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Impaired T-cell responses in domestic pigs and wild boar upon infection with a highly virulent African swine fever virus strain

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Abstract

Since African swine fever (ASF) first appeared in the Caucasus region in 2007, it has spread rapidly and is now present in numerous European and Asian countries. In Europe, mainly wild boar populations are affected and pose a risk for domestic pigs. In Asia, domestic pigs are almost exclusively affected. An effective and safe vaccine is not available, and correlates of protection are far from being understood. Therefore, research on immune responses, immune dysfunction and pathogenesis is mandatory. It is acknowledged that T cells play a pivotal role. Thus, we investigated T-cell responses of domestic pigs and wild boar upon infection with the highly virulent ASF virus (ASFV) strain 'Armenia08'. For this purpose, we used a flow cytometry-based multicolour analysis to identify T-cell subtypes (cytotoxic T cells, T-helper cells, γδ T cells) and their functional impairment in ASFV-infected pigs. Domestic pigs showed lymphopaenia, and neither in the blood nor in the lymphoid organs was a proliferation of $CD8^+$ effector cells observed. Furthermore, a T-bet-dependent activation of the remaining CD8 T cells did not occur. In contrast, a T-cell response could be observed in wild boar at 5 days post-inoculation in the blood and in tendency also in some organs. However, this cytotoxic response was not beneficial as all wild boars showed a severe acute lethal disease and a higher proportion died spontaneously or was euthanized at the humane endpoint.

KEYWORDS

African swine fever virus, domestic pig, experimental infection, T-cell response, wild boar

1 | INTRODUCTION

African swine fever (ASF) is one of the most devastating diseases of domestic pigs and wild boar with lethality rates up to 100% (Kleiboeker, 2002; Penrith & Vosloo, 2009). The ASF virus (ASFV), a large double-stranded DNA virus, is the only known member of the *Asfarviridae* family (Alonso et al., 2018; Dixon et al., 2005). Since its onset in the Caucasus and Russia in 2007, ASF has spread widely and now affects several European countries. In autumn 2018, the virus also reached China, the world's largest pig producer, and spread subsequently to several Asian countries (Dixon, Sun, & Roberts, 2019). Within Europe, the abundant wild boar population plays an important role in the maintenance of ASFV. Wild boars serve as a reservoir for ASFV and present a risk factor for the

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introduction into the domestic pig population (Jori & Bastos, 2009; Pietschmann et al., 2016).

To date, no effective vaccine is available. Moreover, previous studies demonstrated dysregulated immune responses, which are still poorly understood. ASFV encodes several factors that mediate immune modulation and evasion (Dixon, Islam, Nash, & Reis, 2019). Multiple genes in the ASFV genome encode for type I interferon and apoptosis inhibitors during early stages of infection (Dixon, Islam, et al., 2019). However, infected macrophages might also induce TNF-α-mediated apoptosis in bystander lymphocytes during later stages (Dixon, Islam, et al., 2019).

Professional antigen-presenting cells (APCs), such as macrophages and dendritic cells, are the main targets for ASFV (Franzoni, Dei Giudici, & Oggiano, 2018). Consequently, the ability of APCs to present antigen could be disturbed and the induction of an effective immune response might be impaired (Franzoni, Graham, et al., 2018; Galindo & Alonso, 2017; Sanchez-Torres et al., 2003). Additionally, reduction in lymphocyte counts because of virus-mediated apoptosis is a well-known characteristic during ASFV infections (Dixon, Sanchez-Cordon, Galindo, & Alonso, 2017; Oura, Powell, Anderson, & Parkhouse, 1998; Ramiro-Ibanez, Ortega, Ruiz-Gonzalvo, Escribano, & Alonso, 1997). Besides, little is known about the mechanisms of the immune response against ASFV. Cytokines and chemokines are important factors orchestrating the immune system's response against viral infections. For ASF, an involvement of cytokines (TNF-α, IFN-α, IL-12, IL-1β) in immunopathology, especially tissue destruction, is described (Lacasta et al., 2015). Additionally, some authors found a correlation of protection against ASFV infections with an increase in IFN-γ, although the cellular source remained unknown (King et al., 2011; Sanchez-Cordon, Montoya, Reis, & Dixon, 2018). However, other authors could not confirm the correlation of these responses (Carlson et al., 2016). The cytokine profile of infected macrophages differs depending on the ASFV strain virulence (Franzoni et al., 2017). Unfortunately, the number of in vivo studies on ASFV infection, which could further investigate those questions, is limited (King et al., 2003; O'Donnell et al., 2015; Reis et al., 2016). Whether antibodies generated against ASFV proteins are neutralizing remains a matter of controversy. It was demonstrated that antibodies alone cannot protect from ASFV. Moreover, they are not predictive for disease outcome (Escribano, Galindo, & Alonso, 2013; Neilan et al., 2004; Onisk et al., 1994; Schlafer, Mebus, & McVicar, 1984; Zsak, Onisk, Afonso, & Rock, 1993).

T cells—especially $CD8\alpha^+$ T cells—play an important role in protective immunity against ASFV (Oura, Denyer, Takamatsu, & Parkhouse, 2005). After exposure to the avirulent ASFV strain OUR/T88/3, pigs were depleted of $CD8\alpha^+$ lymphocytes with monoclonal anti-CD8 antibodies. A subsequent challenge with homologous but virulent OUR/T88/1 revealed that depleted animals suffered from severe acute ASF and died, whereas undepleted animals showed only mild clinical signs and survived. Since CD8α is expressed on different T-cell populations such as cytotoxic T cells (CTL), γδ T cells, NK cells, invariant T cells or memory helper T cells (Saalmüller, Pauly, Hohlich, & Pfaff, 1999; Schäfer et al., 2019; Yang & Parkhouse, 1997), it remains unclear which of these subpopulations might mediate the described protection. Most of the findings on the immune response against ASFV were obtained from in vitro studies, often limited to the blood of infected animals. As antigen contact, antigen presentation and T-cell activation take place in lymphoid organs, investigation of these lymphoid tissues is indispensable. Furthermore, only little is known about the kinetics of the immune response, neither in blood nor in lymphoid organs, and whether T cells other than cytotoxic $CD8^+$ cells are involved in the immune response. Finally, it remains to be elucidated whether the disease is due to a failure to mount protective immunity or even displays an immunopathologic process. These gaps in knowledge highlight the necessity to understand ASFV modulation of immune responses, especially since increased efforts to develop new vaccine technologies still have not been successful. The aim of this study was to investigate the significance of the cellular immune response in more detail, in order to clarify which cell components are affected during ASFV infection. Therefore, we have chosen a comparative approach in which we inoculated domestic pigs and wild boar with the highly virulent ASFV 'Armenia08' in order to investigate the phenotype of different T-cell populations in blood and lymphatic organs as well as their kinetics and functionality in the course of the infection.

2 | MATERIALS AND METHODS

2.1 | Experimental design

The study included 16 German landrace pigs of both sexes aged three months at the start of the trial and 16 subadult/adult European wild boars aged 1–2 years. All wild boars were clinically unremarkable and were treated with anti-parasitics. Domestic pigs were obtained from a commercial pig farm, and the wild boars were provided by wildlife parks in Mecklenburg-Western Pomerania. For the experiment, the animals were transferred into the high containment facilities of the Friedrich-Loeffler-Institut (L3+). After one week of acclimatization, 12 domestic pigs and 12 wild boars were oronasally inoculated with 2 ml cell culture supernatant containing $10^{6.25}$ haemadsorbing units (HAU)/ml of ASFV 'Armenia08'. Four domestic pigs and wild boars each were separated prior to infection, remained untreated and served as controls.

Clinical signs of domestic pigs and wild boars were recorded daily over the course of the trial. Rectal temperatures were only measured in domestic pigs in order to avoid frequent immobilization of wild boars. Fever was defined as a body temperature over 40°C for at least two consecutive days. Based on the adapted scoring system by Mittelholzer *et al.* (Mittelholzer, Moser, Tratschin, & Hofmann, 2000), the clinical score was evaluated as previously described (Petrov, Forth, Zani, Beer, & Blome, 2018). It comprised the parameters liveliness, bearing, breathing, gait, skin, eyes, faeces and feed uptake, reaching from 0 (asymptomatic) to 3 points each

(severe). The sum of the points was recorded as a clinical score. Fifteen score points were defined as humane endpoint. Five and 7 days post-infection (dpi), four domestic pigs and four wild boars were euthanized by intracardial injection of pentobarbital (Release, Wirtschaftsgenossenschaft deutscher Tierärzte) after deep anaesthesia with tiletamine/zolazepam (Zoletil ®, Virbac) and xylazine (Rompun® 2%, Bayer HealthCare). Necropsy was performed on all animals. For immunological investigations and to assess levels of viraemia, blood, lymphoid tissue from spleen, gastro-hepatic lymph nodes (ghLN, *Lymphonodi hepatici* or *gastrici*) and liver were collected.

2.2 | Virus detection

For virus back titration of the culture supernatant used for infection, the haemadsorption test (HAT) was performed by endpoint titration on macrophages derived from peripheral blood monocytic cells of healthy donor pigs as previously described (Pietschmann et al., 2015).

All tissue samples were homogenized using a TissueLyser II (Qiagen) at 30 Hz for 3 min in a 2-ml reaction tube in the presence of one 5-mm stainless steel bead and 300 µl sterile PBS. For qPCR, viral DNA was extracted from tissue samples using the NucleoMag VET Kit (Macherey-Nagel) and KingFisher® extraction platform (Thermo Scientific). The qPCR analysis was performed on a CFX96 Real-Time PCR Detection System (Bio-Rad) according to the assay previously described (King et al., 2003), with slight modifications (addition of a heterologous internal control). Genome copy numbers were estimated using an in-house standard. Standard was prepared as followed: A culture of peripheral blood mononuclear cell (PBMC)-derived macrophages was infected with ASFV strain 'Armenia08' and incubated at 37°C in a humidified atmosphere containing 5% CO2 for 72 hr. Following a freeze–thaw cycle, the cell culture supernatant was collected and cleared by centrifugation at 650 x g for 10 min. Viral nucleic acids were extracted from the supernatant using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. Subsequently, the DNA concentration was determined by spectrophotometry using a NanoDrop 2000c (Thermo Fisher Scientific, Schwerte, Germany) and the exact number of DNA molecules was calculated using open-access online tools (http://molbiol.edu.ru/eng/scripts/01_07.html).

2.3 | Haematological analyses

Differential leucocyte counts were determined from fresh EDTA blood samples using an automated haematological analyzer (ProCyte Dx Hematology Analyzer, IDEXX). The data were used to calculate the total number of lymphocyte subpopulations using the frequencies obtained by flow cytometry.

2.4 | Preparation of single-cell suspensions

Single-cell suspensions from spleen and ghLN were generated by mechanically disrupting tissue with a steel strainer. Lymphocytes from liver tissue were isolated after perfusion with ice-cold PBS-EDTA. Subsequently, perfused regions were minced and resuspended in PBS-EDTA supplemented with 100 µM $CaCl_2$ and digested with collagenase D (1 mg/ml; Sigma-Aldrich) for 40 min at 37°C. The remaining tissue was removed via short centrifugation, and the cell pellet was resuspended in PBS-EDTA and used for flow cytometry.

2.5 | Flow cytometry

At indicated time points, 50 µl whole blood and 50 µl single-cell suspensions (corresponding to approximately 1×10^6 cells) of spleen, ghLN and liver were stained for flow cytometric analyses. All incubation steps with monoclonal antibodies (mAbs) targeting extracellular antigens were carried out for 15 min at 4°C in the dark. Between each antibody staining, a washing step was performed. Before intracellular labelling, erythrocytes in blood samples were lysed with red blood cell lysis buffer (1.55 M NH_4Cl, 100 mM $KHCO_3$, 12.7 mM Na_4EDTA, pH 7.4, in *Aqua destillata*). Subsequently, samples were fixed and permeabilized with the True-Nuclear Transcription Factor Buffer Set (BioLegend, USA) according to the manufacturer's instructions. All incubation steps for intracellular staining were carried out for 30 min at 4°C in the dark. Antibodies and conjugates used for flow cytometry are shown in Table 1. Appropriate controls (isotype and fluorescence minus one, FMO) were used throughout the establishment of the stainings. Anti-pig antibodies were used for wild boar as well, because both subspecies (wild boar, *Sus scrofa scrofa*, and domestic pig, *Sus scrofa domesticus*) exhibit high genetic consensus (Groenen et al., 2012). Moreover, anti-pig antibodies have successfully been used by other groups (Beltran-Beck et al., 2014; Cabezon et al., 2017). Flow Cytometer BD FACS Canto II with FACS DIVA Software (BD Bioscience) was used for all analyses. Living lymphocytes were gated based on their forward-scatter (FSC) and side-scatter (SSC) properties and further separated into different T-cell subpopulations. At least 10,000 live, single lymphocytes were acquired.

2.6 | Statistical analysis

GraphPad Prism7 (GraphPad Software Inc.) was used for statistical analysis and graph creation. Normality was verified using the Shapiro–Wilk test. To investigate statistically significant differences between infected and uninfected animals, ordinary one-way ANOVA with Dunnett's correction for multiple comparisons was used. Since both species displayed different cell frequencies even before the infection, differences between both species during the study period

TABLE 1 Antibodies used in this study

Marker	Clone	Isotype	Conjugate	Source	Dilution
CD2	MSA4	Mouse IgG2a	—	In-house	1:100
CD3ε	PPT3	Mouse IgG1	PE	Southern Biotech	1:500
CD3ε	PPT3	Mouse IgG1	—	In-house	
CD4a	74-12-4	Mouse IgG2b	PerCp-Cy5.5	BD	1:100
CD8a	76-2-11	Mouse IgG2a	FITC	Southern Biotech	1:100
CD8b	PG164A	Mouse IgG2a	—	In-house	1:1,000
CD79a	HM47	Mouse IgG1	PE	ebioscience	1:200
CD172a	74-22-15	Mouse IgG1	—	In-house	1:100
EOMES	WD1928	Mouse IgG1	PE	Invitrogen	1:50
Foxp3	236A/E7	Mouse IgG1	Biotin	ebioscience	1:200
gdTCR	PPT16	Mouse IgG2b	—	In-house	1:100
Ki-67	B56	Mouse IgG1	BV421	BD Biosciences	1:40
Perforin	dG9	Mouse IgG2b	Alexa 647	BioLegend	1:20
T-bet	4B10	Mouse IgG1	APC	BioLegend	1:500
IgG1	RMG1–1	Rat IgG	APC-Cy7	BioLegend	1:250
IgG1	RMG1–1	Rat IgG	BV421	BioLegend	1:400
IgG2a	Polyclonal	Goat IgG	APC-Cy7	Southern Biotech	1:250
IgG2a	Polyclonal	Goat IgG	APC	Jackson Immuno	1:1,000
IgG2b	Polyclonal	Goat IgG	PE-Cy7	Southern Biotech	1:400
Streptavidin	—	—	BV510	BioLegend	1:1,000

were not tested. Survival was analysed using the Mantel–Cox test. Each dot represents one animal with a bar indicating mean. Statistical significance was defined as $p < .05$ (*).

3 | RESULTS

3.1 | Clinical course

Back titration of the virus suspension used for inoculation verified the administered titre of $1 \times 10^{6.25}$ HAU/ml (data not shown). All tissue samples from inoculated domestic pigs and wild boars were positive for ASFV genome in qPCR. Subsequent to oronasal inoculation, domestic pigs developed severe clinical signs. Parallel to the onset of fever 5 dpi, loss of appetite, general depression and huddling were observed. Haemorrhages at the tips of the ears and lower legs of domestic pigs as well as conjunctivitis occurred in the final phase of the experiment. Similar signs of disease were observed in infected wild boars (depression, loss of appetite). Comparison of clinical signs among domestic pigs and wild boars was hampered by the fact that wild boar conceal disease by minimized sickness behaviour more than domestic pigs (Tizard, 2008). Moreover, assessment of clinical signs, that is fever, in wild boar, would have required to narcotize the animals. Such severe manipulations would have distorted the overall outcome and were, therefore, omitted. It has to be taken into account that survival kinetics alone do not represent the clinical course in the animals, especially because 4 animals each were randomly selected for necropsy 5 dpi and 7 dpi. Given the small group size, it cannot be ruled out that some of the animals euthanized for necropsy would have survived the infection. Still, early lethality indicated a severe disease course in wild boars. One wild boar died spontaneously overnight from 5 dpi to 6 dpi, two more from 6 dpi to 7 dpi. Of the domestic pigs, three were euthanized at the humane endpoint 8 dpi. Overall, there was no significant difference in the survival of domestic pigs and wild boar (data not shown). Due to the progressing clinical signs and the associated insufficient quality of the blood and tissue sample material, it was not possible to analyse all samples at every scheduled time. Nevertheless, data from remaining animals were integrated into the study to show trends. During necropsy, typical ASF lesions were observed in all animals, with increasing severity from 5 dpi to 7 dpi (data not shown). Lesions were highly variable and included cutaneous reddening, haemorrhages in lymph nodes, diffuse renal cortical and medullar haemorrhages or petechiae in kidneys, splenomegaly, lung oedema and gastritis. The remaining animals were kept to investigate later time points but had to be euthanized 8 dpi and were thus not included in this study.

3.2 | T-cell responses in peripheral blood

Because ASF is often associated with lymphopaenia and leucocyte apoptosis, we investigated which cell populations are directly affected and whether there are differences between domestic pigs and wild boars. In domestic pigs, the total number of leucocytes did not change during infection. However, a decrease in $CD3^+$ (T cells)

and CD79a$^+$ (B cells) lymphocytes 5 dpi and an increase in monocytes 7 dpi were observed (Figure 1 upper panel). In contrast to previous studies in domestic pigs (Dixon et al., 2017; Oura et al., 1998; Ramiro-Ibanez et al., 1997), we did not detect a decrease in lymphocyte counts in wild boars. Lymphocyte counts increased temporarily at 5 dpi, while total leucocytes and monocytes increased over the study period (Figure 1 lower panel). In line with higher lymphocyte counts, T and B cells of wild boars increased on 5 dpi in contrast to domestic pigs (Figure 1 lower panel). Besides, we detected no changes in the numbers of circulating granulocytes in domestic pigs (Figure 1 upper panel) but granulocytosis in wild boar (Figure 1 lower panel).

Studying the main subpopulations of T cells (αβ T cells and γδ T cells) revealed a tendency to decrease in total number of αβ T cells (CD3$^+$γδTCR$^-$) of domestic pigs (Figure 2 left panels). In wild boars, however, αβ T cells were temporarily increased at 5 dpi. The total amount of γδ T cells (CD3$^+$γδTCR$^+$) decreased in both, domestic pigs and wild boars, during infection (Figure 2 right panels). The αβ T cells were further divided into three subsets, CD4$^+$ helper T cells (CD3$^+$CD4$^+$CD8$^-$), double-positive (DP) T cells (CD3$^+$CD4$^+$CD8$^+$) and CD8$^+$ effector T cells (CD3$^+$CD4$^-$CD8$^+$). Analysis of these αβ T-cell subpopulations showed that the total number of CD4$^+$ T cells of domestic pigs decreased. In wild boars, on the other hand, first an increase at 5 dpi with a subsequent decrease at 7 dpi, even below their initial level, was observed. This reaction has to be viewed with caution as at 7 dpi only one animal could be investigated. The total number of DP T cells increased significantly in domestic pigs but remained unchanged in wild boars during the experiment. Numbers of CD8$^+$ effector T cells decreased in domestic pigs but displayed a temporary increase at 5 dpi in wild

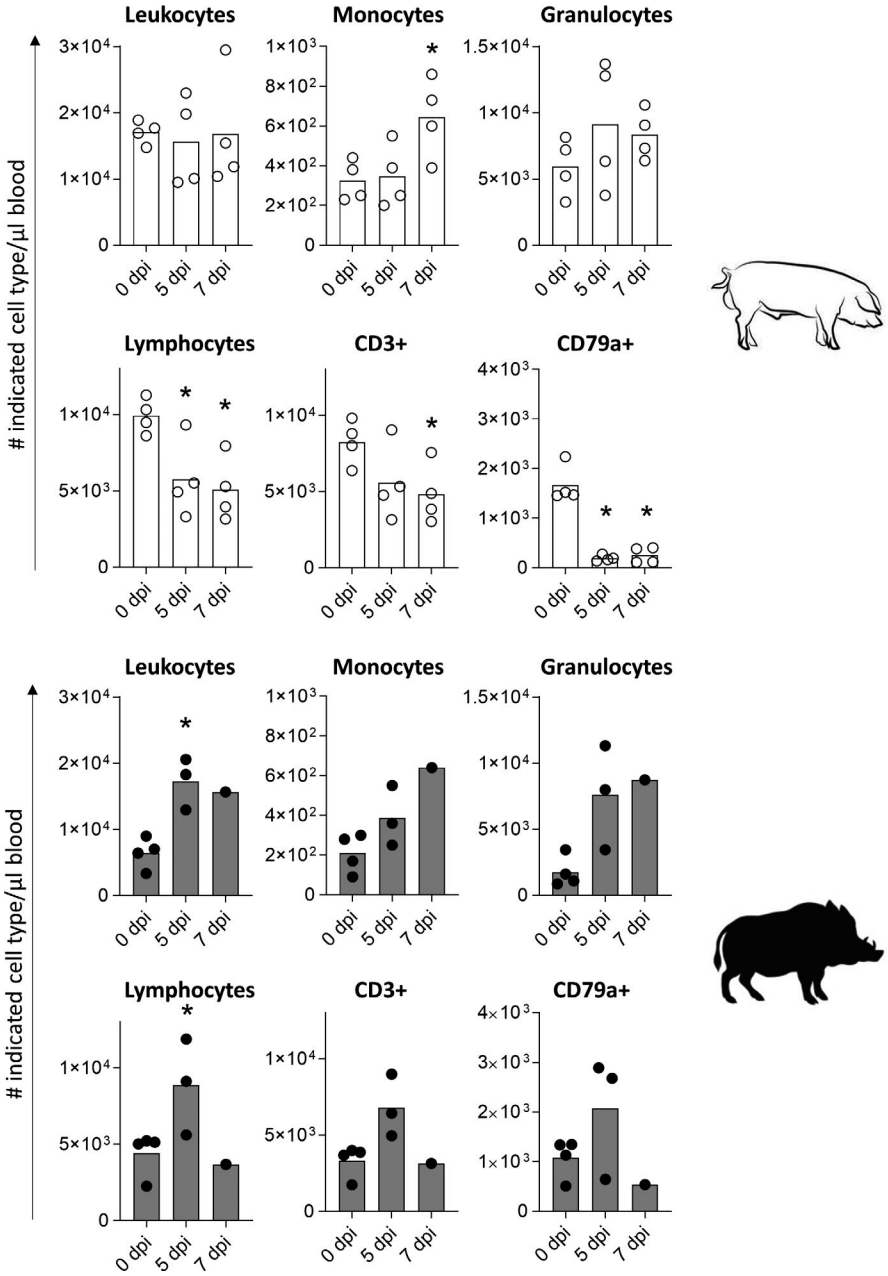

FIGURE 1 T cells in blood of domestic pigs decreased, while in wild boars increasing numbers of T cells occurred. Total number of leucocytes, lymphocytes, subdivided into T cells (CD3$^+$) and B cells (CD79a$^+$), monocytes and granulocytes from blood of domestic pigs (upper panel, open circles, white bars) and wild boars (lower panel, closed circles, grey bars) upon infection with ASFV 'Armenia08'. At indicated time points, pigs were euthanized and blood was collected. Blood counts were determined by using the IDEXX ProCyte Dx Haematology Analyzer. To calculate the total number of leucocyte subpopulations, the flow cytometry data were compared to data obtained from the blood counting device. Each point represents the value of a single pig, while the horizontal lines represent the means for the designated time points. *$p < .05$

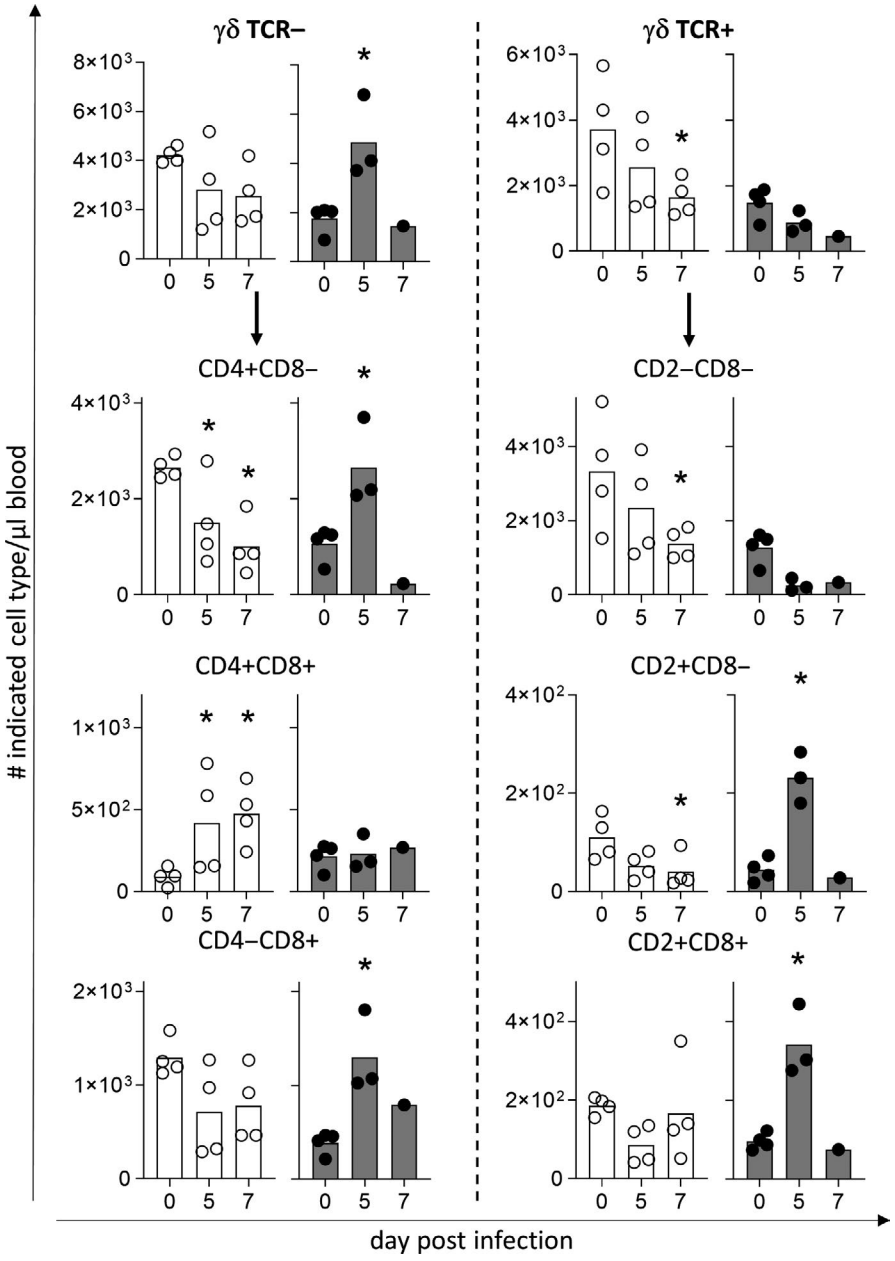

FIGURE 2 Distribution and phenotype of T-cell subsets in blood were different between domestic pigs and wild boars. Distribution and phenotype of different T-cell subsets from domestic pigs (open circles, white bars) and wild boars (closed circles, grey bars) upon ASFV 'Armenia08' infection. T cells from blood (Figure 2), ghLN (Figure 3), spleen (Figure 4) and liver (Figure 5) were analysed by flow cytometry. At indicated time points, pigs were euthanized and lymphocytes of indicated tissues were isolated. Lymphocytes were gated on $CD3^+$ T cells and grouped into γδ TCR^- (αβ T cells, left panels) and γδ TCR^+ (γδ T cells, right panels) T cells. αβ T cells were subdivided into T-helper cells ($CD4^+CD8^-$), double-positive cells ($CD4^+CD8^+$) and effector T cells ($CD4^-CD8^+$). γδ T cells were subdivided into naïve ($CD2^-CD8^-$), activated ($CD2^+CD8^-$) and effector ($CD2^+CD8^+$) cells. Each point represents the value of a single pig, while the horizontal lines represent the means for the designated time points. *$p < .05$

boars. In addition, all γδ T-cell subpopulations, namely naive ($CD2^-CD8^-$), activated ($CD2^+CD8^-$) and effector ($CD2^+CD8^+$) γδ T cells of domestic pigs, decreased during infection, although effector γδ T cells recovered to pre-infection levels 7 dpi. This was in contrast to the increasing numbers of activated and effector γδ T cells in wild boars 5 dpi (Figure 2).

3.3 | Distribution and phenotype of T-cell subsets in lymphoid organs

To investigate the complex cellular immune response to ASFV, we also analysed phenotype and activation status of T cells as well as their distribution in lymphoid organs. We focused on spleen and regionally affected lymph nodes, ghLN, because they are well described as pathomorphologically conspicuous, as well as the liver, an organ with importance in innate and adaptive immunity.

With regard to αβ T cells of domestic pigs, total frequencies of αβ T cells in ghLN (Figure 3) and spleen remained constant (Figure 4). In the liver, a slight decrease in total frequency was detected over the course of infection on 7 dpi (Figure 5). In wild boars, a decrease in frequency of αβ T cells in ghLN was observed (Figure 3). In spleen (Figure 4) of wild boars, αβ T cells increased in frequency from 5 dpi, while they only peaked 5 dpi but returned to baseline at 7 dpi in the liver (Figure 5).

The distribution of the three aforementioned αβ T-cell subsets was analysed more closely. In domestic pigs, frequencies of $CD4^+$ T cells decreased 7 dpi in ghLN (Figure 3) and in spleen from 5 dpi on (Figure 4). In the liver, we observed an increase in the frequency of $CD4^+$ T cells 5 dpi, but 7 dpi the frequencies decreased (Figure 5).

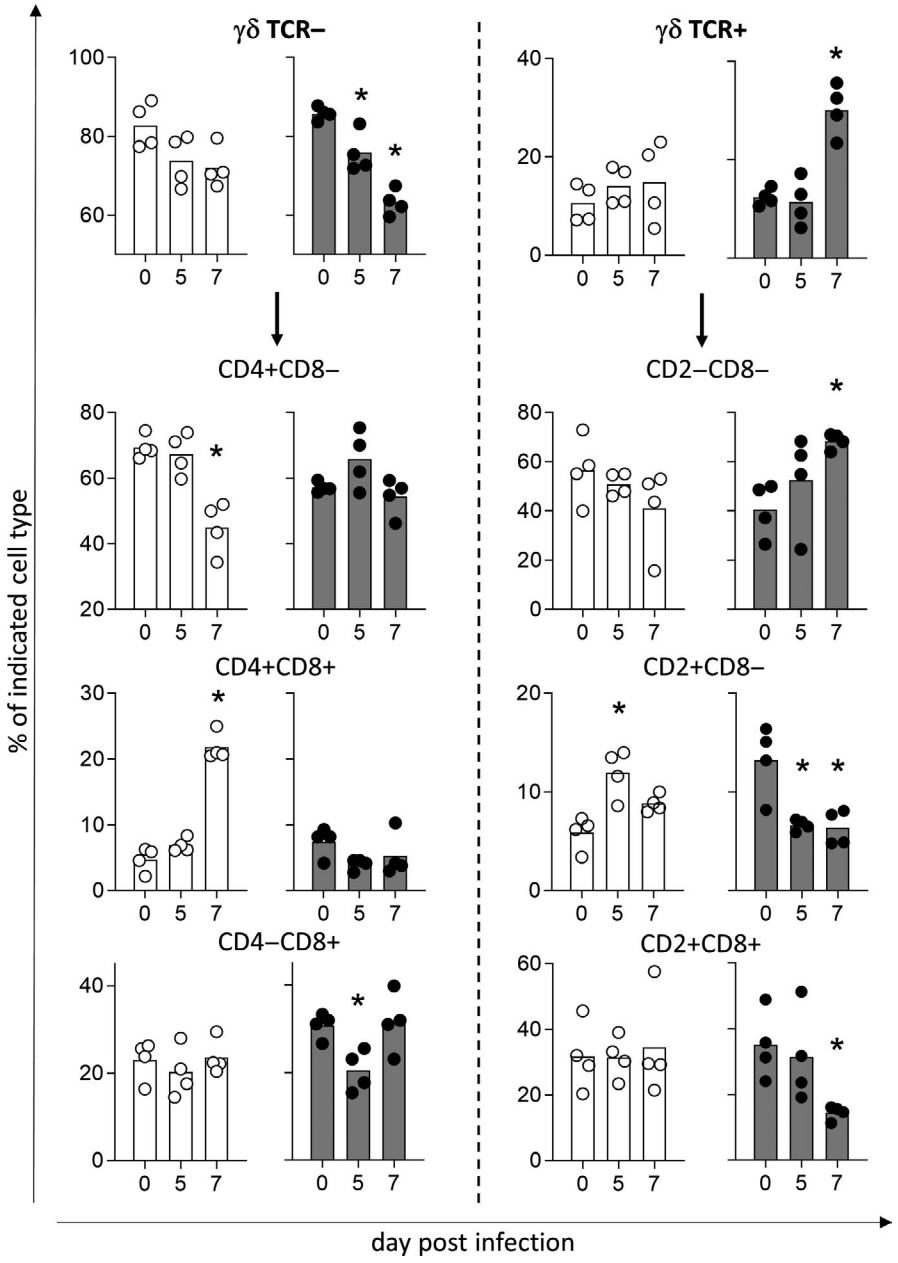

FIGURE 3 Distribution and phenotype of T-cell subsets in ghLN were different between domestic pigs and wild boar. For details, see figure legend of Figure 2

Frequencies of DP T cells in all investigated tissues of domestic pigs inversely mirrored $CD4^+$ T-cell frequencies, with an increase in all investigated organs. The percentage of $CD8^+$ T cells of domestic pigs did not change over time in ghLN (Figure 3) and spleen (Figure 4), while in the liver this subpopulation slightly dropped 5 dpi and normalized again 7 dpi (Figure 5). In wild boars, we detected a temporary decrease in $CD8^+$ T cells 5 dpi in ghLN (Figure 3), a decrease in $CD4^+$ T cells with a corresponding increase in $CD8^+$ T cells 7 dpi in the spleen (Figure 4) and heightened frequencies of $CD4^+$ T cells with a loss of DP T cells 5 dpi in the liver (Figure 5).

Furthermore, total frequencies of γδ T cells in ghLN, spleen and liver of domestic pigs remained unchanged during infection (Figures 3–5); additionally, only minor changes in frequencies of subpopulations of γδ T cells were detected. In ghLN, we detected an increase in activated γδ T cells. In liver and spleen, frequencies of effector γδ T cells tended to increase, with a corresponding loss of activated γδ T cells. In wild boars, total frequencies of γδ T cells were rather the opposite of αβ T cells: they increased in ghLN 7 dpi (Figure 3), whereas in spleen they decreased over time (Figure 4). Of note, increasing frequencies of γδ T cells in ghLN were mainly due to increasing frequencies of naïve γδ T cells. However, in the spleen, effector γδ T cells accumulated over the course of infection. In the liver, frequencies of γδ T-cell subpopulations did not change over time.

3.4 | Proliferative activity of T cells in lymphoid organs

To clarify whether the increasing frequencies in subpopulations of αβ T cells and γδ T cells were caused by infiltration

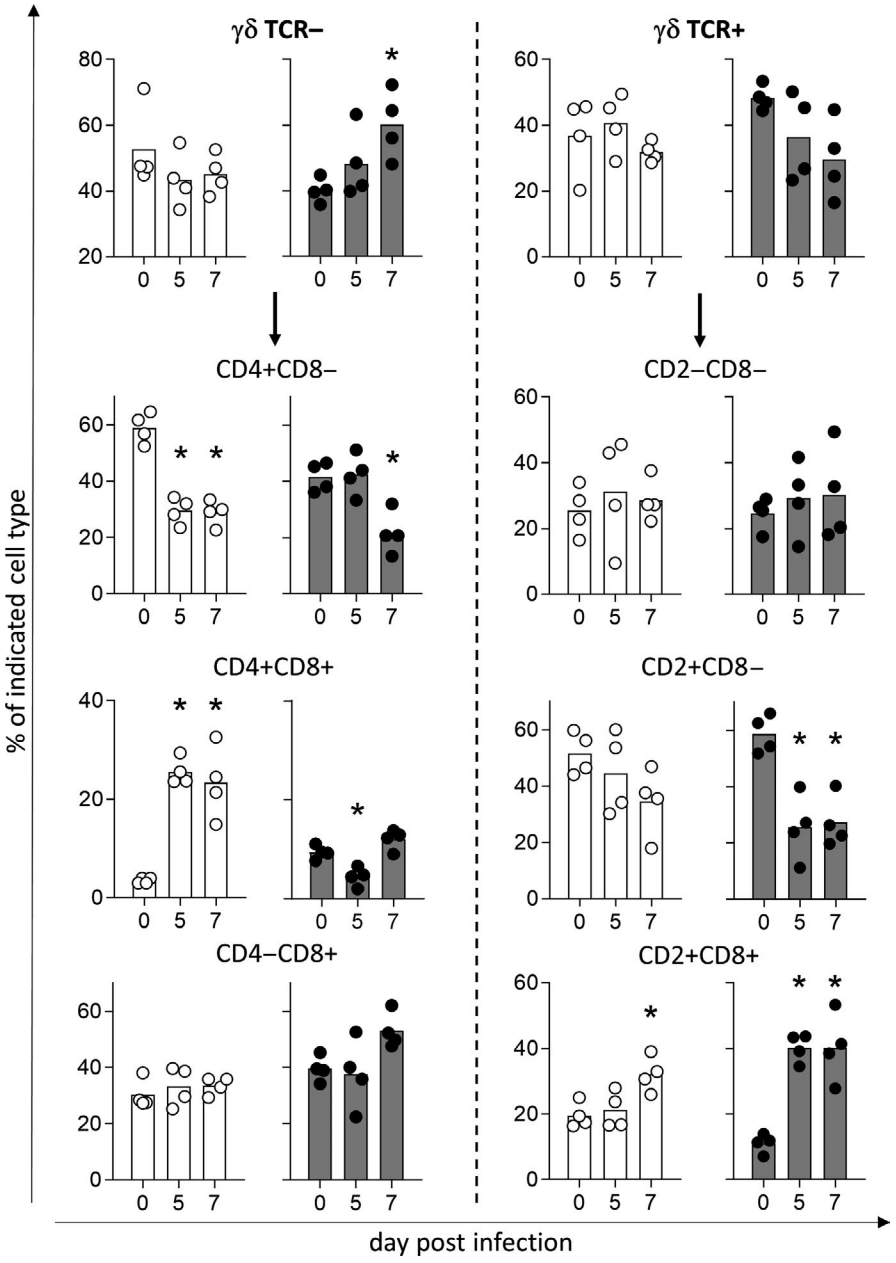

FIGURE 4 Distribution and phenotype of T-cell subsets in spleen were different between domestic pigs and wild boar. For details, see figure legend of Figure 2

or proliferation, we investigated the proliferative activity by Ki-67 detection of freshly isolated lymphocytes (Figure 6). During the course of infection, there were no changes in the expression of Ki-67 in αβ T cells in all investigated tissues of domestic pigs.

In contrast, DP T cells of wild boars in ghLN as well as in the spleen and liver showed an increase from 10% to more than 70% in Ki-67 expressing DP T cells at 5 dpi. Elevated frequencies of Ki-67 expressing DP T cells remained in ghLN and liver, while they declined to basal levels in the spleen. Moreover, we detected slightly increased frequencies of $CD4^+$ and $CD8\alpha\beta^+$ T cells exclusively in the liver of infected wild boars over the course of infection. Additionally, proliferating γδ T cells were only observed in ghLN and the liver of wild boars 5 dpi.

3.5 | Cytotoxic T-cell responses in lymphoid organs

We further analysed the expression of perforin as an effector mechanism of T cells. To determine whether there is any correlation between proliferative activity and expression of effector molecules, perforin in $CD8^+$ and DP αβ T cells and γδ T-cell populations in ghLN, spleen and liver were analysed (Figure 7). We detected a reduced perforin expression in T cells of domestic pigs 5 dpi in almost all investigated tissues; only $CD8\alpha\alpha^+$ T cells in spleen increased perforin expression over time. Especially, $CD8\alpha\beta^+$ T cells displayed a nearly complete loss of perforin in all tissues at 5 dpi, recovering at 7 dpi. The consumption of perforin at 5 dpi was also seen in wild boars; however, it was less pronounced with 50% of $CD8\alpha\beta^+$ T cells still expressing perforin. The loss of perforin expression was also detected

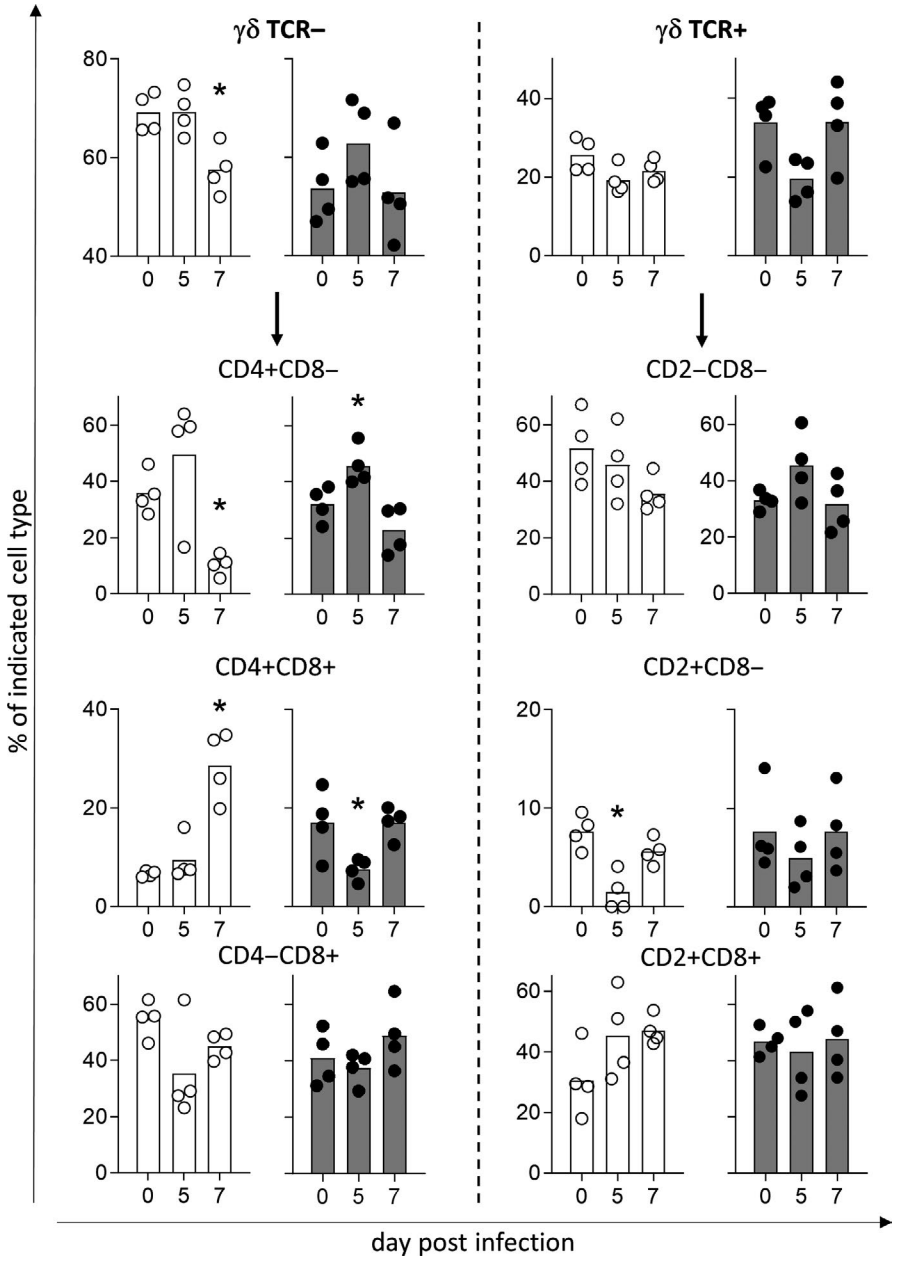

FIGURE 5 Distribution and phenotype of T-cell subsets in liver were different between domestic pigs and wild boar. For details, see figure legend of Figure 2

in DP T cells of domestic pigs, while DP T cells of wild boars in contrast increased perforin expression. The same was essentially true for $CD8^+$ γδ T cells.

3.6 | T-helper cell differentiation in lymphoid organs

The expression of T-bet in $CD8^+$ and $CD4^+$ T cells was investigated as a marker for activation.

T-bet expression of $CD8^+$ T cells (Figure 8a) from blood in domestic pigs decreased at 5 dpi but returned to basal expression levels at 7 dpi. In spleen and ghLN, no changes in the frequency of T-bet-expressing $CD8^+$ T cells were detectable. In contrast, in wild boars, the frequency of T-bet-expressing $CD8^+$ T cells increased especially in the blood over the course of infection, although to a lesser extent than in domestic pigs. T-bet expression of $CD4^+$ T cells ($CD3^+CD8^-$, Figure 8b) increased significantly 7 dpi in peripheral blood, ghLN and spleen of domestic pigs, whereas T-bet expression in $CD4^+$ T cells of wild boar was hardly detectable at any time point during the study period.

3.7 | Regulatory T-cell responses in blood and lymphoid organs

Further, the expression of FoxP3, as a marker for regulatory T cells (Käser, Gerner, & Saalmüller, 2011), was investigated to determine the induction of a regulatory immune response (Figure 9). High numbers of $FoxP3^+$ T cells in domestic pigs occurred pre-dominantly in blood and spleen, and to a lesser extent in ghLN at 7 dpi. In wild boars, regulatory T cells appeared at 5 dpi, again primarily in blood and spleen.

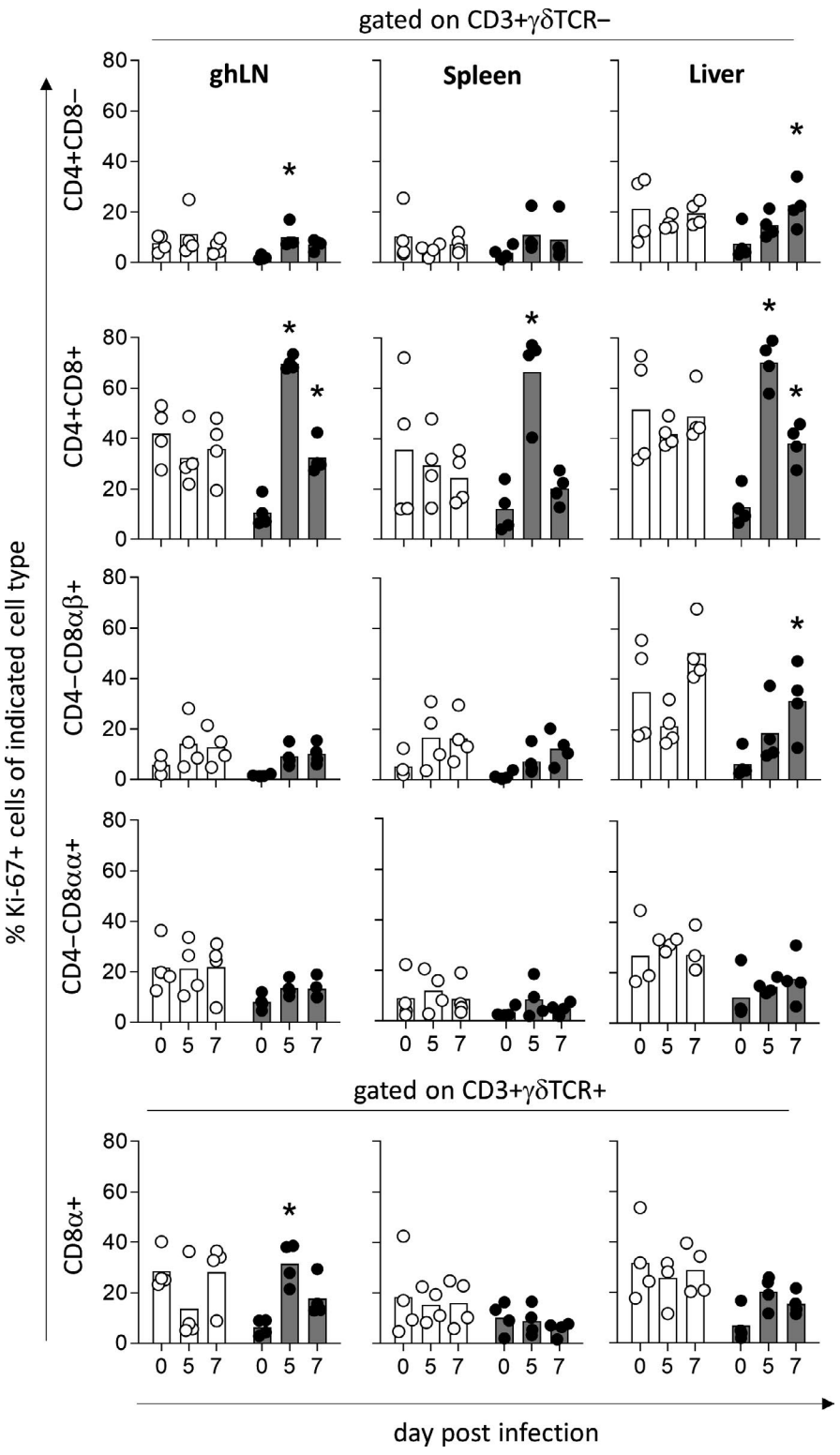

FIGURE 6 Proliferative activity of T cells from domestic pigs appeared to be impaired in contrast to T cells from wild boars. Proliferative activity of different T-cell subsets was measured by Ki-67 expression in ghLN, spleen and liver. Each point represents the value of a single pig, while the horizontal lines represent the means for the indicated time points. Domestic pigs (open circles, white bars) and wild boars (closed circles, grey bars). *$p < .05$

4 | DISCUSSION

The major focus of our study was to characterize the T-cell-mediated responses in ASFV-infected domestic pigs compared with wild boars. $CD8\alpha^+$ T cells, consisting of cytotoxic T cells (CTL), γδ T cells, NK cells, invariant T cells or memory helper T cells (Saalmüller et al., 1999; Schäfer et al., 2019; Yang & Parkhouse, 1997), have been shown to be of pivotal importance for anti-viral immunity after priming with homologous but attenuated ASFV strains (Oura et al., 2005). Furthermore, macrophages are described to be the main target cells and replication site of ASFV (Franzoni, Dei Giudici, et al., 2018; Gomez-Villamandos, Bautista, Sanchez-Cordon, & Carrasco, 2013). Therefore, we hypothesized that during infections with virulent ASFV strains, T-cell

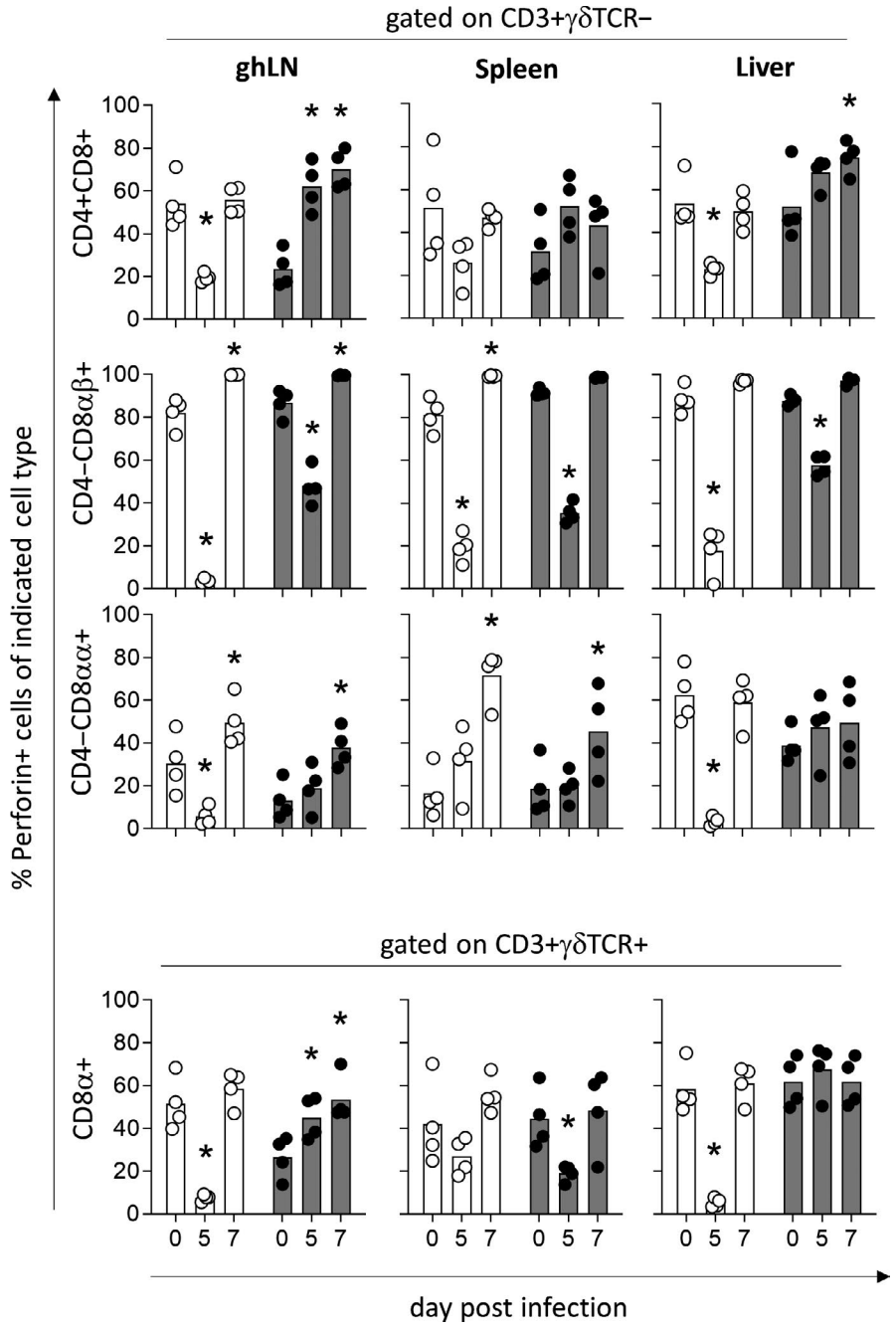

FIGURE 7 ASFV led to an impaired perforin expression of T cells in domestic pigs, while wild boar T cells appeared to counteract. Cytotoxic activity of different T-cell subsets was measured by the expression of perforin in ghLN, spleen and liver at indicated time points. Each point represents the value of a single pig, while the horizontal lines represent the means for the indicated time points. Domestic pigs (open circles, white bars), wild boars (closed circles, grey bars). *$p < .05$

responses might be impaired due to insufficient antigen presentation of infected macrophages.

During an acute viral infection, MHC class I-restricted antigen presentation leads to T-box transcription factor TBX21 (T-bet)-dependent differentiation of naïve $CD8^+$ T cells into CTL. This activation is characterized by cell proliferation, expression of pro-inflammatory cytokines (e.g. IFN-γ or IL-2) and upregulation of effector molecules such as perforin and granzyme B (Barry & Bleackley, 2002; Cobb et al., 2009; Intlekofer et al., 2005; Pearce et al., 2003). Our results show that this protective immune response does not occur in virulent ASFV infection. However, we detected an enormous decrease in perforin expression in $CD8\alpha\alpha^+$ and $CD8\alpha\beta^+$ T cells, pre-dominantly in domestic pigs.

There are various possible explanations for the observed loss of perforin expression. The transforming growth factor (TGF)-β induces regulatory T-cell (Treg) responses (Chen et al., 2003) but has also been shown to reduce perforin expression in cytotoxic cells (Thomas & Massague, 2005). Moreover, TGF-β impairs proliferation of antigen-reactive lymphocytes by cell cycle arrest (Tiemessen et al., 2003). This is in line with our findings, but the underlying mechanisms require further investigation. Release of pro-inflammatory cytokines might result in a polyclonal activation, which ultimately leads to loss of perforin due to consumption and T-cell exhaustion. Exhausted T cells are usually thought to occur after multiple antigen encounters during chronic infections (Kurachi, 2019), but T cells with an exhaustion-like phenotype have been found during

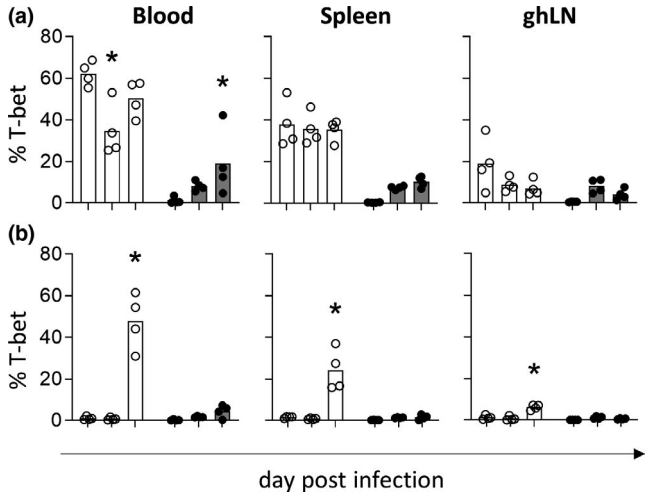

FIGURE 8 CD8$^+$ T cells downregulated T-bet expression and CD4$^+$ T cells differentiated in Th1 cells in domestic pigs but not wild boars. Expression of T-bet in (a) CD8$^+$ and (b) CD8- T cells of blood, spleen and ghLN of domestic pigs (open circles, white bars) and wild boar (closed circles, grey bars) is shown for indicated time points. Each point represents the value of a single pig, while the horizontal lines represent the means for the indicated time points. *$p < .05$

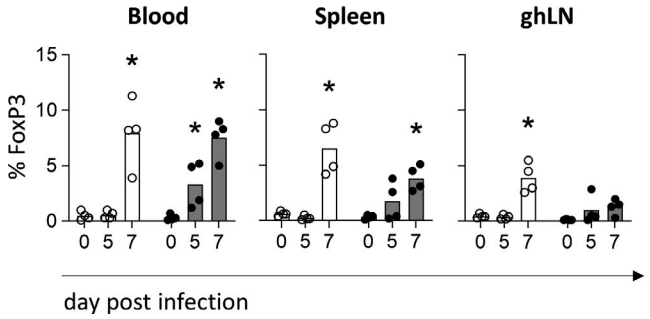

FIGURE 9 FoxP3$^+$ regulatory T cells were induced in domestic pigs and wild boars after ASFV infection. Expression of FoxP3 in CD3$^+$ γδ TCR$^-$ CD8$^-$ CD4$^+$ T cells of blood, spleen and ghLN of domestic pigs (open circles) and wild boar (closed circles) is shown for the indicated time points. Each point represents the value of a single pig, while the horizontal lines represent the means for the indicated time points. *$p < .05$

acute infections in humans and mice (Erickson et al., 2015). These cells displayed characteristics of both effector and exhausted T cells and are thought to minimize tissue damage and immunopathology (Erickson et al., 2015). Since T-cell exhaustion is Eomes-dependent (Li, He, Hao, Ni, & Dong, 2018), this hypothesis has to be investigated in future studies. A more technical explanation supporting the consumption hypothesis is based on the antibody clone used in this study. Clone dG9 has been shown to detect perforin in granula only, missing detection of newly synthesized and immediately secreted perforin molecules (Hersperger, Makedonas, & Betts, 2008). Finally, it is also possible that the cytotoxic response switched from perforin-mediated to Fas/FasL-mediated killing (Meiraz, Garber, Harari, Hassin, & Berke, 2009). Since antibodies for other porcine cytotoxic proteins are missing, this switch remains undetectable.

In contrast to domestic pigs, we found T-bet$^+$CD8$^+$ T cells in blood and increasing frequencies of T-bet$^+$CD8$^+$ T cells in spleen and ghLN of wild boars. Even though a decrease in perforin$^+$CD8αβ$^+$ T cells in wild boars occurred in ghLN and in the liver, it was only half as pronounced as in domestic pigs. Moreover, we even observed an increase in perforin$^+$CD8αα$^+$ T cells in these organs over the time of infection, indicating a functional activation. Taken together, CD8$^+$ T cells in both species did not proliferate upon ASFV infection, but in contrast to domestic pigs, CD8$^+$ T cells from wild boars developed an activated phenotype with cytotoxic potential in the course of infection. However, this was measured by T-bet expression only, and classical activation markers such as CD69 are still missing. Activation could also be shown using in vitro assays to determine the release of IFN-γ by CD8$^+$ and CD4$^+$CD8$^+$ T cells in response to ASFV, as increasing numbers of IFN-γ-producing cells have been shown to correlate with protection (Takamatsu et al., 2013). Moreover, this could investigate functional differences between domestic pigs and wild boars. The detected cytotoxic T-cell response in wild boar was not accompanied by a milder disease course or elimination of virus. This phenomenon needs further investigation.

Another important component for cellular immune responses are MHC class II-restricted CD4$^+$ T-helper (Th) cells. Activated CD4$^+$ Th cells differentiate into different functional subsets, depending on cytokines from the antigenic environment (Gerner et al., 2015). Upon ASFV infection, we observed a strong increase in T-bet$^+$CD4$^+$ T cells in blood and lymphoid organs of domestic pigs 7 dpi, demonstrating a strong cytotoxic Th1 response (Szabo et al., 2000). However, the proliferation of CD4$^+$ T cells could not be detected, indicating that ASFV infection in domestic pigs causes activation but not expansion of pro-inflammatory Th1 cells. Whether these cells sufficiently stimulate CTL and macrophages has to be determined. In wild boars, there was no increase in T-bet expression of CD4$^+$ T cells. A disturbed or non-existent differentiation of Th1 cells further inhibits downstream activation of macrophages and CTL and anti-viral immune responses (Pritchard, Kedl, & Hunter, 2019). However, since Eomes and T-bet show functional redundancy (Intlekofer et al., 2008), lack of T-bet expression alone does not necessarily indicate a non-functioning Th1 response.

Pigs are known to hold a significant amount of CD4$^+$CD8$^+$ double-positive (DP) T cells (Saalmüller, Reddehase, Buhring, Jonjic, & Koszinowski, 1987). We observed an increase in DP T-cell frequency with a corresponding decrease in CD4$^+$ T-cell frequency in peripheral blood and lymphoid organs of domestic pigs, but no proliferative activity or proper perforin expression. Strikingly, we detected an enormous loss of perforin expression 5 dpi in DP T cells, which was restored two days later. DP T cells in wild boar showed a high proliferative activity. DP T cells are playing a distinct role in porcine immune responses (Okutani, Tsukahara, Kato, Fukuta, & Inoue, 2018; Saalmüller, Hirt, & Reddehase, 1989; Zuckermann, 1999) and are often described as memory or effector T cells (Denyer, Wileman, Stirling, Zuber, & Takamatsu, 2006; Gerner, Kaser, & Saalmüller, 2009; Saalmüller, Werner, & Fachinger, 2002; Zuckermann, 1999). Since only ASFV-naïve pigs

were infected and the trial ended 8 dpi, a memory response can be excluded. Previous studies demonstrated CD8α upregulation on porcine CD4 T cells after various in vitro stimulations (Reutner et al., 2013; Saalmüller et al., 1987, 2002). Other studies, using in vitro re-stimulation with viruses or viral peptides, showed proliferation or cytokine production in DP T cells but not conventional $CD4^+$ T cells (Blanco et al., 2000; Lefevre et al., 2012; Reutner et al., 2013; Saalmüller et al., 2002). DP T cells are capable of expressing perforin and granzyme (Chung et al., 2018; Herndler-Brandstetter, Schwanninger, & Grubeck-Loebenstein, 2007; Suni et al., 2001), also indicating a role as cytotoxic effector cells. Together, these studies indicate an activation-dependent expression of CD8α on $CD4^+$ T cells and underline the effector functions of DP T cells.

γδ T cells are often described not only as a bridge between the innate and adaptive immune system but also as cytotoxic effector cells (Charerntantanakul & Roth, 2006; Gerner et al., 2009; Takamatsu et al., 2006; Takamatsu, Denyer, & Wileman, 2002). Previously, it has been shown that γδ T cells of ASFV immune pigs were able to present viral antigen (Takamatsu et al., 2006). Moreover, higher frequencies of circulating γδ T cells correlated with increased survival in an infection with moderately virulent ASFV (Post, Weesendorp, Montoya, & Loeffen, 2017). However, the results of our study suggest that γδ T cells are also affected by highly virulent ASFV. In both species, the numbers of γδ T cells decreased during infection. We did not see any correlation between γδ T-cell frequencies and survival in our study. This might be explained by the higher virulence of the ASFV strain in our study and age differences of the animals compared with previously published reports (Post et al., 2017). Furthermore, there was no change in γδ T-cell subsets in domestic pigs. In contrast, in wild boars, the remaining γδ T cells in blood and spleen differentiated into $CD2^+CD8^-$ and $CD2^+CD8^+$ cells, respectively. Previous studies described $CD2^+CD8^+$ γδ T cells as terminally differentiated effector cells with cytotoxic and anti-viral effector functions (Lopez Fuertes et al., 1999; Stepanova & Sinkora, 2012; Takamatsu et al., 2006; Thielke et al., 2003), indicating a strong pro-inflammatory response. Interestingly, porcine $CD2^+CD8^+$ cells have also been discussed as regulatory cells (Wen et al., 2012) and their appearance correlates with the increase in $FoxP3^+CD4^+$ Tregs in wild boars. This might indicate an anti-inflammatory response in these animals. $CD2^-CD8^-$ and $CD2^+CD8^-$ γδ T cells are thought to elicit pro-inflammatory responses (Wen et al., 2012). These cells were found pre-dominantly in lymph nodes of infected wild boars and might suggest pro-inflammatory responses at the site of infection. In general, the diverse subsets and functions of porcine γδ T cells need further research.

$FoxP3^+$ Tregs are pivotal immune regulators and are needed to prevent immunopathology and tissue damage (Veiga-Parga, Sehrawat, & Rouse, 2013). Tregs appeared earlier in wild boars than in domestic pigs, which might be the reason for the non-development of $CD4^+$ Th1 cells and lack of activation of CTL. This is in line with recent findings by Sanchez-Cordon et al. Here, they showed that increased Treg frequencies and heightened levels of regulatory IL-10 after immunization with an attenuated ASFV strain impair anti-viral responses after virulent ASFV challenge (Sanchez-Cordon, Jabbar, Chapman, Dixon, & Montoya, 2020). Treg responses during acute viral infections are multifaceted. During human dengue fever, Tregs have been shown to proliferate and function normally. Still, because their frequency was too low, they were unable to reduce immunopathology (Lühn et al., 2007), demonstrating that pathological hyper-inflammation is not necessarily associated with impaired Treg functions (Boer, Joosten, & Ottenhoff, 2015). Mild disease, however, was associated with increased numbers of Tregs (Lühn et al., 2007). Therefore, the mere appearance of Tregs during ASFV infection does not exclude immunopathology. Moreover, during acute West Nile fever virus infection in mice, Tregs facilitated enhanced memory T-cell formation at the cost of prolonged antigen presence (Graham, Da Costa, & Lund, 2014). In the case of ASFV infection, prolonged presence of viruses might cause a higher immunopathology and, eventually, lethality. On the other hand, during acute herpes simplex virus infection in mice, Tregs have been suggested as pivotal orchestrators for the migration of inflammatory cells to and production of pro-inflammatory cytokines at the site of infection, thereby facilitating protective immunity (Lund, Hsing, Pham, & Rudensky, 2008). In general, the influence of Tregs on the clinical course and immune response during acute ASFV infection needs further investigation.

Taken together, the experimental ASFV infection with the highly virulent 'Armenia08' strain led to severe clinical courses in domestic pigs and wild boars. The immune response failed in both species and most animals died latest 8 dpi, although a few animals might have survived the infection. Apparently, the ASFV infection suppressed the proliferation of $CD8^+$ T cells, $CD4^+$ T cells and γδ T cells. However, in wild boars we detected $CD8^+$ T cells and γδ T cells with an activated phenotype and partial cytotoxic potential. No activation, measured by T-bet, was observed in domestic pigs. The reaction of DP T cells after ASFV infection was striking in both species: domestic pigs reacted with a differentiation of $CD4^+$ T cells into DP T cells. However, their proliferation remained impaired. The DP T cells of wild boars showed massive proliferative activity and increased perforin expression. Tregs appeared in both species but earlier in wild boars. Still, the immune responses in domestic pigs and wild boars were not able to counteract ASFV infection. Whether the outcome was caused by an inefficient or excessive immune response has to be determined in future studies, for which our study paves the way.

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ETHICAL APPROVAL

In this study, all applicable animal welfare regulations, including EU Directive 2010/63/EC and institutional guidelines, were taken into consideration. The animal experiment was approved by the State Office for Agriculture, Food Safety and Fishery in Mecklenburg-Western Pomerania (LALFF M-V), under reference number LALLF 7221.3–1.1-064/17.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

SB and UB conceived and designed the experiments. JH, LZ, JS, TS and SB acquired animal samples. JH and AS processed samples. JH, AS, TS and UB analysed and interpreted the data. JH, AS, TS, TCM and UB prepared the manuscript. All authors reviewed and approved the final version of the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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