMRA displayed a series of different gene expressing profiles, some similar to TEM, others clearly distinct[31]. There also exist populations of memory T cells that permanently reside in tissues after infection, namely tissue-resident memory T cells (TRM)[32].



CD45RO	-	-	-	-	+	+
CD45RA	+	+	+	+	-	-
CCR7	+	+	+	+	+	-
CD62L	+	+	+	+	+	-
CD27	+	+	+	+	+	+/-
CD28	+	?	+	+	+	+/-
CD31	+/-	-	+/-	+	-	-
CD95	-	?	+/-	+	+	+
CD49d	-	?	+	?	+/-	+
CXCR3	-	+/-	+/-	+	+	+
CD11a	-	+/-	?	+	+	+
CD122	-	?	?	+	+	+/-
CD127	+	?	+	+	+	-
TCR diversity	+	+/-	+	+/-	+/-	+/-
Effector functions	-	+	+	+	+	+
Proliferation potential	+/-	+/-	?	+	+/-	-

Figure 1. Hypothetical model of human CD4^{+} T-cell differentiation. Here are among naïve, central and effector memory subsets, three atypical effector/memory cells with naïve-like phenotypes described, giving a brief hint at the differentiation complexity. The naïve receptor T cell (TNR) being the earliest stage display a very much alike phenotype to TN while exhibiting some effector functions like production of IL-4 or IFN-γ. The cytokine-producing (TCNP) and the stem cell memory (TSCM) also produce cytokines like IFN-γ and TNF-α after stimulation[29].

1.3. CD4⁺ T-cell maturation and differentiation in pig

One of the biggest problems in biomedical research is the selection of right experimental animal reference models. Because of its physiological and immunological similarities to humans and susceptibility to human or human-related pathogens, swine are handled as good large animal model for humans compared to other species[33]. However not being human, the porcine immune system differs which makes it an important subject of study for later experimental adjustments. Most important, it is necessary to get better knowledge on porcine immune-cell subsets for studying immune responses after vaccination and infection with veterinary relevant targets.

Similarities in human and porcine T-cell biology were observed as the T cells similarly mature in thymus to CD4⁺ and CD8⁺ naïve stages. Furthermore, after antigen contact, porcine αβ T cells differentiate into effector cells and can develop into memory cells for providing long-lasting immunity[34,35].

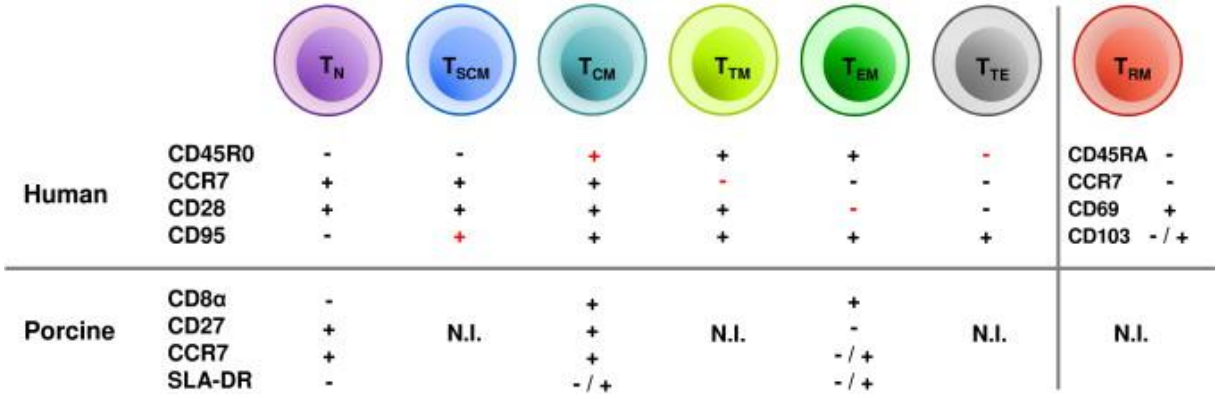


Figure 2. Immunophenotyping of CD4⁺ T-cell differentiation stages in human (up) and swine (low) with TN, TSCM, TCM, TTM, TEM, TTE (TEMRA) and TRM. The proposed model of T-cell differentiation by Mahnke et al.[27] was applied on the porcine memory T lymphocytes with different markers CD8α, CD27 and SLA-DR (MHC-II). Only TN, TCM and TEM were characterized in detail as the other subsets were not yet identified in the pig[34].

CD45R and its isoforms have also been studied in the pig. At least four different isoforms were reported including CD45RA, CD45RC and CD45R0[36]. CD45RC⁺ cells are already present on the day of birth in contrast to human[37]. However, porcine CD4⁺ T lymphocytes were reported upregulating CD8α upon antigen encounter in contrast to human[38,39]. CD8 is, like CD4 for helper T cells, a coreceptor in cytotoxic CD8⁺ T cells. Being exclusively present in its dimeric structure of α- and β-chain in cytotoxic T cells[40], CD8α expression in CD4⁺ lymphocytes makes it unique for swine[41]. CD8α frequency in the pig also increases with age[37] and after *in vitro*

stimulation most of the T cells express it[42,43] which makes CD8α (comparable of CD45R0 in humans) a marker of porcine antigen-experienced $CD4^+$ T cells.

CD27 is a part of the tumor necrosis factor receptor superfamily and is an important T-cell costimulatory molecule, also in the pig[44]. The marker is mainly expressed on naïve peripheral T lymphocytes, upon contact with CD70 helps in proliferation, survival and cytokine production. However, it is downregulated after clonal expansion and during differentiation[45] which makes it a solid candidate for discrimination of memory subsets. An overlapping expression pattern of CCR7 (important in human memory cell immunophenotyping) and CD27 was observed by Reutner et al., therefore the group used CD8α and CD27 for determination of porcine memory subsets. $CD8\alpha^-CD27^+$ cells represented TN, $CD8\alpha^+CD27^+$ TCM and $CD8\alpha^+CD27^-$ TEM $CD4^+$ T cells[42]. Functional data like higher proliferation in TN and ability to produce cytokines after antigen recall stimulation in TCM and TEM confirmed this classification[42]. Despite that, there are also emerging works using CD8α/CCR7 expression pattern[46–49] which leaves the choice of the most suitable marker combination as subject for future studies. A summary of investigated $CD4^+$ differentiation stages in the pig is provided in Figure 2.

1.4. Importance of porcine $CD4^+$ T cells in vaccination and infection

The overall immune response to an infection is dependent on humoral and cellular immunity. Being natural hosts for pathogens like influenza A virus, classical swine fever virus and porcine reproductive and respiratory syndrome virus, pigs were also reported to be a “mixing vessel” – a species providing a suitable environment for reassortments between avian and mammalian influenza virus strains that can lead to generation of new virulent strains[50]. This way, pig production could be disturbed by infections linked to a drop of meat production and economy, therefore research of porcine immune system is needed to provide targeted protection against pathogens to protect the world population from pandemic and famine.

Vaccination is an essential method for a successful establishing of porcine immune protection against pathogenic invasions[51]. In human viral diseases, multifunctional $CD4^+$ T lymphocytes were reported to have an important role in establishing protection[11]. These cells can perform more functions at once such as degranulation and cytokine production simultaneously. Porcine multifunctional $CD4^+$ T lymphocytes were also studied and identified in the influenza A infection, being able to produce two or three of the cytokines IFN-γ, TNF-α and IL-2 at once[34,50]. Therefore, the understanding of $CD4^+$ T-cell mediated immune mechanisms and their teamwork with other compartments of the immune system is an important step in the

development of the suitable treatment. Efficient vaccines should ultimately result in a rapid pathogen recognition, expansion of effector cells to fight and kill the pathogen, and provide long-lasting immunity in the form of memory cells. A crucial decision is also the choice of a suitable adjuvant as these substances can enhance the immune response and boost the establishment of certain immunological components as well. In this regard, carbomer adjuvant Carbopol® was reported to induce porcine early IFN-γ-producing cells and potentially to drive T-cell differentiation[52]. However, to target specific T-cell responses and subsequentially improve the vaccine efficacy, a phenotypic characterization of porcine T-cell subsets in healthy and diseased individuals is of a great importance, moreover since there are little studies regarding this topic[34,50–52].

2. Aim of this study

The $CD4^+$ T-cell differentiation in humans is very complex and heterogenous. According to latest studies, naïve T lymphocytes (TN) undergo differentiation starting with the naïve receptor (TNR), followed by cytokine-producing (TCNP) and stem-cell memory (TSCM) T cells, all having naïve phenotype and, apart from TN, effector functions. The differentiation continues with lymph-node-homing central memory T cells (TCM), intermediate stage of transitional (TTM) and lastly effector memory (TEM) T lymphocytes. Some TEM can re-express CD45RA, making up a category of TEMRA cells. Since $CD4^+$ T-cell differentiation is a complex topic and important for immune response research and treatment development, it is therefore crucial to study this in pig, a large animal model. The aim of this study was in-depth characterization of porcine naïve and memory $CD4^+$ T-cell subsets. Our focus was the basic T-cell discrimination with porcine CD45R isoforms CD45RA and CD45RC in combination with CD8α, CD27, CD28, CD62L, CD95, MHC-II and CCR7 since there are little studies investigating this important topic. We expected a similar T-cell heterogeneity as in humans. This study also should provide more insight into porcine T-cell composition and the opportunity of comparing porcine T-cell differentiation stages to the human ones.

3. Materials and Methods

3.1. Reagents and solutions

Table 1. Reagents and solutions used in the study.

Reagent/Solution	Source
Lymphocyte Separation Medium (LSM)	
• Pancoll human (density 1.077 g/ml)	PAN-Biotech (Aidenbach, Germany)
PBS (without Ca^{2+}, Mg^{2+})	PAN-Biotech
Washing Medium	
• RPMI 1640 with stable glutamine	PAN-Biotech
• 100 IU/ml penicillin and 0.1 mg/ml streptomycin	PAN-Biotech
• 5 % (v/v) FCS	Sigma-Aldrich (Vienna, Austria)
Tissue Culture Medium	
• RPMI 1640 with stable glutamine	PAN-Biotech
• 100 IU/ml penicillin and 0.1 mg/ml streptomycin	PAN-Biotech
• 10 % (v/v) FCS	Sigma-Aldrich
Freezing Medium	
• 50 % (v/v) RPMI 1640 with stable glutamine	PAN-Biotech
• 100 IU/ml penicillin and 0.1 mg/ml streptomycin	PAN-Biotech
• 40 % (v/v) FCS	Sigma-Aldrich
• 10 % (v/v) DMSO	Sigma-Aldrich
FCM buffer with porcine plasma	
• 10 % (v/v) heat-inactivated porcine plasma	in-house
• PBS (without Ca^{2+}, Mg^{2+})	PAN-Biotech

Table 2. Primary monoclonal antibodies (mAbs) used in FCM analysis.

Antigen	Fluorochrome /Biotin	Clone	Isotype	Labeling	Source
CD3	PE-Cy7	BB23-8E6-8C8	mouse IgG2a	direct	BD Biosciences (San Jose, CA, USA)
CD4	-	74-12-4	mouse IgG2b	indirect	in-house
CD8α	Biotin	11/295/33	mouse IgG2a	Biotin-Streptavidin	in-house
CD27	Alexa 647	b30c7	mouse IgG1	direct	in-house
CD28	-	3D11	mouse IgG1	indirect	in-house[1)]
CD45RA	FITC	MIL13	mouse IgG1	direct	BioRad (Vienna, Austria)
CD45RC	-	3a56	mouse IgG1	indirect	in-house
CD62L	-	CC32	mouse IgG1	indirect	BioRad
CD95	-	DX3	mouse IgG2a	indirect	OriGene Technologies (Herford, Germany)
MHC-II	-	MSA3	mouse IgG2a	indirect	in-house
CCR7	BrilliantViolet 711	3D12	mouse IgG2a	direct	BD Biosciences

1) Hybridoma kindly provided by Niklas Beyersdorf, JMU Würzburg, Germany[53]

Table 3. Secondary Abs and reagents used in FCM analysis.

Antibody	Fluorochrome	Source
rat-anti-mouse IgG1	PE/Dazzle594	BioLegend (San Diego, CA, USA)
goat-anti-mouse IgG2a	BrilliantViolet 421	Jackson Immuno Research (Cambridgeshire, United Kingdom)
goat-anti-mouse IgG2b	PE	Southern Biotech (Birmingham, AL, USA)
Streptavidin	BrilliantViolet 510	BioLegend

3.2. Isolation of porcine PBMCs

Blood was derived from healthy animals from a slaughterhouse, in accordance with Austrian welfare regulations. For isolation of porcine PBMCs, density gradient centrifugation was used. First, the blood was mixed and diluted with approximately the same amount of sterile PBS at room temperature (RT). Alongside 50 ml tubes were prepared with 15 ml LSM each. For 100 ml blood a total of six gradients were prepared. The blood/PBS mixture was carefully overlaid on top of the LSM and filled up to 50 ml. The tubes were centrifuged for 30 min at 920 × g, RT, acceleration 4, deceleration 1. After centrifugation the porcine plasma (upper layer) was collected in a separate bottle for FCM buffer preparation. The mononuclear layer was collected and transferred into beforehand prepared ice cooled 50 ml tubes which were then filled up to 50 ml with PBS. The solutions were spun down for 10 min at 470 × g, 4 °C and the supernatant was discarded. Remaining cell pellets were resuspended in residual PBS. Cells from the same animal were pooled into one tube, filled up with cold PBS and centrifuged as above. Next, cells were treated as above but this time with cold washing medium. After another centrifugation round the cell pellet was resuspended in tissue culture medium and cells were counted on a Sysmex XP-300 cell counter (Sysmex Corporation, Kobe, Japan). The cells were frozen for long-term storage as described in 3.3.

3.3. Freezing and defrosting of porcine PBMCs

For freezing, isolated porcine PBMCs were centrifuged for 8 min at 470 × g, 4 °C. The cryo-vials used for freezing were placed on a pre-cooled rack alongside. Later, the supernatant was discarded, and the cells were resuspended in freezing medium in concentrations of 3×10^7 – 6×10^7 PBMCs per 1 – 1.5 ml freezing medium and quickly transferred into the cryo-vials. The cryo-vials were initially frozen at -80 °C and transferred to -150 °C after one or two days for later usage.

For defrosting of porcine PBMCs, one 15 ml tube per cryo-vial was filled with 10 ml FCM buffer and put alongside with the cryo-vials in the water bath at 37 °C. The cryo-vials were removed once the cell suspension was almost completely defrosted. Then, 1 ml of the pre-warmed FCM buffer was slowly added to the cell suspension and the cells were transferred to the corresponding tube. The mixture was spun down for 8 min at 470 × g and RT, the supernatant was discarded, and the cell pellet was resuspended in FCM buffer and counted as described above. Cell concentration was set to 2×10^7 cells/ml for FCM analysis.

3.4. FCM staining of porcine PBMCs

The FCM staining was done in 96-well round-bottom microtiter-plates. 4×10^6 cells per sample were stained in a volume of 25 µl according to the panels shown in Table 4. All incubation steps were performed for 20 min at 4 °C in the dark. After each incubation step, cells were washed by adding 200 µl of FCM buffer per well and centrifugation for 4 min at 4 °C. Then the supernatant was discarded over the sink and the pellet resuspended on a plate shaker. This process was repeated once more before continuing with the next staining step. In general, for the first step antigen-specific unlabeled primary mAbs were added, followed by isotype-specific secondary reagents in the second step. In the third step all free antigen binding sites were blocked by whole mouse IgG molecules (mIgG, ChromPure, Jackson Immuno Research), followed by directly conjugated primary mAbs and Streptavidine conjugates. In addition, in the last step, cells were stained for live/dead discrimination by Fixable Viability Dye eFluor780 (VDeF780, Thermo Fisher Scientific) according to manufacturer's protocol. Once the staining was done, the cells were washed twice again, resuspended in 250 µl of FCM buffer and transferred into tubes for measuring of samples.

Table 4. Staining protocol for FCM analysis.

ID	1. step	2. step	3. step	4. step	5. step
1	unstained	unstained	unstained	unstained	unstained
2	CD45RC MHC-2 CD4	anti-IgG1-PE/Dazzle-594 anti-IgG2a-BV421 anti-IgG2b-PE	mIgG	CD3-PE-Cy7 CD8α-Biotin CD27-A647 CD45RA-FITC CCR7-BV711	VDeF780 Strep.-BV510
3	CD62L CD95 CD4	anti-IgG1-PE/Dazzle-594 anti-IgG2a-BV421 anti-IgG2b-PE	mIgG	CD3-PE-Cy7 CD8α-Biotin CD27-A647 CD45RA-FITC CCR7-BV711	VDeF780 Strep.-BV510
4	CD28 CD4	anti-IgG1-PE/Dazzle-594 anti-IgG2b-PE	mIgG	CD3-PE-Cy7 CD8α-Biotin CD27-A647 CD45RA-FITC CCR7-BV711	VDeF780 Strep.-BV510

3.5. Data analysis

FCM data was analyzed using a Cytek® Aurora flow cytometer with SpectroFlo® software (Fremont, CA, USA). The stopping gate was set on lymphocytes and 100,000 cells were recorded for each sample. Further analysis was done in FlowJo™ V10.8.1 (FlowJo Software, Ashland, OR, USA) following the gating strategy shown in Fig. 3A. The general gating strategy was as following: gating on lymphocytes according to FSC-A/SSC-A light scatter properties, followed by gating on single cells by a FSC-A/FSC-H gate and exclusion of dead cells by gating on VDeF780 negative cells. The scatter plots were created via GraphPad Prism V9.5.1 (GraphPad Software, San Diego, CA, USA). Due to high heterogeneity between animals, no statistical analysis were performed yet. This will be done, when data of more animals is collected.

4. Results

4.1. CD8α/CD27-defined subsets in porcine PBMCs

For characterization of porcine $CD4^+$ T-cell differentiation stages, the gating strategy described in 3.5 (Figure 3A) was followed. Plotting CD3 (T-lymphocyte marker) against CD4 resulted in three distinct populations: $CD3^-CD4^-$ non-T cells, $CD3^+CD4^-$ and $CD3^+CD4^+$ T lymphocytes, the latter resembling the population of interest. $CD4^+$ T cells were then divided by CD8α/CD27 marker expression frequently used for pig[42] into $CD8\alpha^-CD27^+$ naïve (TN), $CD8\alpha^+CD27^+$ central memory (TCM) and $CD8\alpha^+CD27^-$ effector memory (TEM) $CD4^+$ T cells (Figure 3B). The distribution of each population was very similar in most of the animals. The vast majority of $CD4^+$ T cells were TN (about 46 %), the TCM and TEM made up between 25–30 % both. The only exception was one pig that showed lower frequencies of TN linked with an increase in TCM and TEM.

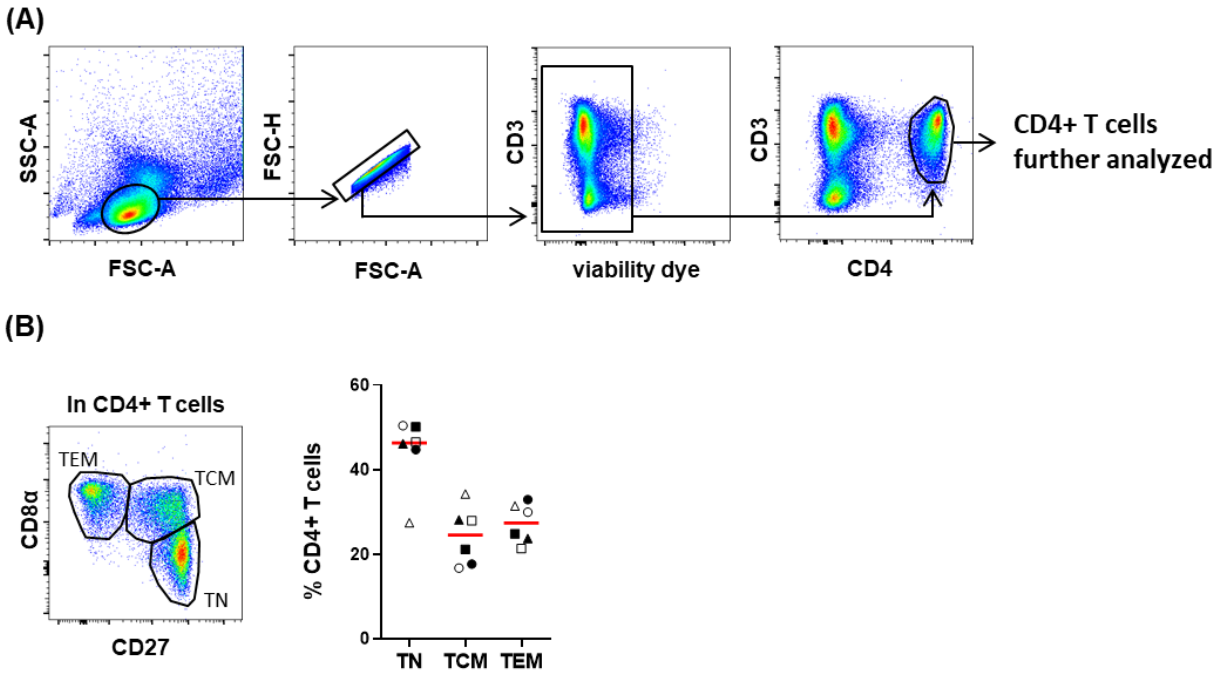


Figure 3. The fundamental analysis of $CD4^+$ T cells. The plots in the study were depicted by one representative animal. (A) The gating strategy of $CD4^+$ T cells. First, the lymphocytes were selected (FSC-A/SSC-A), followed by exclusion of doublets (FSC-A/FSC-H). Next, we gated on the live cells (viability dye negative) and lastly specifically on the $CD4^+$ T cells. (B) The naïve and memory $CD4^+$ T-cell subsets and their distribution. The $CD3^+CD4^+$ cells were analyzed by CD8α/CD27 expression resulting in portrayal of three distinct subsets - the naïve (TN), the central memory (TCM) and the effector memory (TEM) T cells. On the right is a scatter plot showing the exact frequencies of these subsets in six pigs (represented by individual symbols) including median in red.

4.2. CCR7/CD45RA expression in CD8α/CD27 subsets

Next analysis was done by gating on CCR7 and CD45RA (a marker combination used for human $CD4^+$ T lymphocytes) in each CD8α/CD27 subset. CCR7 is an important lymph node homing receptor and CD45RA was reported to be expressed on naïve T cells. Using these markers together results in the discrimination of human naïve and memory $CD4^+$ T cells[13]. The goal was to check the applicability of this marker combination on porcine PBMCs. In the $CD8α^-$ $CD27^+$ TN population emerged three subpopulations (Figure 4A). The majority (about 49 %) were $CCR7^+CD45RA^+$ (7+RA+), which in humans correspond to the naïve phenotype. $CCR7^+CD45RA^-$ (7+) accounted for 40 % and therefore the next prominent subpopulation that resemble TCM in humans. A very small portion of $CCR7^-CD45RA^-$ (7-RA-, normally human TEM) lymphocytes was also detected. However, as they only made up only 5 % of all porcine-defined TN, they were omitted for further analysis. Looking at porcine $CD8α^+CD27^+$ TCM, the composition of CCR7/CD45RA expressing cells differed from TN (Figure 4B). Apart from the visible reduction of 7+RA+ to only 15 %, we observed an increase of 7+ to 55 % and in 7-RA- up to 25 %. Regarding $CD8α^+CD27^-$ TEM, the animals displayed more variable composition in contrast to TN and TCM (Figure 4C). Although TEM comprised mostly 7-RA- (~53 %) and a bit of 7+ (15 %) cells, a significant $CD45RA^+$ (RA+) population emerged. It was observed that the number of RA+ varied from animal to animal. Most of the pigs had about 22 % RA+ but one pig had almost 60 %, indicating that the size of the RA+ population could be dependent on the individual.

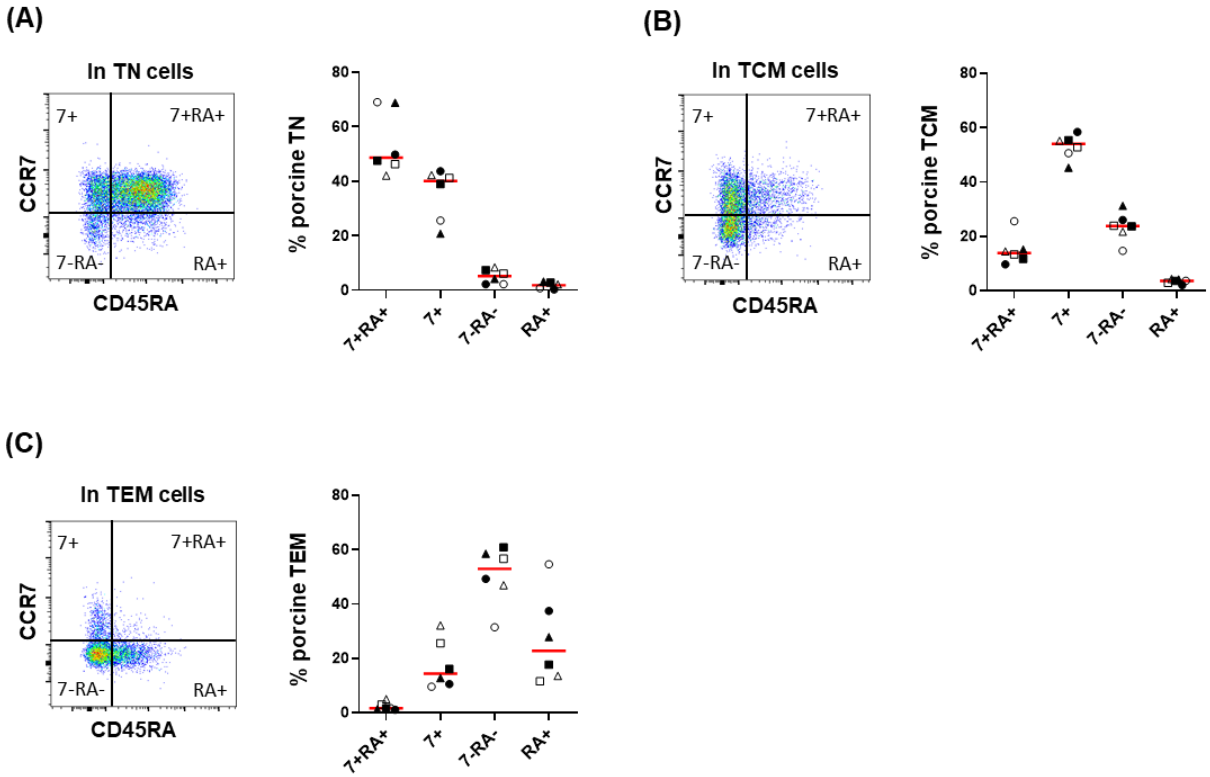


Figure 4. The CCR7/CD45RA expression in (A) CD8α$^-$CD27$^+$ TN, (B) CD8α$^+$CD27$^+$ TCM and (C) CD8α$^+$CD27$^-$ TEM subsets with their scatter plots showing the actual percentage composition in different animals (individual symbols) with median in red. Comparing the two markers gave us four populations in total – CCR7$^+$CD45RA$^+$ (7+RA+), CCR7$^+$CD45RA$^-$ (7+), CCR7$^-$CD45RA$^-$ (7-RA-) and CCR7$^-$CD45RA$^+$ (RA+), their distribution and size dependent on the beforehand gated CD8α/CD27 expressing subset.

4.3. CD28 expression in CD8α/CD27 subsets

Regarding the expression of the costimulatory molecule CD28, all TN were clearly CD28$^+$. The same applied to TCM, where the vast majority was CD28$^+$ as well. This accounted for the porcine CD8α/CD27 as well as the human CCR7/CD45RA defined subsets (Figure 5A+B). On the other hand, the TEM population showed much higher heterogeneity (Figure 5C). All 7+ TEM cells expressed CD28. Besides CD28$^+$ cells within 7-RA- TEM population, a prominent subset of T lymphocytes that downregulated CD28 was also observed, although high variations of individual animals were observed. The RA+ TEM were mostly CD28$^{-/dim}$ with animal-to-animal variations similar to the 7-RA- population.

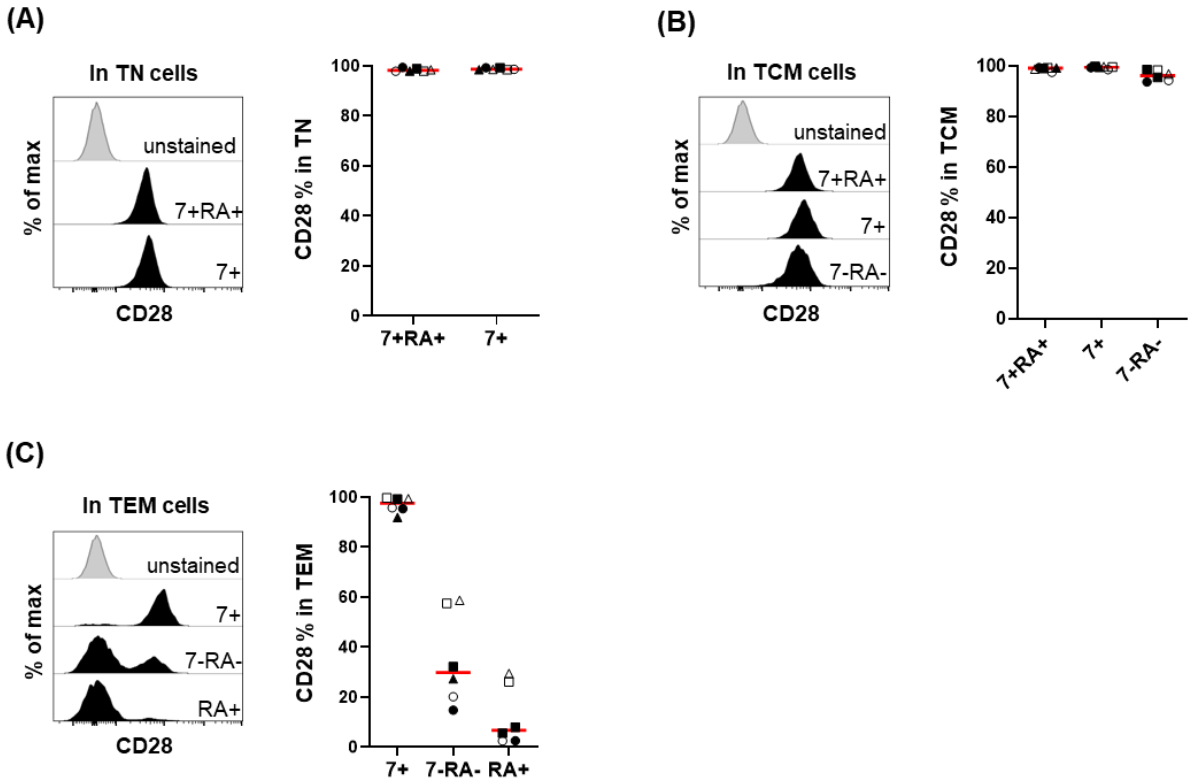


Figure 5. The CD28 expression in (A) CD8α$^-$CD27$^+$ TN, (B) CD8α$^+$CD27$^+$ TCM and (C) CD8α$^+$CD27$^-$ TEM subsets. Left graph resembles overlay histograms of CD28 expression within each selected CCR7/CD45RA population from 4.2. On the right is the corresponding scatter plot showing the percentages of six individual animals (shown by different symbols) with median in red. Almost all naïve and central memory CD4$^+$ T cells expressed CD28. The population of effector memory cells became heterogenous as the CD28 expression dropped in most of the 7-RA- and in all RA+ T cells apart from 7+ subset.

4.4. Expression of MHC-II, CD45RC, CD62L and CD95

4.4.1. In CD8α$^-$CD27$^+$ naïve CD4$^+$ T cells

Based on the results of CCR7/CD45RA expression we focused then on the characterization of 7+RA+ and 7+ populations within the porcine TN with markers mentioned in the 4.4 title. As shown in Figure 6 the subsets displayed different phenotypes. Regarding MHC-II, CD62L and CD95 expression, the vast majority of cells in both subsets showed no MHC-II and CD95 expression and upregulated CD62L (85–90 %). Except for one animal that interestingly had only about 35 % of cells expressing CD62L (Figure 6B+D, open triangle). However, the overall expression of CD45RC differed. Both subsets displayed high expression of CD45RC which is characteristic for naïve CD4$^+$ T cells[23]. Nevertheless, about 35 % of 7+ cells downregulated CD45RC, which could be the sign of the start of T-cell differentiation.

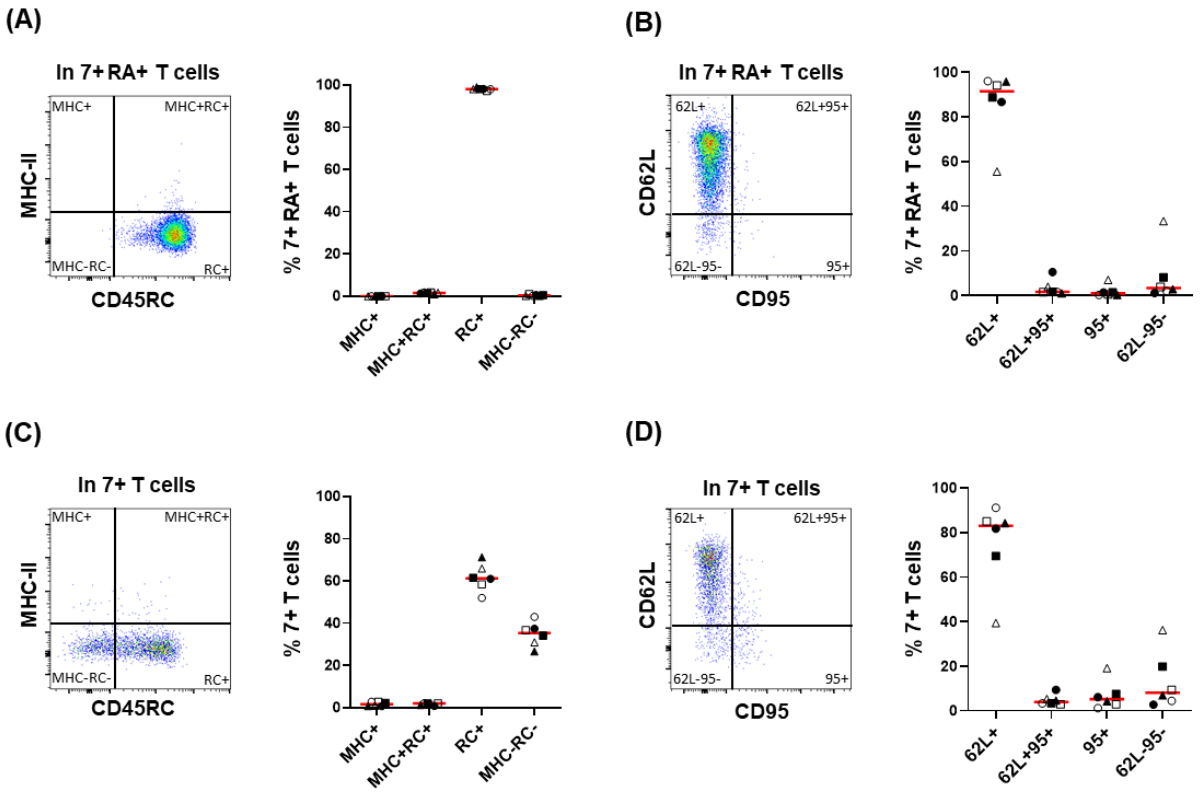


Figure 6. The expression of MHC-II, CD45RC, CD62L and CD95 in CD8α$^-$CD27$^+$ TN of different animals (individual symbols) in density and scatter plots with median in red. The MHC-II and CD45RC expression is in (A) for 7+RA+ and (C) for 7+ T cells. CD62L and CD95 results are shown in (B) for 7+RA+ and (D) for 7+ T cells. Comparing the expression of MHC-II/CD45RC and CD62L/CD95 gave us four populations each, their distribution and size dependent on the gating before. The first-combination populations were MHC-II$^+$CD45RC$^-$ (MHC+), MHC-II$^+$CD45RC$^+$ (MHC+CD45RC+), MHC-II$^-$CD45RC$^+$ (RC+) and MHC-II$^-$CD45RC$^-$ (MHC-RC-). The second combination resulted in CD62L$^+$CD95$^-$ (62L+), CD62L$^+$CD95$^+$ (62L+95+), CD62L$^-$CD95$^+$ (95+) and CD62L$^-$CD95$^-$ (62L-95-) populations.

4.4.2. In CD8α$^+$CD27$^+$ central memory CD4$^+$ T cells

Looking at porcine TCM, the defined T-cell subsets became more heterogenous than before. Here we analyzed three populations – 7+RA+, 7+ and 7-RA-. The 7+RA+ population contained mostly CD45RC$^+$ cells (Figure 7A). Besides 7+RA+MHC-II$^-$ cells, about 15 % cells that upregulated MHC-II were also observed. In comparison, about two thirds of 7+ TCM cells lost their CD45RC phenotype, though there were no differences observed in the expression of MHC-II (Figure 7C). Regarding the 7-RA- subset, the loss of CD45RC was more prominent than in the other two populations (80 % cells were stained negative). As the cells differentiated further, they also started expressing a bit more MHC-II (up to 35 % of total 7-RA- TCM).

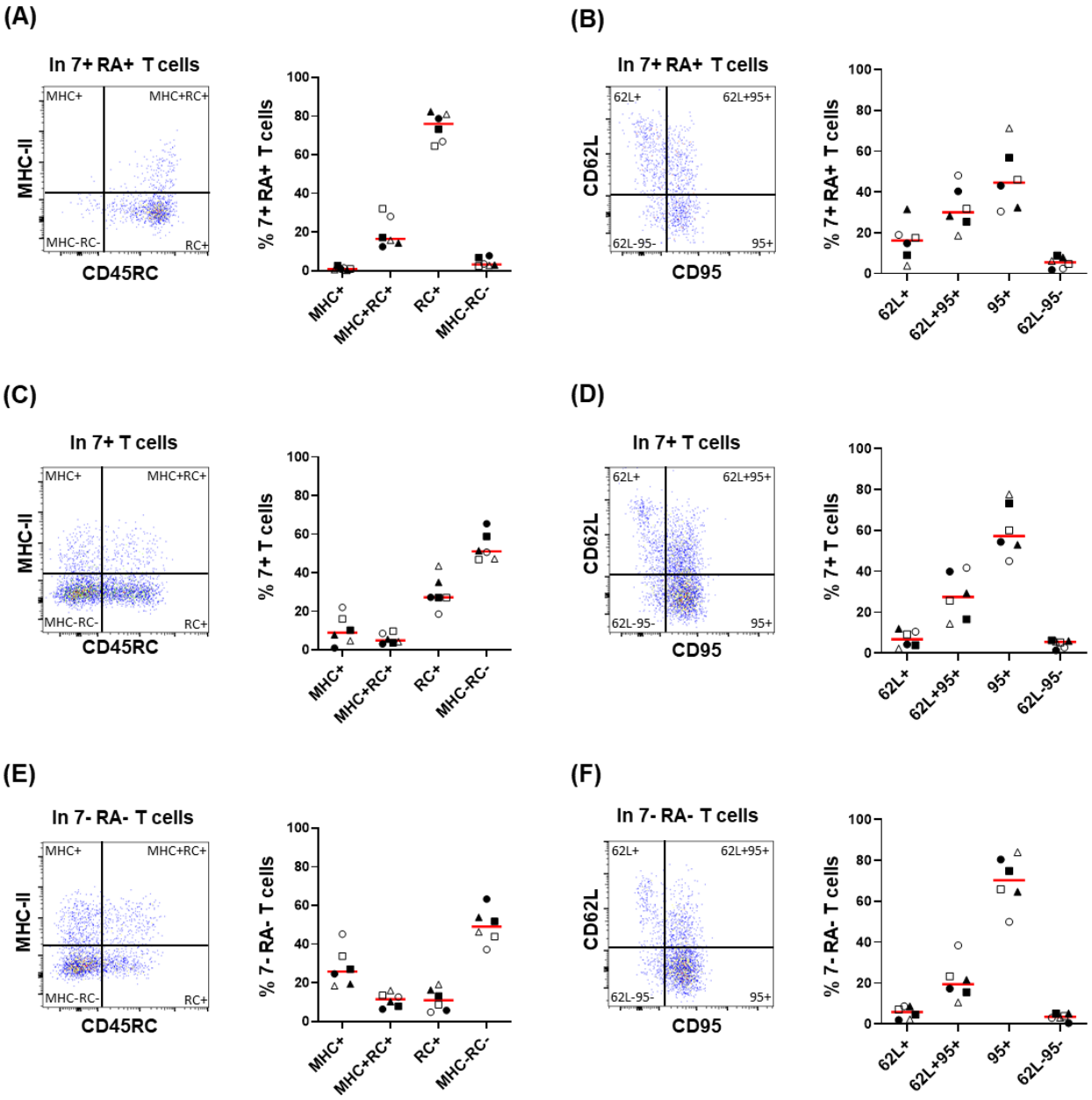


Figure 7. The expression of MHC-II, CD45RC, CD62L and CD95 in CD8α^{+}CD27^{+} TCM in density and scatter plots with median in red. The MHC-II and CD45RC expression is in (A) for 7+RA+, (C) for 7+ and (E) for 7-RA- T cells. CD62L and CD95 results are shown in (B) for 7+RA+, (D) for 7+ and (F) for 7-RA- T cells. Comparing the expression of MHC-II/CD45RC and CD62L/CD95 gave us four populations each, their distribution and size dependent on the gating before. The first-combination populations were MHC-II^{+}CD45RC^{-} (MHC+), MHC-II^{+}CD45RC^{+} (MHC+CD45RC+), MHC-II^{-}CD45RC^{+} (RC+) and MHC-II^{-}CD45RC^{-} (MHC-RC-). The second combination resulted in CD62L^{+}CD95^{-} (62L+), CD62L^{+}CD95^{+} (62L+95+), CD62L^{-}CD95^{+} (95+) and CD62L^{-}CD95^{-} (62L-95-) populations.

The CD62L/CD95 expression pattern looked very much the same between all three populations. In all there were some cells with CD62L$^+$ phenotype (~20 % in 7+RA+, 5–10 % in 7+ and 7-RA- population). The second most prominent subset included CD62L$^+$CD95$^+$ T lymphocytes (~30 % in 7+RA+ and 7+, 20 % in 7-RA-). Most of the cells (~50 % in 7+RA+ and 7+, 70 % in 7-RA-) were positive. However, it is important to state that the data in each subset was very heterogenous, possibly due to individual differences.

4.4.3. In CD8α$^+$CD27$^-$ effector memory CD4$^+$ T cells

The porcine TEM subset displayed a very different composition of lymphocytes compared to naïve and central memory T cells. Based on results from 4.2, the focus was set on 7+, 7-RA- and RA+ porcine-defined TEM. The CCR7 expressing population (Figure 8A) consisted of mainly MHC-II$^-$CD45RC$^-$ (approx. 60 %) among the other three phenotypes worth mentioning (MHC-II$^+$CD45RC$^-$, MHC-II$^+$CD45RC$^+$, MHC-II$^-$CD45RC$^+$), each making up to about 15 % cells. The 7-RA- subset (Figure 8C) could be divided into three distinct populations – MHC-II$^+$CD45RC$^-$, MHC-II$^+$CD45RC$^+$ and MHC-II$^-$CD45RC$^-$, the latter having the smallest cell count concluding that the MHC-II expression increased. We also saw CD45RC marker reappearing here on MHC-II positive cells. This MHC-II$^+$CD45RC$^+$ population was detected in the RA+ TEM as well as the one and only prominent subset there (Figure 8E).

Looking at CD62L and CD95, the CCR7 expressing subset (Figure 8B) showed a small proportion of CD62L$^+$CD95$^+$ cells (20 %) and a much bigger population with CD95$^+$ phenotype (80 %). The 7-RA- and RA+ subsets (Figure 8D+F) displayed very similar CD62L/CD95 expression pattern as they both had their majority of cells lacking the CD62L homing receptor. Interestingly in both cases the populations were moderately expressing CD95 as approx. 20 % of total cells were CD95$^-$ and the rest was CD95$^+$, although mentioning the little scattered data owing to pig differences is important.

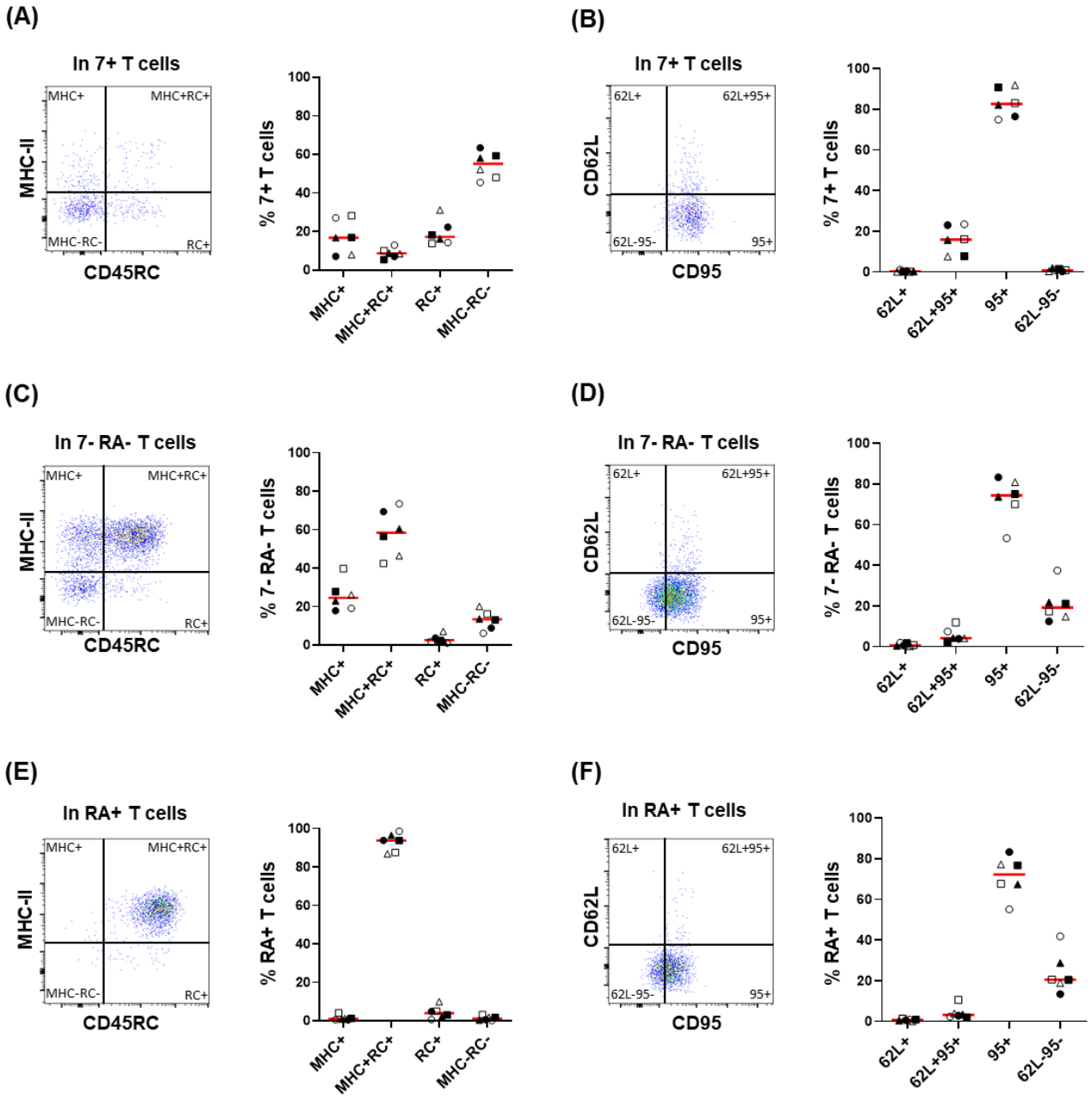


Figure 8. The expression of MHC-II, CD45RC, CD62L and CD95 in CD8α^{+}CD27^{-} TEM in density and scatter plots with median in red. The MHC-II and CD45RC expression is in (A) for 7+, (C) for 7-RA- and (E) for RA+ T cells. CD62L and CD95 results are shown in (B) for 7+, (D) for 7-RA- and (F) for RA+ T cells. Comparing the expression of MHC-II/CD45RC and CD62L/CD95 gave us four populations each, their distribution and size dependent on the gating before. The first-combination populations were MHC-II^{+}CD45RC^{-} (MHC+), MHC-II^{+}CD45RC^{+} (MHC+RC+), MHC-II^{-}CD45RC^{+} (RC+) and MHC-II^{-}CD45RC^{-} (MHC-RC-). The second combination resulted in CD62L^{+}CD95^{-} (62L+), CD62L^{+}CD95^{+} (62L+95+), CD62L^{-}CD95^{+} (95+) and CD62L^{-}CD95^{-} (62L-95-) populations.

5. Discussion

In this study we investigated the differentiation stages of porcine naïve and memory $CD4^+$ T-cell subsets by immunophenotyping. We have found that the differentiation in the pig is as complex and heterogeneous as in humans according to recent studies and thus we introduce a hypothetical $CD4^+$ T-lymphocyte differentiation model. The $CD4^+$ T cells were firstly analyzed by CD8α/CD27 expression, a pattern used for porcine naïve and effector/memory subset discrimination, resulting in three distinct populations of $CD8\alpha^-CD27^+$ TN, $CD8\alpha^+CD27^+$ TCM and $CD8\alpha^+CD27^-$ TEM[42]. In a next step the CCR7/CD45RA expression was investigated, frequently used in humans/mice to define naïve and memory cell subsets[13].

5.1. Porcine $CD8\alpha^-CD27^+$ naïve $CD4^+$ T-cell subset

According to our results, the porcine $CD4^+CD8\alpha^-CD27^+$ TN subset displayed a very heterogenous composition after investigating the CCR7/CD45RA expression pattern. The cells dispersed mainly across two populations of $CCR7^+CD45RA^+$ (naïve in human) and $CCR7^+CD45RA^-$ (central memory in human) T cells. The T cells still displayed a naïve phenotype having no expression of CD95, an important receptor mediating cell lysis[54], and upregulated CD62L expression, mediating the homing of the T cell to SLOs[24]. MHC-II was also nonexistent in this subset and CD28 on the other hand was upregulated, all supporting the fact that the cells show a phenotype of porcine naïve T lymphocytes despite the lack of CD45RA of some cells. The $MHC\text{-}II^-CD28^+$ phenotype was already shown for the CD8α/CD27 TN subset, as well as $CD62L^+$ on mRNA level[42,53]. However, the differentiation became complex again looking at the expression of CD45RC. $CCR7^+CD45RA^+$ T cells expressed CD45RC, nevertheless, $CCR7^+CD45RA^-$ subset was divided into $CD45RC^+$ and $CD45RC^-$ cells. It is known that CD45RC is present in young pigs[37] but a $CD45RC^-$ subset within TN has also been observed[42]. Nevertheless, the data could suggest starting differentiation with the first loss of CD45RA followed by the loss of CD45RC.

5.2. Porcine $CD8\alpha^+CD27^+$ central memory $CD4^+$ T-cell subset

Porcine TCM composition clearly differed from TN, but also showed a high heterogeneity, having T cells dispersed into main three subsets of $CCR7^+CD45RA^+$ (human naïve), $CCR7^+CD45RA^-$ (human central memory) and $CCR7^-CD45RA^-$ (human effector memory), the second being the most eminent. CD28 was still expressed in all subsets. The CD62L/CD95 profile in all populations looked very similar to each other suggesting that with time the $CD62L^+$ cells started expressing CD95 and with further differentiation lost CD62L, indicating that these

cells could have been able to leave the lymph node by losing their SLOs-homing receptor[24]. Regarding MHC-II expression, our data showed some MHC-II expressing T cells already in the CCR7$^+$CD45RA$^+$ population, increasing within CCR7$^+$CD45RA$^-$ and CCR7$^-$CD45RA$^-$ populations, though the most cells did not express MHC-II. Saalmüller et al. observed CD4$^+$ T cells with an MHC-II$^+$ phenotype in both primary and secondary immune response[43]. That could mean that gating on CD8α$^+$CD27$^+$ contains effector/memory T cells, potentially with the ability of antigen-presentation due to the expression of MHC-II. A small subset expressing MHC-II within porcine CD8α/CD27 defined TCM was also reported by Reutner et al.[42] The CD45RC expression pattern in CCR7$^+$CD45RA$^+$ and CCR7$^+$CD45RA$^-$ was comparable to TN. Most of these cells had the latter phenotype, and CD45RC in CCR7$^-$CD45RA$^-$ TCM was mostly downregulated, supporting the theory of primary losing CD45RA and then CD45RC. This differentiation seems, however, not dependent on antigen priming as this phenotype can be already found within CD8α$^-$CD27$^-$ TN. On the other hand, increasing MHC-II expression seems linked to the CD8α expression since TN were negative for both.

5.3. Porcine CD8α$^+$CD27$^-$ effector memory CD4$^+$ T-cell subset

Regarding our data on the TEM subset, CCR7$^+$CD45RA$^-$ (human central memory), CCR7$^-$CD45RA$^-$ (human effector memory) and CCR7$^-$CD45RA$^+$ (human TEMRA) populations were found, the second being the biggest. In contrast to previous subsets, the CD28 expression was heterogenous across the cells. In CCR7$^+$CD45RA$^-$ TEM CD28 expression was still high but started to change with CCR7$^-$CD45RA$^-$ T cells. They were divided into two groups – one still expressing CD28, and the second group showed downregulation of CD28. It would be interesting to observe the MHC-II/CD45RC phenotype within this CCR7$^-$CD45RA$^-$CD28$^-$ population but was not possible in the same staining sample. Looking at CCR7$^-$CD45RA$^+$ TEM, there was no CD28 detected. The data suggest that CD28 downregulation is a sign towards terminally differentiated effector memory cells, supporting previous data[53]. The CCR7$^+$CD45RA$^-$ population consisted mainly of CD62L$^-$CD95$^+$ cells, with a small population of cells expressing both markers. The other two CD95$^+$ populations displayed no CD62L expression, all supporting the theory that after the gain of CD95 the loss of CD62L followed. The MHC-II/CD45RC expression pattern looked differently across the TEM populations. Starting with CCR7$^+$CD45RA$^-$, some cells expressed CD45RC, some MHC-II only and most of them none of the markers. However, that changed with CCR7$^-$CD45RA$^-$, having a few double negative cells but a great amount of MHC-II$^+$CD45RC$^-$ and even greater amount of double positive T cells. This could suggest following MHC-II expression, CD45RC reappears. This

hypothesis was further supported as the cells differentiated towards CCR7$^-$CD45RA$^+$MHC-II$^+$CD45RC$^+$ TEMRA, known for very low expansion potential in humans[13]. The loss of CD28 and the gain of CD95 on human T cells has also been reported to be linked to further differentiated cells and aging[55] which also supports our TEMRA findings. However, porcine TEMRA have been suggested as CD8α$^-$CCR7$^-$ cells[33], which contradicts our differentiation theory. Regarding these CD8α$^-$CCR7$^-$ T cells, being not antigen-experienced could hint at virtual memory[56], or tissue resident memory T cells[32], being mainly found in the lung, lymph node and bronchoalveolar lavage. For the full understanding further research is needed.

5.4. Outlook

To sum up, this study provides more insight into the differentiation of porcine naïve and memory CD4$^+$ T cells. The outliers in our results could indicate that immunosenescence could also have an impact on the porcine cellular T-cell composition like in humans[55]. However, the animals being young (6–8 months), it is hard to support this theory. Our data and better understanding of memory subsets could be helpful in the development of vaccines against porcine pathogens to protect pig industries and global population against emerging new pathogens and thus preventing pandemics. Nevertheless, further studies of naïve and effector/memory T cells in different animal age groups and in different tissues are needed, as we cannot omit CD8α$^-$CCR7$^-$ tissue-associated T-cell population[33]. It would also be interesting to look at MHC-II/CD45RC phenotype within CCR7$^-$CD45RA$^-$CD28$^-$ TEM population since we could not combine CD28 with MHC-II and CD45RC in our staining panel. Important data could also provide *in vitro* stimulation of each subset to confirm our differentiation model by measuring induced cytokines. Analysis of cytokine production could provide more insight, especially in the naïve subset that has been reported in human to consist of naïve-like T cells having various effector functions whilst residing in the naïve subset[29]. Analysis of cytolytic functions and cytokine production could also precisely clarify multifunctional CD4$^+$ T-cell subsets, known for aiding in viral infections[34,50]. An antibody against porcine CD45R0 would also answer some questions in immunophenotyping since human CD45R0 has been reported as memory T-cell marker[21]. Also, the discrimination between effector and memory T cells still evades the scientific understanding but with extensive research it should be only a question of time.

6. Summary

$CD4^+$ T cells are an important component of the adaptive immune response. It is generally acknowledged that after antigen exposure they differentiate from naïve to memory $CD4^+$ T cells. In 1999 two main subsets of human memory T lymphocytes were described, the central memory and effector memory T cells. However, recent studies showed that the human $CD4^+$ T-cell population is much more heterogenous, hinting there may be more differentiation stages than formerly described. More insight into this topic can be provided by multi-color FCM, combining specific markers associated with T-cell differentiation. As swine is being considered as a potential biomedical animal model, further studies of its immune system are needed. Similarities between human and porcine lymphocytes have been observed, therefore for our study on porcine $CD4^+$ T cells we also expected a high subset heterogeneity. The goal was to establish an applicable complex multi-color staining panel for better characterization of the porcine naive and effector/memory $CD4^+$ T-cell subsets with cell surface markers CD4, CD8α, CD27, CD28, CD45RA, CD45RC, CD62L, CD95, CCR7, and MHC-II. For the study peripheral blood mononuclear cells were isolated from blood of six 6-months old healthy animals by gradient centrifugation. The expression of the markers mentioned above was analyzed by multi-color FCM. The naïve and memory subsets were defined by CD8α/CD27 expression – according to literature on porcine cells, and further analyzed by CCR7/CD45RA expression – a marker combination used for human T cells. Our results indicate the presence of various subpopulations of naïve and effector/memory $CD4^+$ T lymphocytes. Besides $CCR7^+CD45RA^+$ cells within $CD8\alpha^-CD27^+$ naive, a prominent subset of cells that downregulated CD45RA was observed. $CD4^+CD8\alpha^+CD27^+$ TCM consisted of $CCR7^+CD45RA^-$, $CCR7^-CD45RA^-$, as well as a minor subset with a $CCR7^+CD45RA^+$ phenotype. $CD4^+CD8\alpha^+CD27^-$ TEM mainly had a $CCR7^-CD45RA^-$ phenotype, but also $CCR7^+CD45RA^-$ or $CCR7^-CD45RA^+$ subpopulations were observed. The latter representing TEMRA. CD28 expression was high in naïve and TCM subsets and declined in TEM and TEMRA defined stages. CD62L expression on the other hand declined already in TCM subpopulations and was absent in TEM and TEMRA subsets, while CD95 expression was only observed within TCM subsets and MHC-II was upregulated only towards the TEM stage. Expression of CD45RC showed a more heterogenous expression within the diverse $CD4^+$ subsets. Together, this data highlights the high variability of porcine naïve and memory/effector $CD4^+$ T cells which may cast light on diverse transitional stages of T-cell differentiation in the pig.

7. Zusammenfassung

$CD4^+$ T-Zellen sind ein wichtiger Bestandteil der adaptiven Immunantwort. Es ist allgemein anerkannt, dass sie sich nach Antigenexposition von naiven zu $CD4^+$ T-Gedächtniszellen differenzieren. Im Jahr 1999 wurden zwei Hauptuntergruppen menschlicher T-Gedächtniszellen beschrieben: die Zentralen und die Effektor T-Gedächtniszellen. Jüngste Studien haben jedoch gezeigt, dass die Population der menschlichen $CD4^+$ T-Zellen viel heterogener ist, was auf eine mögliche Existenz mehrerer Differenzierungsstadien hindeutet. Die Mehrfarben-Durchflusszytometrie, in der spezifische Marker für die T-Zell-Differenzierung kombiniert werden können, kann weitere Erkenntnisse zu diesem Thema liefern. Da das Schwein als vielversprechendes biomedizinisches Tiermodell beschrieben ist, sind weitere Studien über das porzine Immunsystem erforderlich. In vergangenen Studien wurden Parallelen zwischen humanen und porzinen Lymphozyten festgestellt, daher erwarteten wir für unsere Studie über porzine $CD4^+$ T-Zellen auch eine hohe Heterogenität der Subpopulationen. Ziel dieser Studie war es, ein komplexes Mehrfarben Panel für die Durchflusszytometrie zur genaueren Charakterisierung der $CD4^+$ naiven und T-Effektor/Gedächtnis-Zellsubsets vom Schwein zu entwickeln. Dazu wurden die Zelloberflächenmarker CD4, CD8α, CD27, CD28, CD45RA, CD45RC, CD62L, CD95, CCR7 und MHC-II untersucht. Für die Studie wurden periphere mononukleäre Blutzellen aus dem Blut von sechs Monate alten gesunden Tieren durch Gradientenzentrifugation isoliert. Die naiven und die T-Gedächtniszellen wurden anhand der Expression von CD8α/CD27 definiert - entsprechend der Literatur über Schweinezellen. In einem weiteren Schritt wurden die Zellen anhand der Expression von CCR7/CD45RA analysiert - einer Markerkombination, die für die menschlichen T-Zellen verwendet wird. Unsere Ergebnisse weisen auf das Vorhandensein vielfältiger Subpopulationen von naiven und $CD4^+$-Effektor-/Gedächtnis-T-Lymphozyten hin. Neben $CCR7^+CD45RA^+$ Zellen innerhalb der $CD4^+CD8\alpha^-CD27^+$ naiven T-Zellen wurde eine Population von Zellen gefunden, die CD45RA herunterreguliert hatte. $CD4^+CD8\alpha^+CD27^+$ Zentrale T-Gedächtniszellen bestanden aus $CCR7^+CD45RA^-$ und $CCR7^-CD45RA^-$ Zellen, sowie einer kleineren Untergruppe mit einem $CCR7^+CD45RA^+$ Phänotyp. $CD4^+CD8\alpha^+CD27^-$ Effektor T-Gedächtniszellen hatten hauptsächlich einen $CCR7^-CD45RA^-$ Phänotyp, aber es wurden auch geringere Frequenzen von $CCR7^+CD45RA^-$ oder $CCR7^-CD45RA^+$ Subpopulationen beobachtet. Letzter Phänotyp repräsentiert final differenzierte Effektor T-Gedächtniszellen. Die CD28 Expression war hoch in naiven und Zentralen T-Gedächtniszellen und nahm in Effektor T-Gedächtniszellen ab. Die Expression von CD62L hingegen nahm bereits in den Subpopulationen der Zentralen T-

Gedächtniszellen ab und war in den verschiedenen Subpopulationen der Effektor T-Gedächtniszellen nicht vorhanden. Die Expression von CD95 wurde dagegen nur in den Subpopulationen der Zentralen T-Gedächtniszellen gemessen und MHC-II wurde ausschließlich im Differenzierungsstadium der Effektor T-Gedächtniszellen hochreguliert. Die Expression von CD45RC war dagegen sehr heterogen innerhalb der verschiedenen $CD4^+$ Subpopulationen. Zusammenfassend heben diese Daten die große Variabilität der $CD4^+$ naiven und T-Gedächtnis/Effektorzellen beim Schwein hervor, die uns die verschiedenen Übergangsstadien der T-Zell-Differenzierung beim Schwein erklären könnte.

8. Abbreviations

Abs	antibodies
APCs	antigen-presenting cell
CD	Cluster of Differentiation
FCM	Flow Cytometry
FCS	Foetal Calf Serum
FSC	Forward Scatter
Ig	Immunoglobulin
LSM	Lymphocyte Separation Medium
mAbs	monoclonal antibodies
MHC	Major Histocompatibility Complex
mIgG	mouse IgG molecule
PBMCs	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
RT	room temperature
SLOs	secondary lymphoid organs
SSC	Side Scatter
Strep.	Streptavidin
TCM	central memory T cells
TCNP	cytokine-producing T cells
TCR	T-cell receptor
TEM	effector memory T cells
TEMRA	effector memory T cells re-expressing CD45RA
TN	naive T cells
TNR	naive receptor T cells
TRM	tissue-resident memory T cells
TSCM	stem cell memory T cells
TTM	transitional memory T cells
VDeF780	Viability Dye eFluor780

9. References

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10. List of figures and tables