Phenotype analysis of $CD3^+CD16^+$ lymphocytes in the peripheral blood of pigs

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Summary

The phenotype of porcine peripheral blood T cells and natural killer (NK) cells has been well-studied over the past three decades, though porcine peripheral blood lymphocytes with mixed T/NK-cell phenotype within perforin- and NKp46-positive $CD3^+$ populations have also been identified. Despite the mixed phenotype, both populations showed *in vitro* NK cell-like major histocompatibility complex-unrestricted cytolysis. In this study, the peripheral blood lymphocytes of 15 crossbreed, 12-week-old pigs of both sexes, were analysed by flow cytometry for the expression of leukocyte surface antigens (cluster of differentiation, CD) that can be found on porcine T cells (CD3, TCR-γδ and CD4), NK cells (CD16) or on both cell populations (CD8α and SLA-DR). We found the presence of a minor population of $CD3^+CD16^+$ cells within peripheral blood lymphocytes (2.84%). Peripheral blood $CD3^+CD16^+$ lymphocytes consisted of all four subpopulations with respect to the expression of surface antigens CD4 and CD8α; most were $CD4^-CD8\alpha^+$ (60.64%) and $CD4^-CD8\alpha^-$ (36.77%). While the proportion of SLA-DR^+ cells within both subpopulations was similar (8.01% of $CD3^+CD16^+CD4^-CD8\alpha^+$ lymphocytes and 7.41% of $CD3^+CD16^+CD4^-CD8\alpha^-$ lymphocytes), the proportion of TCR-$\gamma\delta^+$ cells was noticeably higher within $CD3^+CD16^+CD4^-CD8\alpha^+$ (43.48%) than $CD3^+CD16^+CD4^-CD8\alpha^-$ (16.55%) lymphocytes. When the expression of individual surface antigens was analysed on peripheral blood $CD3^+CD16^+$ lymphocytes, most were $CD8\alpha^+$ (62.44%), though some were also TCR-$\gamma\delta^+$ (32.56%), SLA-DR^+ (7.55%) or $CD4^+$ (2.59%). Expression of CD8α on $CD3^+CD16^+$ lymphocytes was not related to co-expression of other surface antigens, though most $CD3^+CD16^+$TCR-$\gamma\delta^+$ lymphocytes (81.04%) and most $CD3^+CD16^+CD4^+$ lymphocytes (69.50%) expressed CD8α. Expression of SLA-DR was not related to the co-expression of TCR-γδ or CD8α, or to the co-expression of both antigens (TCR-γδ and CD8α) on $CD3^+CD16^+CD4^-$ lymphocytes. The results also showed the presence of peripheral blood lymphocytes with the combined phenotype

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of T cells and NK cells in three-month old pigs. Though a functional analysis of the investigated cells was not performed in this study, future investigations should provide more insight about the functional properties of porcine peripheral blood $CD3^+CD16^+$ lymphocytes with distinct phenotypic characteristics, especially concerning antigen-specific responses and whether the results presented here are solely age-related.

Key words: *T cells*; *NK cells*; *swine*; *phenotype analysis*

Introduction

Among domestic animal species, particularly farm animals, swine has been the most thoroughly studied regarding leukocyte differentiation antigens (i.e. cluster of differentiation or CD antigens). Only laboratory rodents and humans have received more attention (Haverson et al., 2001). Subsequently, over the past three decades, the phenotypes of different porcine T-cell subpopulations and natural killer (NK) cells have been studied intensely (Mair et al., 2014; Gerner et al., 2015). Both T cells and NK cells of pigs can express the same surface CD antigens, such as CD2, C8α, CD27, major histocompatibility complex (MHC) class II (MHC II) and perforin, and some porcine T-cell subsets, such as gamma delta (γδ) T cells, show the function of cells of the innate immune system, like NK cells (Gerner et al., 2009). However, porcine NK cells, unlike T cells, express CD16 and never express the T-cell lineage marker CD3 (Mair et al., 2012). As in humans and cattle (Lin et al., 2006; Connelley et al., 2014), cells with mixed T/NK-cell phenotype have been also identified in pigs (Denyer et al., 2006; Mair et al., 2016). Denyer et al. (2006) showed that some peripheral blood $CD3^+CD2^+CD8\alpha^+$ lymphocytes co-expressing perforin (the cytolytic protein found on porcine NK cells and cytolytic T cells) can additionally express other porcine T-cell surface antigens, like CD5 and CD8β, but can also express the NK-cell phenotype, *i.e.* including the expression of CD11b and CD16 and the lack of expression of T-cell receptor (TCR) composed of γ and δ chains (TCR-γδ), CD4 and CD6. Mair et al. (2016) also showed expression of CD2, CD8α, CD8β, CD16 and perforin, but on peripheral blood $CD3^+$ lymphocytes that co-express NKp46, a specific receptor that can be found only on NK cells of pigs and of other domestic animal species (Mair et al., 2012). When triggered, it increases IFN-γ production and cytolytic activity. However, additional porcine T-cell surface CD antigens, such as CD6, TCR-γδ and CD27 (Mair et al., 2016) and swine leukocyte CD antigens class II (SLA II) (Lunney et al., 2009) have been identified. Despite the mixed T/NK-cell phenotype, $perforin^+CD3^+TCR\text{-}\gamma\delta^-CD2^+CD4^-CD5^+CD6^-CD8\alpha^+CD8\beta^\pm CD11b^+CD16^+$ lymphocytes or $CD3^+NKp46^+$ lymphocytes showed *in vitro* functional similarity to NK cells (MHC-unrestricted cytolysis towards human leukaemia cell line K562). Both groups of authors suggested that these subsets could belong to porcine NKT cells. Expression of CD16 was previously demonstrated on porcine peripheral blood $CD3^+$ lymphocytes. This is the Fc receptor (FcγRIII) for binding of the complement involved in antibody-dependent cell-mediated cytotoxicity (ADCC), which includes NK cells (Kacskovics, 2004). However, its expression has only been analysed within certain populations of $CD3^+$ lymphocytes.

The purpose of this study was to investigate the proportion of phenotypes within the entire population of porcine peripheral blood $CD3^+CD16^+$ lymphocytes with respect to the distribution of their surface CD antigens found on por-

cine T cells and NK cells (CD8α and SLA-II), but also of surface CD antigens found on porcine T cells (TCR-γδ and CD4), in addition to CD3.

Materials and methods

Animals

Fifteen crossbred (Large White x Swedish Landrace x German Landrace) pigs of both sexes, 12 weeks of age, and weighing approximately 25 kg were randomly selected for this study. They were weaned at 4 weeks of age, reared under standard husbandry conditions and clinically monitored during the study. Pigs remained clinically healthy throughout the study and were fed *ad libitum* with commercial pig fattening feed.

Sampling

Blood samples (6 mL) were taken by puncturing the anterior *vena cava* and collected into tubes containing anticoagulant lithium heparin. The study was conducted in accordance with ethical principles and approved by the institutional ethics committee and ethics committee of the Croatian Ministry of Agriculture.

Isolation of peripheral blood mononuclear cells

Blood samples were diluted with saline 1:1 (v/v) and peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using lymphocyte separation medium (Lymphoprep™, Alere Technologies AS, Norway). Isolated PBMCs were washed twice in RPMI 1640 medium and stored in cryoprotective medium at the temperature of liquid nitrogen.

Monoclonal antibodies for labelling PBMCs

All mAbs used were commercially available and mAbs specific for porcine CD3, CD4 and CD16 were directly

Table 1. mAbs specific for porcine leukocyte surface CD antigens and fluorochromes used for labelling of PBMCs isolated from 12-week-old pigs

Antigen	mAb clone	mAb isotype	Fluorochrome	mAb source
CD3ε	PPT3	IgG1κ	Alexa Fluor® 405[a]	Novus Biologicals, USA
TCR-δ	PGBL22A	IgG1	Alexa Fluor™ 750 - Allophycocyanin[b]	Washington State University – Monoclonal Antibody Center, USA
CD4	74-12-4	IgG2bκ	Spectral Red™ (R-phycoerithrin/cyanin 5)[a]	Southern Biotech, USA
CD8α	76-2-11	IgG2a	Alexa Fluor™ 488[c]	Washington State University – Monoclonal Antibody Center, USA
CD16	G7	IgG1κ	R-phycoerythrin[a]	Bio-Rad Laboratories, USA
SLA-DR	2E9/13	IgG2b	Allophycocyanin[d]	Bio-Rad Laboratories, USA

[a] mAb is directly conjugated with fluorochrome
[b] Zenon™AlexaFluor™ 750-Allophycocyanin (Thermo Fisher Scientific, USA) containing secondary antibodies conjugated with fluorochrome
[c] Zenon™AlexaFluor™ 488 (Thermo Fisher Scientific, USA) containing secondary antibodies conjugated with fluorochrome
[d] Lightning-Link® Allophycocyanin (APC) Conjugation Kit (Innova Biosciences, UK)

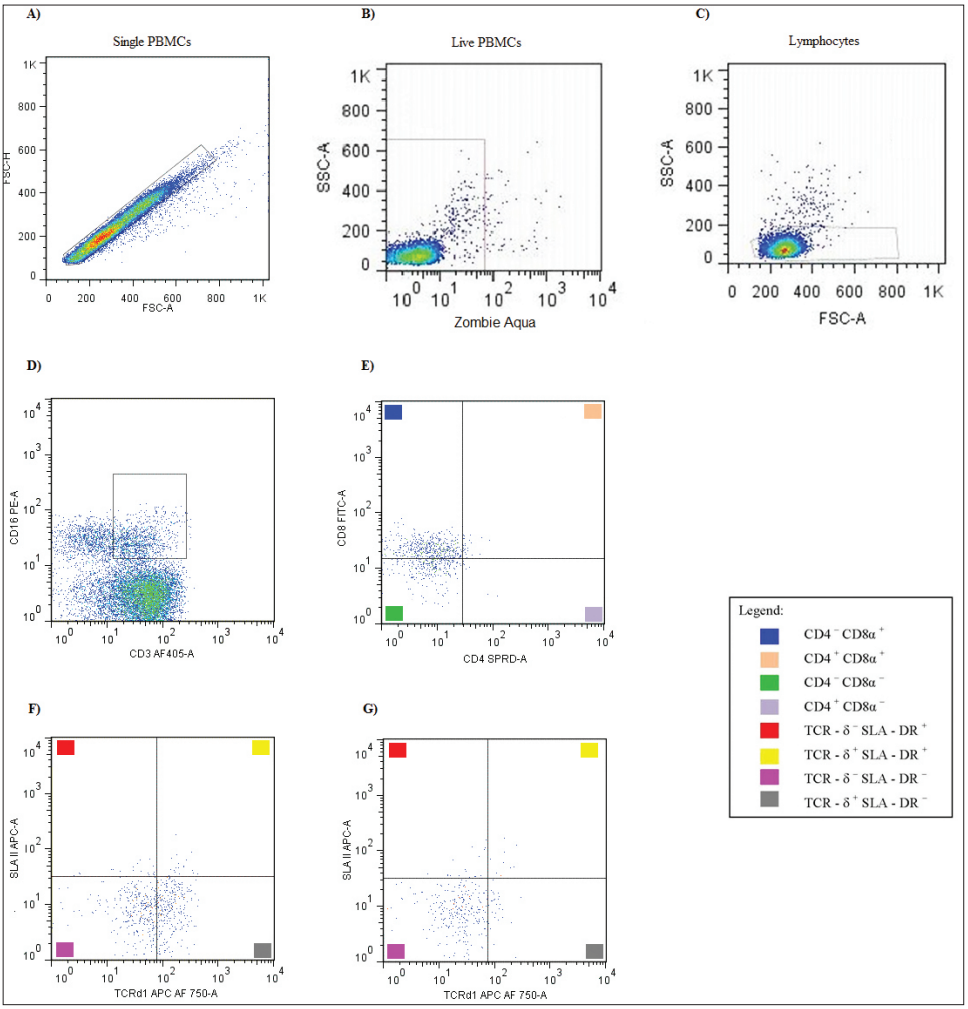

Figure 1. Gating strategy for flow cytometry analysis. Gating was first applied to (A) single PBMCs based on FSC-H (Forward Scatter-Hight) and FSC-A (Forward Scatter-Area), then to (B) live PBMCs based on labelling with Zombie Aqua™, then to (C) lymphocytes based on SSC-A (Side Scatter) and FSC-A. (D) Lymphocytes were then analysed for expression of CD3 (CD3 AF 405 - Alexa Fluor® 405) and CD16 (CD16 PE), then (E) the $CD3^+CD16^+$ subpopulation for expression of CD4 (CD4 SPRD) and CD8α (CD8α AF488), then (F) the $CD3^+CD16^+CD4^-CD8\alpha^+$ subpopulation, and (G) $CD3^+CD16^+CD4^-CD8\alpha^-$ subpopulation for expression of TCR-δ (TCR-δ APC AF750) and SLA-DR (SLA II APC)

stained with fluorochromes. Suitable commercially available labelling kits were used for staining mAbs specific for porcine TCR-δ, CD8α and SLA-DR according to the manufacturers' instructions. Detailed information about mAbs, fluorochromes and labelling kits used for labelling of PBMCs are summarized in Table 1.

Labelling of PBMCs

Isolated PBMCs were defrosted in a water bath (38 °C), washed twice in RPMI 1640 medium and counted in each sample

using a Bürker-Türk haemocytometer. The samples of PBMCs were then washed in PBS and dead cells were stained with Zombie Aqua™ Fixable Viability Dye (BioLegend, USA) according to the manufacturer's instructions. After washing in PBS, the samples were adjusted to 2.5 x 10^6 PBMCs/mL and 180 µL of each PBMC sample was transferred to FACS tubes. PBMCs were then mixed with mouse serum IgG (Invitrogen, USA) to block unspecific staining and mAbs specific for CD3, CD4, CD16 TCR-δ, CD8α and SLA-DR were added. After 30 minutes of incubation at 4 °C, the labelled PBMCs were washed in PBS and fixed with 2% (w/v) formaldehyde in PBS (pH=7.4).

Flow cytometry analysis

Flow cytometry analysis of labelled PBMCs was performed using a BD LSR II flow cytometer (BD Biosciences, USA) equipped with three lasers (405, 488 and 640 nm).

Per sample, 1 x 10^4 of live, individual lymphocytes were acquired using FACSDiva Software Version 5.0.3 (BD Biosciences). Lymphocytes were analysed for expression of CD3 and CD16, and then the $CD3^+CD16^+$ subpopulation for expression of CD4 and CD8α, and then each subpopulation ($CD3^+CD16^+CD4^-CD8\alpha^+$, $CD3^+CD16^+CD4^+CD8\alpha^+$, $CD3^+CD16^+CD4^+CD8\alpha^-$ and $CD3^+CD16^+CD4^-CD8\alpha^-$) for the expression of TCR-δ and SLA-DR. The gating strategy for flow cytometry analysis is shown in Figure 1. Raw data were analysed by Flow Jo Software Version 7.6.5 (FlowJo, LCC, USA).

Table 2. Individual and average proportion of the $CD3^+CD16^+$ subpopulation within peripheral blood lymphocytes and of peripheral blood $CD3^+CD16^+$ lymphocyte subpopulations with respect to the expression of CD4 and CD8α in the studied pigs

Pig	$CD3^+CD16^+$ (%)*	$CD3^+CD16^+$			
		$CD4^-CD8\alpha^+$ (%)**	$CD4^+CD8\alpha^+$ (%)**	$CD4^+CD8\alpha^-$(%)**	$CD4^-CD8\alpha^-$ (%)**
1	3.64	50.30	0.78	0.52	48.40
2	1.20	58.70	1.90	2.40	37.00
3	5.75	62.40	2.10	0.70	34.80
4	5.02	59.20	0.90	0.00	39.90
5	0.91	79.50	4.80	0.60	15.10
6	4.71	54.10	1.65	0.35	43.90
7	1.31	41.70	1.63	0.97	55.70
8	3.38	66.40	2.00	0.60	31.00
9	3.06	55.20	1.46	0.74	42.60
10	1.87	80.30	0.90	0.00	18.80
11	3.28	85.90	1.30	0.30	12.50
12	1.94	48.40	1.80	2.20	47.60
13	3.70	52.00	1.20	0.30	46.50
14	1.49	52.10	2.50	1.60	43.80
15	1.36	63.40	2.15	0.55	33.90
Average	2.84	60.64	1.80	0.79	36.77

* Proportion in peripheral blood lymphocytes
** Proportion in the peripheral blood $CD3^+CD16^+$ lymphocyte subpopulation

Results and Discussion

Table 3. Individual and average proportion of peripheral blood $CD3^+CD16^+CD4^-CD8\alpha^+$ lymphocyte subpopulations with respect to the expression of TCR-γδ and SLA-DR in the studied pigs

Pig	$CD3^+CD16^+CD4^-CD8\alpha^+$			
	TCR-$\gamma\delta^-$SLA-DR^+ (%)*	TCR-$\gamma\delta^+$SLA-DR^+ (%)*	TCR-$\gamma\delta^+$SLA-DR^-(%)*	TCR-$\gamma\delta^-$SLA-DR^- (%)*
1	2.30	4.90	43.60	49.20
2	2.40	10.70	58.20	28.70
3	2.85	3.35	45.20	48.60
4	1.20	1.20	18.30	79.30
5	9.85	9.85	22.00	58.30
6	5.26	8.14	33.00	53.60
7	2.35	6.25	34.40	57.00
8	1.90	2.10	40.30	55.70
9	1.10	1.60	26.90	70.40
10	3.70	1.50	39.90	54.90
11	3.10	4.30	40.70	51.90
12	5.74	9.46	40.50	44.30
13	2.60	2.30	39.70	55.40
14	2.45	1.85	35.00	60.70
15	0.80	5.10	61.90	32.20
Average	3.17	4.84	38.64	53.35

* Proportion in the peripheral blood $CD3^+CD16^+CD4^-CD8\alpha^+$ lymphocyte subpopulation

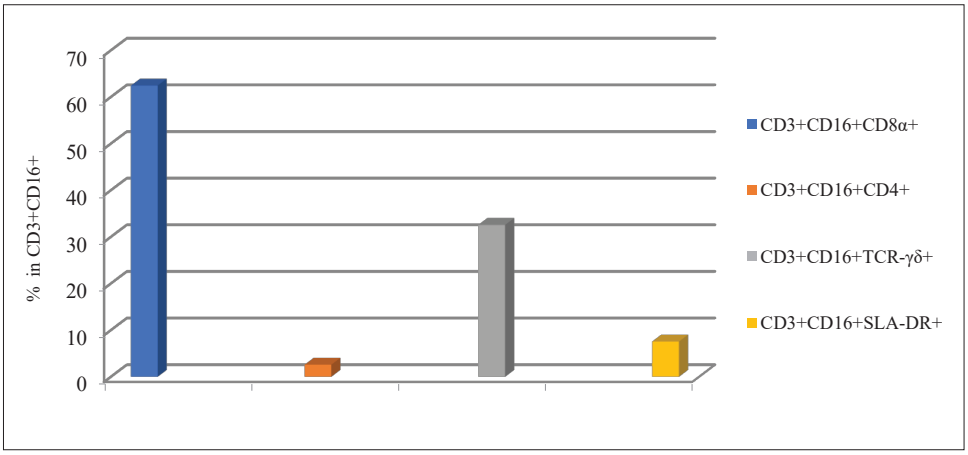

Figure 2. Average proportion of peripheral blood $CD3^+CD16^+$ lymphocyte subpopulations expressing CD8α, CD4, TCR-γδ or SLA-DR within peripheral blood $CD3^+CD16^+$ lymphocytes of the studied pigs

Table 4. Individual and average proportion of peripheral blood CD3+CD16+CD4-CD8α- lymphocyte subpopulations with respect to the expression of TCR-γδ and SLA-DR in the studied pigs

Pig	CD3+CD16+CD4-CD8α-			
	TCR-γδ-SLA-DR+ (%)*	TCR-γδ+SLA-DR+ (%)*	TCR-γδ+SLA-DR- (%)*	TCR-γδ-SLA-DR- (%)*
1	3.25	2.95	12.60	81.20
2	10.40	3.90	6.50	79.20
3	4.60	2.80	10.20	82.40
4	1.02	1.18	11.40	86.40
5	4.00	8.00	16.00	72.00
6	5.70	2.55	5.85	85.90
7	5.84	0.58	4.68	88.90
8	5.00	1.82	6.38	86.80
9	3.15	2.55	6.30	88.00
10	3.10	0.00	25.00	71.90
11	9.55	9.55	10.70	70.20
12	3.75	2.75	17.20	76.30
13	1.30	0.35	3.25	95.10
14	2.19	2.91	30.70	64.20
15	1.60	4.80	34.90	58.70
Average	4.30	3.11	13.44	79.15

* Proportion in peripheral blood CD3+CD16+CD4-CD8α- lymphocyte subpopulation

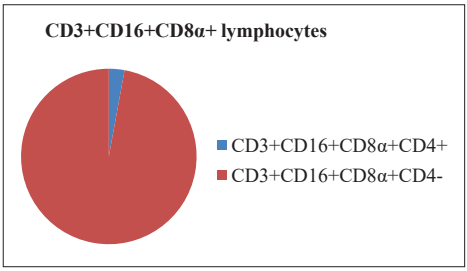

Figure 3. Average proportion of CD3+CD16+CD8α+CD4+ and CD3+CD16+CD8α+CD4- subpopulations within the peripheral blood CD3+CD16+CD8α+ lymphocytes of the studied pigs

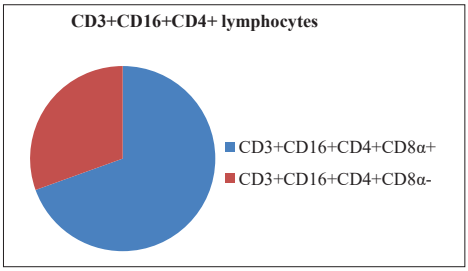

Figure 4. Average proportion of CD3+CD16+CD4+CD8α+ and CD3+CD16+CD4+CD8α- subpopulations within the peripheral blood CD3+CD16+CD4+ lymphocytes of the studied pigs

The proportion of the CD3+CD16+ subpopulation within peripheral blood lymphocytes as obtained in our study was between 0.91% and 5.75% (2.84% in average, Table 2). These findings correspond more to the distribution of NK

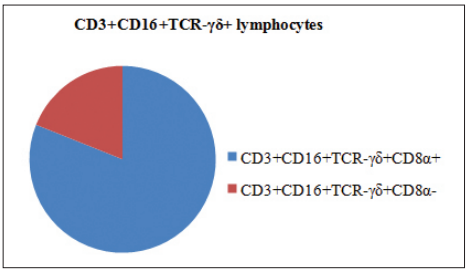

Figure 5. Average proportion of $CD3^+CD16^+TCR\text{-}\gamma\delta^+CD8\alpha^+$ and $CD3^+CD16^+TCR\text{-}\gamma\delta^+CD8\alpha^-$ subpopulations within the peripheral blood $CD3^+CD16^+TCR\text{-}\gamma\delta^+$ lymphocytes of the studied pigs

cells than T cells in the peripheral blood of slightly older pigs as reported in the literature (Stepanova et al., 2007; Mair et al., 2012). In the current study, peripheral blood $CD3^+CD16^+$ lymphocytes consisted of all four subpopulations with respect to the expression of surface antigens CD4 and CD8α (Table 2). Most were $CD4^-CD8\alpha^+$ (60.64% on average) and $CD4^-CD8\alpha^-$ (36.77% on average), while proportions of $CD4^+CD8\alpha^+$ and $CD4^+CD8\alpha^-$ subpopulations were low (1.80% and 0.79% on average). Analysis of surface antigens TCR-γδ and SLA-DR on peripheral blood $CD3^+CD16^+CD4^-CD8\alpha^+$ and $CD3^+CD16^+CD4^-CD8\alpha^-$ lymphocytes showed different distribution patterns (Tables 3 and 4). The proportion of the $TCR\text{-}\gamma\delta^+$ subpopulation was noticeably higher within $CD3^+CD16^+CD4^-CD8\alpha^+$ lymphocytes than within $CD3^+CD16^+CD4^-CD8\alpha^-$ lymphocytes (43.48% *vs* 16.55% on average) and the proportion of the $TCR\text{-}\gamma\delta^-$ subpopulation was noticeably lower within $CD3^+CD16^+CD4^-CD8\alpha^+$ lymphocytes than $CD3^+CD16^+CD4^-CD8\alpha^-$ lymphocytes (56.52% *vs* 83.45% on average). On the contrary, the proportion of $SLA\text{-}DR^+$ and $SLA\text{-}DR^-$ subpopulations within $CD3^+CD16^+CD4^-CD8\alpha^+$ lymphocytes (8.01% *vs* 91.99% on average, Table 3) and within $CD3^+CD16^+CD4^-CD8\alpha^-$ lymphocytes (7.41% *vs* 92.59% on average, Table 4) was similar.

The present study also analysed the expression of individual surface antigens (CD8α, CD4, TCR-γδ and SLA-DR) on porcine peripheral blood $CD3^+CD16^+$ lymphocytes. The surface antigen CD8 on cytolytic T cells is a coreceptor for MHC class I (MHC I) antigens, and it is important to this cell subset for the recognition of endogenous antigens presented in complex with MHC I (Tizard, 2000). However, apart from porcine cytolytic alpha beta (αβ) T cells ($CD3^+TCR\text{-}\gamma\delta^-$), this surface antigen can be also found on other porcine T cells (helper αβ T cells and γδ T cells) and NK cells (Yang and Parkhouse, 1997; Piriou-Guzylack and Salmon, 2008; Summerfield and McCullough, 2009; Šinkora and Butler, 2009). In this study, CD8 with an α chain (CD8α) was also found on the majority of peripheral blood $CD3^+CD16^+$ lymphocytes (> 50% in 14/15 pigs) or 62.44% on average (Table 2, Figure 2). These values are lower than those obtained by Mair et al. (2016). However, they did not analyse the whole population of peripheral blood $CD3^+CD16^+$ lymphocytes (but only $NKp46^+$) and they used slightly older pigs than in this study. Surface antigen CD8α is not expressed on all porcine T cells, such as γδ T cells ($CD3^+TCR\text{-}\gamma\delta^+$) and $CD4^+$ αβ T cells (Gerner et al., 2009; Šinkora and Butler, 2009; Talker et al., 2013), but according to most authors, all porcine NK cells express CD8α (Denyer et al., 2006; Mair et al., 2012). It is not yet clear whether our results are representative only for three-month old pigs, or whether the expression of CD8α on peripheral blood $CD3^+CD16^+$ lymphocytes increases with age, as was found for helper ($CD4^+$) αβ T cells (Zuckermann and Husmann, 1996; Talker et al., 2013). However, our findings suggest that the expression of CD8α on $CD3^+CD16^+$ lymphocytes more closely resembles the phenotype pattern of porcine T cells than NK cells.

Even though Denyer et al. (2006) and Mair et al. (2016) did not find the expression of CD4 on peripheral blood perforin+CD3+CD16+ lymphocytes or on peripheral blood CD3+NKp46+ lymphocytes (which were mostly CD16+), our study that analysed the whole peripheral blood CD3+CD16+ lymphocyte population, showed the expression of CD4 in all studied pigs, though in a low percentage (2.59% on average, Table 2, Figure 2). Surface antigen CD4 is a receptor for MHC II antigens and it is important to helper T cells for the recognition of processed antigens and their presentation in the MHC II complex (Tizard, 2000). This surface antigen can also be expressed on porcine regulatory T cells and plasmacytoid dendritic cells in peripheral blood (Summerfield and McCullough, 2009; Käser et al., 2011). Whether CD4 has the same function on CD3+CD16+ lymphocytes as on helper T cells is not clear. Even though a strong majority of the CD3+CD16+CD8α+ lymphocytes in this study did not express CD4 (97.12% on average, Figure 3), it appears that CD3+CD16+ lymphocytes were phenotypically more similar to T cells with respect to the expression of CD4, as this surface antigen is absent from porcine NK cells (De Bruin et al., 1997; Denyer et al., 2006). Another similarity between porcine peripheral blood T cells and CD3+CD16+ lymphocytes in this study was the presence of the CD4-CD8α- subset (Table 2), which was not found on porcine peripheral blood NK cells (Gerner et al., 2009). Most CD3+CD16+CD4-CD8α- lymphocytes in this study were also TCR-γδ- and SLA-DR- (Table 4). As in the porcine peripheral blood helper CD4+ T-cell population, CD8α was not expressed on all CD4-positive CD3+CD16+ lymphocytes. However, co-expression of CD8α was found on most CD3+CD16+CD4+ lymphocytes (69.50% on average, Figure 4).

Similar to the findings of Mair et al. (2016), we have also found expression of TCR-γδ, though on the whole population of peripheral blood CD3+CD16+ lymphocytes and in a higher proportion (32.56% on average, Figure 2). This result was similar to the distribution pattern of TCR-γδ+ cells in the population of peripheral blood CD3+ lymphocytes for slightly younger (Štěpánová and Šinkora, 2012) and older pigs (Sedlak et al., 2014). Even though TCR is responsible for antigen recognition (Yang et al., 2005), previous studies have shown that porcine γδ T cells mostly possess functions of cells of the innate immune system (Yang and Parkhouse, 1997; De Bruin et al., 2000). The antigen-specific immune functions of T cells have mostly been demonstrated for porcine CD3+TCR-γδ- T cells, *i.e.* αβ T cells, as the direct identification of porcine TCR composed of α and β chains (TCR-αβ) is not yet possible to the best of our knowledge. However, Mair et al. (2016) showed that CD3+NKp46+ lymphocytes functionally resemble NK cells despite the low percentage of TCR-γδ+ cells. It is not yet clear whether the expression of TCR-γδ, and the possible expression of TCR-αβ on peripheral blood CD3+CD16+ lymphocytes is involved in antigen-specific responses. In this study, the expression of TCR-γδ was reliably found on CD3+CD16+CD4- lymphocytes and was mostly related to the expression of CD8α on CD4-CD3+CD16+ lymphocytes. On average, 81.04% of CD3+CD16+TCR-γδ+ lymphocytes were also CD8α+, while the rest were CD8α- (Figure 5). These findings are similar to those obtained for porcine peripheral blood γδ T cells (Talker et al., 2013).

Previous studies have shown that the SLA II antigens can be found on many porcine PBMC subsets, including αβ T cells, γδ T cells, NK cells and B cells (Gerner et al., 2009; Šinkora and Butler, 2009; Talker et al., 2013). Our results also showed the expression of SLA-DR (one of

the SLA II molecules, Lunney et al., 2009) on porcine peripheral blood $CD3^+CD16^+$ lymphocytes, though in a low proportion (on average 7.55% of $CD3^+CD16^+$ lymphocytes, Figure 2).

Even though the function of SLA II expression on both αβ T cell subpopulations (helper and cytolytic T cells) and on γδ T cells in peripheral blood of pigs is not clear, it has been suggested that the expression of SLA II, in combination with the expression of CD8α, is a phenotype characteristic of activated/memory helper ($CD4^+$) T cells (Saalmüller et al., 2002) and activated γδ T cells (Šinkora et al., 2005). Expression of SLA II was also found mostly on porcine helper and cytolytic αβ T cells and on γδ T cells expressing CD8α in peripheral blood (Reutner et al., 2013; Talker et al., 2013). However, in this study, SLA-DR was reliably found on $CD3^+CD16^+CD4^-$ lymphocytes, though it was not related to the expression of TCR-γδ or CD8α, because it was found at similarly low levels on $CD3^+C16^+CD4^-$ lymphocytes expressing $CD8\alpha^+$TCR-$\gamma\delta^+$, $CD8\alpha^+$TCR-$\gamma\delta^-$, $CD8\alpha^-$TCR-$\gamma\delta^+$ and $CD8\alpha^-$ TCR^- (Figures 4 and 5). These results indicate that $CD3^+CD16^+$ lymphocytes were equally similar to porcine γδ T cells and NK cells with respect to the expression of SLA-DR (SLA II), because the expression of SLA-DR on peripheral blood NK cells was also previously found in pigs of the same age (Talker et al., 2013) or in slightly older pigs (Pintarič et al., 2008). Expression of TCR-γδ and SLA-DR was also analysed on $CD3^+CD16^+CD4^+CD8\alpha^+$ and $CD3^+CD16^+CD4^+CD8\alpha^+$ subpopulations of peripheral blood lymphocytes. Analysis showed that vast majority of $CD3^+CD16^+CD4^+CD8\alpha^+$ and $CD3^+CD16^+CD4^+CD8\alpha^+$ were TCR-$\gamma\delta^-$ and $SLA\text{-}DR^-$ (data not shown).

This study also showed a mixed T/NK-cell phenotype of porcine peripheral blood $CD3^+CD16^+$ lymphocytes. However, unlike Denyer et al. (2006) and Mair et al. (2016), within the whole population of $CD3^+CD16^+$ lymphocytes, we found a small population of $CD4^+$ cells which were either $CD8\alpha^+$ or $CD8\alpha^-$, like the porcine peripheral blood $CD4^+$ αβ T cells (Gerner et al., 2009). The phenotype and functions of porcine NKT cells have recently been under intense investigation, mainly $CD1d^+$ invariant NKT cells (Schäfer et al., 2019; Yang et al., 2019).

Further investigations should provide deeper insight into the functional properties of porcine peripheral blood $CD3^+CD16^+$ lymphocytes with distinct phenotypic characteristics, especially regarding their antigen-specific responses, and whether or not the results presented here are only age-related.

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Analiza fenotipa $CD3^+CD16^+$ limfocita periferne krvi svinja

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Fenotip T limfocita i NK stanica (engl. *Natural Killer Cells*) periferne krvi svinja dobro je istražen tijekom zadnja tri desetljeća. Međutim, unutar subpopulacija limfocita periferne krvi svinja koje iskazuju površinski antigen CD3 i perforin ili receptor NKp46 ustvrđeni su i limfociti s mješovitim fenotipom T limfocita i NK stanica. Unatoč mješovitom fenotipu, obje subpopulacije limfocita su *in vitro* pokazale citolitički učinak koji nije bio usmjeren na molekule glavnog sustava tkivne podudarnosti, odnosno citolitički učinak sličan NK stanicama. U našem istraživanju protočnom citometrijom analizirani su limfociti periferne krvi od petnaest svinja oba spola, križane pasmine i u dobi dvanaest tjedana, s obzirom na iskazivanje površinskih antigena koji mogu biti prisutni na T limfocitima (CD3, TCR-γδ i CD4), na NK stanicama (CD16) ili na obje navedene populacije stanica svinja (CD8α i SLA-DR). Naše je istraživanje pokazalo da limfociti periferne krvi sadržavaju manji udio subpopulacije koja iskazuje površinske antigene CD3 i CD16 (2,84%). Limfociti periferne krvi s fenotipom $CD3^+CD16^+$ sastojali su se od sve četiri subpopulacije s obzirom na iskazivanje površinskih antigena CD4 and CD8α, od kojih je većina bila $CD4^-CD8\alpha^+$ (60,64%) i $CD4^-CD8\alpha^-$ (36,77%). Iako je udio stanica koje iskazuju SLA-DR u obje navedene subpopulacije bio sličan (8,01 % od $CD3^+CD16^+CD4^-CD8\alpha^+$ limfocita i 7,41 % od $CD3^+CD16^+CD4^-CD8\alpha^-$ limfocita), udio stanica koje iskazuju TCR-γδ je bio primjetno veći u $CD3^+CD16^+CD4^-CD8\alpha^+$ limfocitima (43,48 %), nego u $CD3^+CD16^+CD4^-CD8\alpha^-$ limfocitima (16,55 %). Analiza iskazivanja pojedinih površinskih antigena na $CD3^+CD16^+$ limfocitima periferne krvi pokazala je da ih je većina bila $CD8\alpha^+$ (62,44 %), ali također i TCR-$\gamma\delta^+$ (32,56 %), SLA-DR^+ (7,55 %) i $CD4^+$ (2,59 %). Iskazivanje površinskog antigena CD8α na $CD3^+CD16^+$ limfocitima nije bilo povezano s iskazivanjem drugih površinskih antigena. Međutim, većina $CD3^+CD16^+$TCR-$\gamma\delta^+$ limfocita (81,04 %) i većina $CD3^+CD16^+CD4^+$ limfocita (69,50 %) iskazivali su i površinski antigen CD8α. Iskazivanje molekule SLA-DR nije bilo povezano s iskazivanjem površinskog antigena TCR-γδ ili CD8α, niti s oba površinska antigena (TCR-γδ i CD8α) na $CD3^+CD16^+CD4^-$ limfocitima. Naše je istraživanje pokazalo i prisutnost limfocita s kombiniranim fenotipom T limfocita i NK stanica u perifernoj krvi svinja, u dobi od tri mjeseca, međutim funkcija navedenih stanica nije istraživana. Stoga se nadamo se da ćemo budućim istraživanjima moći pojasniti jesu li dobiveni rezultati vezani uz dob svinja. Nadalje, očekujemo da će dodatna istraživanjima omogućiti bolje spoznaje o funkcijama pojedinih subpopulacija $CD3^+CD16^+$ limfocita periferne krvi svinja različitog fenotipa, posebice o odgovoru navedenih stanica specifičnom za određeni antigen.

Ključne riječi: *T limfociti*, *NK stanice*, *svinja*, *analiza fenotipa*