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Diverse immune cell profiles in ASFV-associated lymphopenia

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Abstract

Pathogenic African swine fever virus (ASFV) remains a lethal causative agent in the domestic pig industry, which poses a burden on the swine market and causes substantial socioeconomic losses worldwide. Currently, there are no commercially efficacious vaccines or specific treatments available for ASF prevention and control. Unfortunately, little is known about the swine immune response upon ASFV infection. Here, we investigated the host immune response discrepancy induced by the field moderately virulent strain ASFV HB-2208 among healthy, diseased and asymptomatic pigs. In the peripheral blood of diseased swine, lymphopenia is caused by the massive loss of bystander lymphocytes, such as γδ T cells, B cells and $CD4^+$ T cells. Conversely, ASFV has a strong tropism for the mononuclear phagocyte system (MPS) and partial dendritic cells (DCs), whose antigen-presenting ability is impeded by the downregulation of CD80 and MHC I. However, no significant difference in the number of $CD8\alpha^{high}$ T cells was detected, whereas the frequencies of NK cells, NKT cells, and regulatory T cells (Tregs) were significantly increased. Additionally, an in vitro model was established with a coculture of primary pulmonary alveolar macrophages (PAMs) and peripheral blood mononuclear cells (PBMCs), which significantly reduced γδ T cells, B cells and $CD4^+$ T cells and increased Tregs. The differentiated immune response might aid in enhancing the understanding of ASFV pathogenesis in suids and provide insights into the mechanism of ASFV-induced lymphopenia for further studies.

Keywords African swine fever virus, Antigen-presenting cells, Lymphocytes, Lymphopenia

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Introduction

African swine fever virus (ASFV) is a large complex double-stranded DNA virus that primarily replicates in swine macrophages and is the only known member of the *Asfarviridae* family (Dixon et al. 1990; Tulman et al. 2009). African swine fever (ASF) caused by different strains of ASFV is the most contagious and lethal disease among domestic pigs and has become a major devastating threat in the pig industry (Farlow et al. 2018). Ever since its reintroduction into Geogia in 2007, continuous outbreaks of ASF have disseminated into Western Europe and Asia (Forth et al. 2020), leading to an unprecedented socioeconomic loss that affects China, the largest pork producer that holds over half of the pig population worldwide (Xu et al. 2019). On the basis of the C-terminal region of the B646L gene, 24 different

genotypes of ASFV have been identified (Nefedeva et al. 2020), and genotype II strains are mainly responsible for the circulation of virulent strains in Asia and Europe (Bosch-Camós et al. 2021). Bush pigs, warthogs and soft ticks serve as natural reservoirs of ASFV, constituting the complex and dynamic transmission of ASF to domestic pigs (Plowright et al. 1969). Symptoms of acute ASF include hemorrhages, cyanosis, high fever, gastrointestinal signs and so on, the mortality rates of which reach nearly 100% (Forth et al. 2019). The emergence of highly lethal genotype I and II recombinant strains of ASFV has made early diagnosis highly difficult and has created new challenges for ASFV control (Sun et al. 2021; Zhao et al. 2023). Although experimental gene-deleted vaccines and subunit vaccines are being developed (Chen, Chen, et al. 2020a, b; Fan et al. 2024), no efficient commercial vaccines are extensively used because of their nonideal efficacy and the risk of virulence reinforcement (Vu and McVey 2024).

With a 170-194 kbp genome, ASFV encodes various kinds of immune-related proteins, some of which can modulate the host's immune response, enabling immune-evasive strategies, such as the inhibition of IFN pathways (Correia et al. 2013) and the induction of apoptosis and autophagy (Brun et al. 1996; Sun et al. 2022). Most related studies have focused on the function of viral proteins (Bonneville et al. 2010) and host innate immune responses, especially the IFN response (Wang et al. 2020; Frączyk et al. 2016). However, ASFV can infect both macrophages and some dendritic cells (DCs) (Vallée et al. 2001), which are simultaneously antigen-presenting cells (APCs) and initiators of adaptive immunity (Franzoni et al. 2018a, b). Nevertheless, research underscoring host adaptive immunity remains in its infancy.

Lymphopenia commonly presents during ASFV infection (Gómez-Villamandos et al. 2013). Unlike human and mouse peripheral blood, porcine peripheral blood contains a remarkable percentage of double-positive $CD4^+CD8\alpha^+$ T lymphocytes and a large portion of γδ T cells (Zuckermann and Husmann 1996). Here, we systemically investigated the profile changes in immune cells in the peripheral blood of the epidemic strain ASFV HB-2208 to better understand the pathogenesis of ASFV. Immune cell populations from the peripheral blood of pigs were analyzed via a comparative approach in blood samples from domestic pigs with disease symptoms or without disease symptoms, and typical lymphopenia was observed in diseased animals, in which substantial loss of γδ T cells, B cells and $CD4^+$ T cells was observed. This research provides a fresh perspective on newly developed vaccines and a comparison of different endemic virulent strains.

Results

ASFV infection elicits leukopenia and lymphopenia

Leukocytes from the control, W/D (clinical symptoms such as subcutaneous hemorrhage, diarrhea, pyrexia, etc.) and WO/D (no clinical symptoms but ASFV p30 antibody-positive serology) groups were counted via trypan blue. According to the gating strategies shown in Fig. 1a, at least 3 individual peripheral blood samples were selected from different groups. Compared with those in the control and WO/D groups, the number of leukocytes in the W/D group was markedly lower, indicating ASFV-induced leukopenia (Fig. 1b, left). Not surprisingly, peripheral blood lymphocytes (PBLs), accounting for approximately 20–40% of leukocytes, were also dramatically lower in the W/D group than in the control and WO/D groups (Fig. 1b, right). Overall, the ASFV HB-2208 strain led to leukopenia and lymphopenia in diseased individuals but not in asymptomatic animals.

ASFV infection increased the proportion of antigen-presenting cells

Single-cell suspensions were prepared from heparinized blood samples via lysis of erythrocytes. The antigen-presenting cells, including dendritic cells ($CD172a^+CD16^+CD14^-$, Fig. 2a), monocyte-containing populations ($CD172a^+CD14^{high}$, Fig. 2b) and macrophage precursors ($CD172a^+CD14^{low}$, Fig. 2b), were analyzed via flow cytometry. Compared with those in the control or WO/D groups, there was a drastic increase in both the monocyte and macrophage proportions in the W/D group (Fig. 2c). The percentage of $CD172a^+CD16^+CD14^-$ DCs in peripheral blood leukocytes was greater in the W/D group than in the WO/D group, especially in the control group (Fig. 2d). The results of the statistical analyses are shown in Fig. 2e, which revealed that the percentage of macrophages increased from an average of 5% to 24% and that the percentage of monocytes increased from an average of 10% to approximately 35% in peripheral blood leukocytes. Similarly, the number of DCs increased from 0.5% to approximately 3%. Given that the number of peripheral blood leukocytes in the W/D group was much lower than that in the control group, the absolute cell numbers of APCs were correspondingly dilated.

ASFV infection impaired the antigen-presenting capacity of APCs

Although the proportion of APCs is increased, ASFV infection may also impair the initiation of the immune response (Franzoni et al. 2018a, b). CD80 is an important costimulatory molecule expressed on APCs that works together with MHC/TCR signaling to initiate the T-cell response. A lack of either CD80 or CD86 may

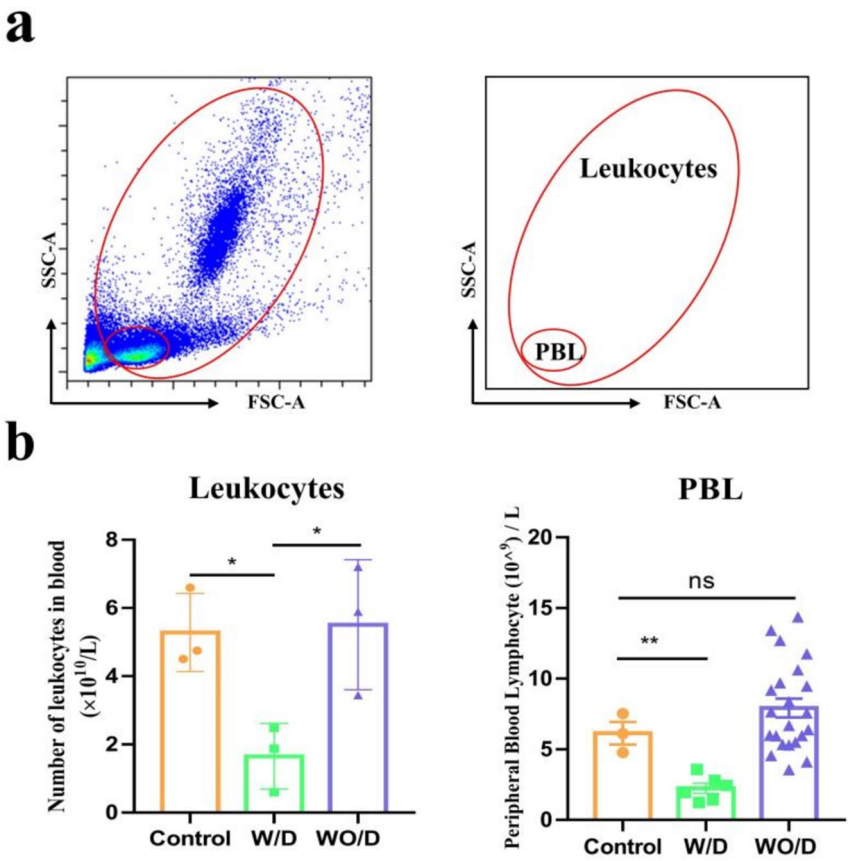


Fig. 1 ASFV infection elicits leukopenia and lymphopenia. **a**, Gating strategy of swine peripheral blood leukocytes and peripheral lymphocytes (PBL). **b**, Cell counts of leukocytes ($n = 3$ in each group) and PBL ($n = 3$, 6 and 22) in Control, W/D and WO/D group (mean ± s.e.m., one-way ANOVA, $^{*}P < 0.05$; $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$)

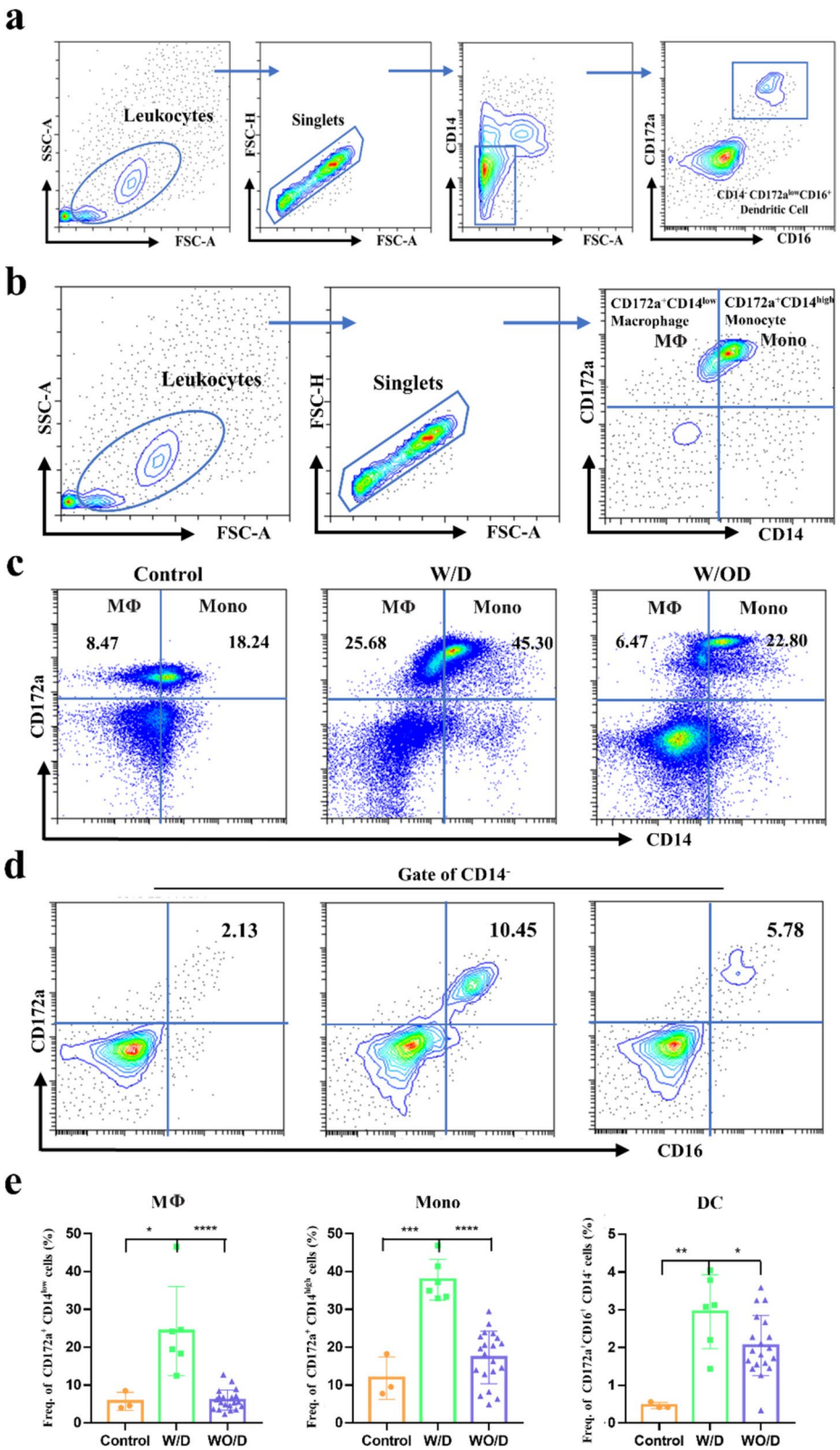
prevent the activation of T cells and enable the immune evasion of viruses. Here, the gated mononuclear phagocyte system (MPS) was analyzed (Fig. 3a). Not surprisingly, we discovered that the expression of both MHC-I (Fig. 3b) and CD80 (Fig. 3c) molecules decreased in the MPS from normal 30% to approximately 15%, which may imply a demolished antigen-presenting function of the MPS. Nonetheless, the expression of MHC-I molecules (Fig. 3d) and CD80 (Fig. 3e) on DCs was significantly suppressed in the W/D group from 30% to lower than 10%, and the abundance of MHC-I molecules and CD80 on APCs was somehow linked to a higher survival rate in a ratio-dependent manner in the WO/D group. Taken together, these data demonstrated that infection with ASFV did not reduce the number of target cells; however, the expression of these markers, which have vital functions in antigen presentation, was compromised in the target cells.

NK and NKT cells are activated during ASFV infection

The CD8α^{high}, NK and NKT cell subsets, which are cytotoxic T lymphocytes (CTLs), exhibit formidable antiviral activity through functional effectors such as perforin, granzyme B, and IFN-γ (Martins et al. 1993). NK cells

(See figure on next page.)

Fig. 2 ASFV infection induced elevated proportion of antigen-presenting cells. **a**, Gating strategy of CD14-CD172alowCD16 + dendritic cells (DCs) in peripheral blood leukocytes. **b**, Gating strategy of CD172a + CD14high monocyte and CD172a + CD14low macrophage in peripheral blood leukocytes. **c**, Representative pseudo-colour plot by five-colour flow cytometry indicative of CD172a + CD14high monocyte and CD172a + CD14low macrophage in peripheral blood leukocytes. **d**, Representative contour plot by five-colour flow cytometry indicative of CD172a + CD16 + dendritic cells (DCs) in gated CD14- cells in peripheral blood leukocytes. **e**, Analysis of macrophage, monocyte and DC percentage among Control, W/D, WO/D groups (mean ± s.e.m., one-way ANOVA, $^{*}P < 0.05$; $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, $n = 3$, 6, 22)



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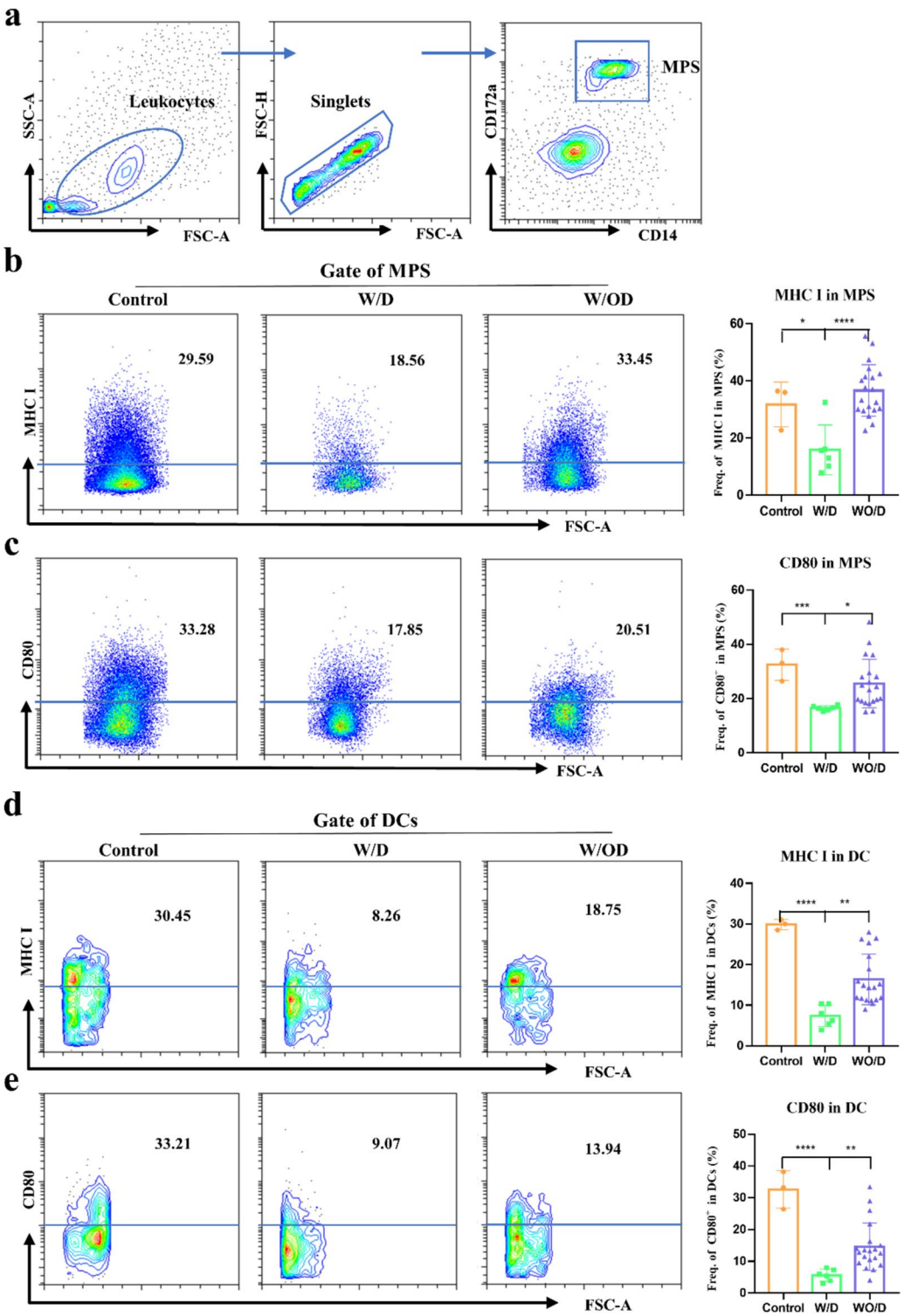

Fig. 3 ASFV infection impaired antigen presenting capacity of APCs. **a**, Gating strategy of CD14 + CD172a + mononuclear phagocyte system (MPS) in peripheral blood leukocytes. **b**, Representative pseudo-colour plot of MHC I molecule expression in MPS. **c**, Representative pseudo colour plot of CD80 molecule expression in MPS. **d**, Representative contour plot of MHC I molecule expression in DCs. **e**, Representative contour plot CD80 molecule expression in DCs among Control, W/D and WO/D groups (mean ± s.e.m., one-way ANOVA, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, $n = 3, 6, 22$)

($CD3^{-}CD8\alpha^{high}$) and NKT cells ($CD3^{+}CD16^{+}$) are perforin-producing innate compartments that are cytotoxic to viral antigens. Intriguingly, perforin secretion by NK and NKT cells was largely expanded in the W/D group (Suppl Fig. 1a & b), and the overlaid histogram clearly revealed enrichment of perforin in the W/D group compared with both the control and WO/D groups (Suppl Fig. 1c), suggesting that positive antiviral activity is mediated by porcine NK and NK T cells.

CTL and DP T-cell proportions were augmented during ASFV infection

As the most powerful effector cells in cellular immunity, $CD3^{+}CD8\alpha^{high}$ cells belong to a CTL subset (Fig. 4a). The ratio of $CD3^{+}CD8\alpha^{high}$ T cells in peripheral blood lymphocytes differed slightly among the different groups. However, due to the decreased number of lymphocytes in the peripheral blood, the absolute number of CTLs may vary. In terms of the cell count, the CTL number was lower in the W/D group than that in the control group (Fig. 4b). Moreover, increased levels of perforin were detected in the W/D and WO/D groups, especially in the W/D group (Fig. 4c), which indicated that perforin is one of the most essential functional effectors of CTLs. Additionally, the secretion of IFN-γ appears to be vital for viral clearance in the early stage and is an indicator of CTL activation. Similarly, the promotion of IFN-γ was greater in the W/D group than that in the control and WO/D groups (Fig. 4d). $CD4^{+}CD8\alpha^{+}$ double-positive (DP) T cells are usually described as progenitor $CD4^{+}$ or $CD8\alpha^{+}$ T cells. Most DP-T cells undergo programmed death in the thymus, and few of them can be exported into the peripheral blood (Hagen et al. 2023). However, increasing evidence has shown that DP T cells can be recruited toward infection or tumor sites in many infectious or tumor models, exhibiting effector and memory functions. Here, we discovered that the frequency of DP T cells was significantly greater in the W/D group than in the control and WO/D groups (Fig. 4e & 4f), which may indicate certain biological functions of DP T cells during ASFV infection.

γδ T cells, B cells and $CD4^{+}$ T cells are involved in lymphopenia in ASFV infection

Leukopenia, more specifically lymphopenia, is observed in the process of many virulent ASFV infections (Zuo et al. 2024). However, no elucidation of the specific mechanism of cell population death has been reported for ASFV infection. γδ T cells have gained increasing attention in recent years owing to their considerable proportion in swine, whose function has not yet been fully illustrated, and CD27 is a commonly acknowledged activation indicator of γδ T cells (Zhu et al. 2019). Our analysis revealed that the percentage of γδ T cells ($CD3^{+}TCR\gamma\delta^{+}$) in PBLs decreased dramatically from approximately 20% in the control group to less than 5% in the W/D group, and the γδ T-cell proportion in the WO/D group was even greater than that in the control group (Fig. 5a). Moreover, the expression of CD27 was significantly upregulated in both the W/D and WO/D groups (Fig. 5b). The percentage of $CD3^{-}CD21^{+}$ B cells also drastically decreased (Fig. 5c) from approximately 10% to less than 2%, suggesting compromised humoral immunity. In the production of neutralizing antibodies, $CD4^{+}$ T cells specialize in assisting T-cell-dependent B-cell proliferation and maturation (Aloulou and Fazilleau 2019). Nevertheless, the ratio of $CD4^{+}$ T cells ($CD3^{+}CD4^{+}$) was distinctly lower in the W/D group (Fig. 5d), while robust expression of IFN-γ was observed in the WD group than in the control and W/OD groups, indicating that a Th1-type response occurred during ASFV infection. CD27, the activation indicator, was strongly upregulated on $CD4^{+}$ T cells in the WO/D group, which was significantly greater than that in the W/D group, suggesting that the durable activity maintenance of $CD4^{+}$ T cells might benefit the survival of ASFV-infected animals. Taken together, these analyses revealed that the lymphopenia induced by ASFV infection was caused by the death of γδ T, B and $CD4^{+}$ T bystander cells and that humoral immunity was severely suppressed.

The proportion of Tregs tended to increase during ASFV infection

Regulatory T cells (Tregs) are a group of T-cell subpopulations that perform regulatory functions in the host immune response through multiple mechanisms, and their long-lasting existence impairs host immunity against infections. In the peripheral blood of diseased swine (W/D group), $CD3^{+}CD4^{+}$ lymphocytes were gated (Fig. 6a). The results of flow cytometric analyses suggested that the percentage of Tregs ($CD25^{+}Foxp3^{+}$ in $CD3^{+}CD4^{+}$) (Fig. 6a right) increased to approximately 14% compared with less than 1% in the other groups (Fig. 6b). These data suggest that ASFV can induce immunosuppression by Tregs, which further leads to failure of the immune response against ASFV.

ASFV-induced increases in lymphopenia and Treg numbers in the coculture system

Next, we established an in vitro coculture system to mimic the occurrence of lymphopenia. First, primary pulmonary alveolar macrophages (PAMs) were recovered in a 37 °C incubator for 2 days until adherence to stable conditions following a 0.1 MOI of the ASFV-HB-2208 strain as the ASFV group (or not the control group), with porcine IL-4

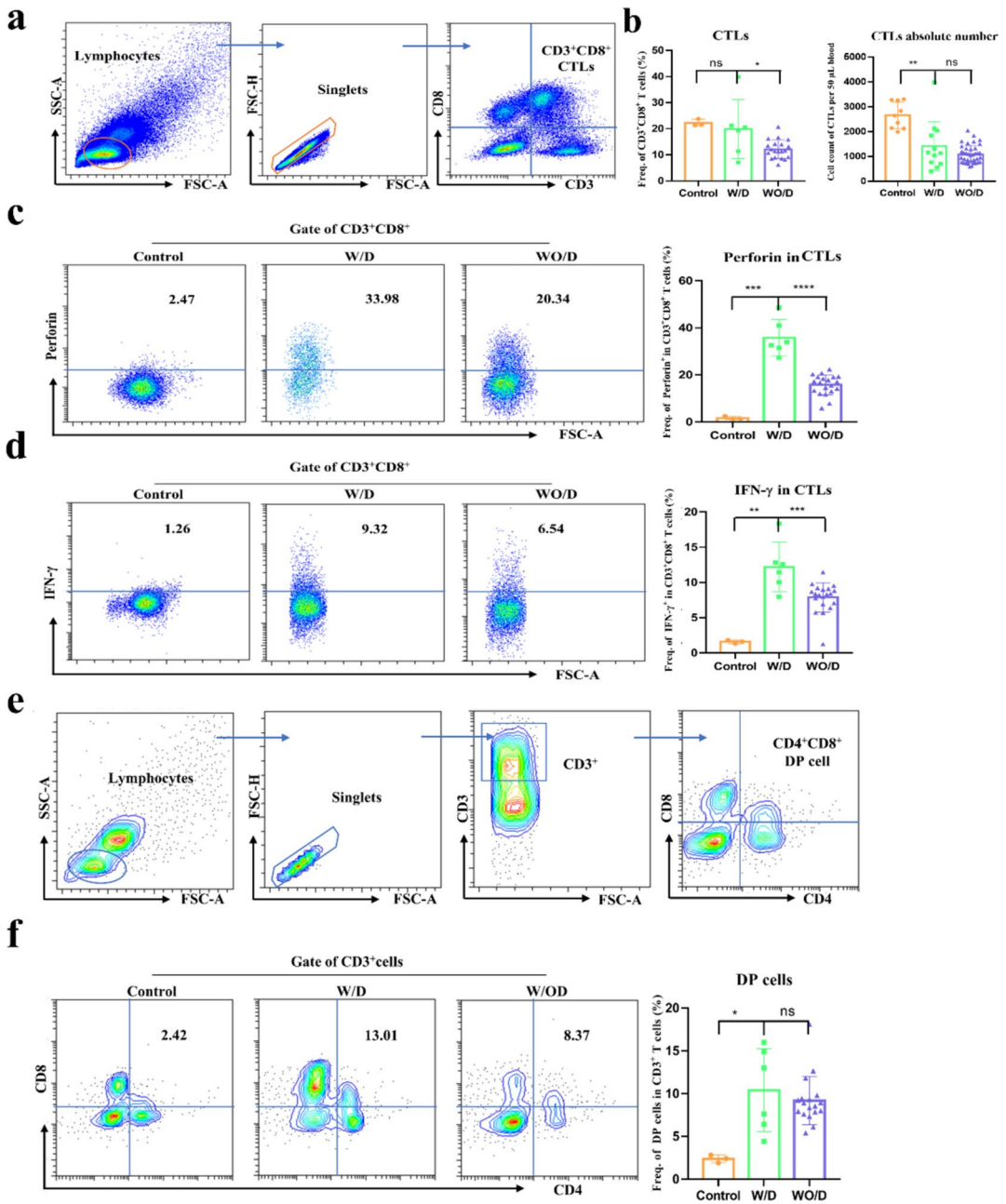

Fig. 4 CTLs and DP T cell proportions were augmented during ASFV infection. **a**, Gating strategy of $CD3^+CD8\alpha^{high}$ CTLs in PBL. **b**, Percentage of $CD3^+CD8\alpha^{high}$ T cells in PBL among Control, W/D and WO/D groups and analysis of CD8 absolute number per 50 μL blood. **c**, Representative contour plot of perforin expression in $CD3^+CD8\alpha^{high}$ CTLs. **d**, Representative contour plot of IFN-γ expression in $CD3^+CD8\alpha^{high}$ CTLs. **e**, Gating strategy of $CD4^+CD8\alpha^+$ double positive (DP) T cells in PBL. **f**, Representative contour plot of DP T cells in PBL and analysis of DP T cells among Control, W/D and WO/D groups (mean ± s.e.m., one-way ANOVA, $^*P < 0.05$; $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, $n = 3, 6, 22$)

and IL-2 added (the supernatant of the porcine IL-2/IL-4-expressing PK15 cell line was previously established in the laboratory, patent No. ZL 2021 1 1,364,146.0). Then, PBMCs were isolated from ASFV-free pigs and cocultured with ASFV-infected PAMs. Interestingly, lymphocyte subpopulations exhibited changes similar to those in vivo. The percentages of $CD4^+$ T cells, B cells and γδ T cells but not those of $CD8\alpha^{high}$ T cells decreased at 7 d.p.i. (Fig. 7a, b). Taking γδ T cells as an example, the sole decrease in γδ T cells amounted to 3000, implying that dramatic cell death occurred in the immune microenvironment after ASFV infection (Fig. 7c).

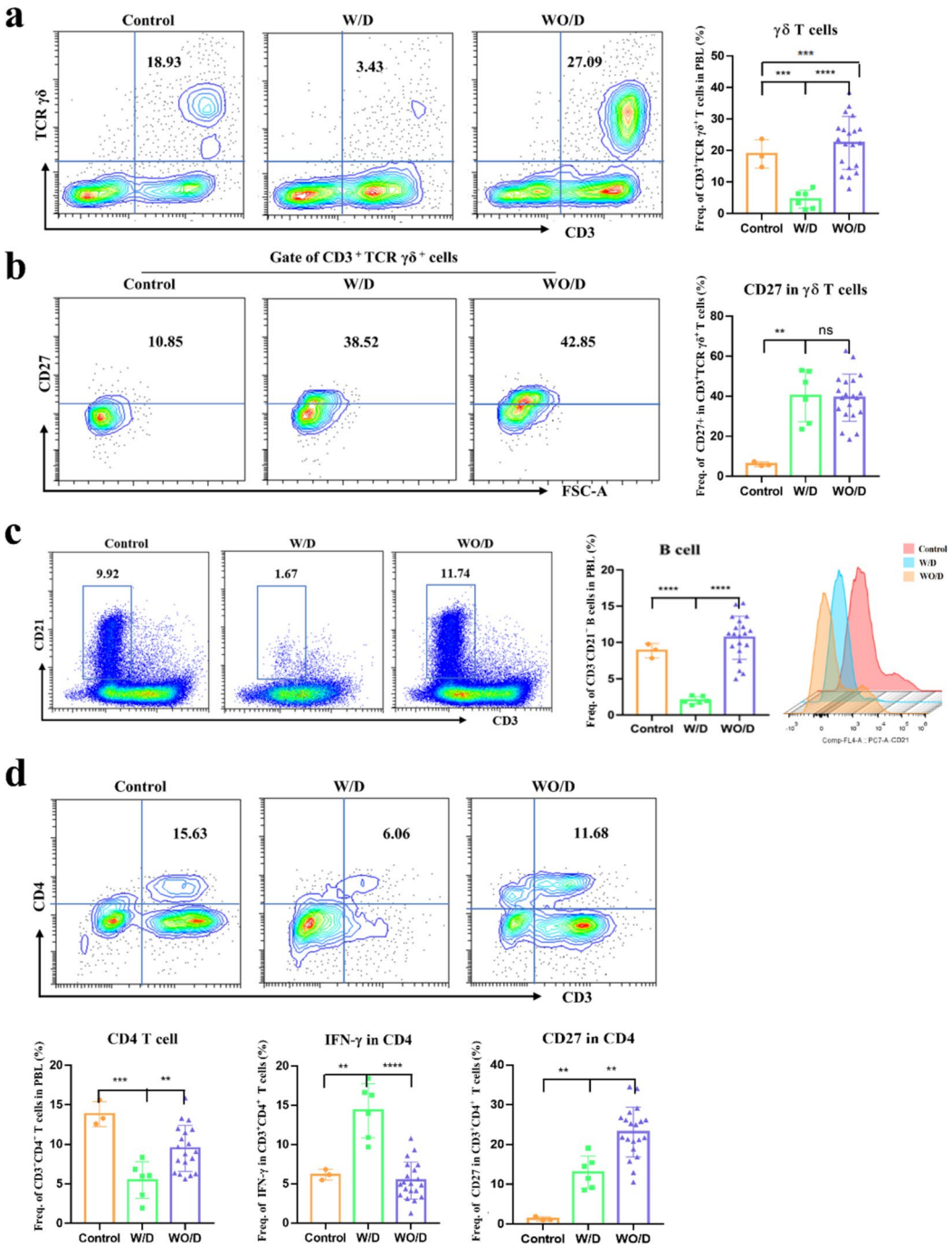

Fig. 5 γδ T cells, B cells and CD4 T cells comprise lymphopenia induced by ASFV infection. **a**, Representative contour plot of $CD3^{+}TCR\ \gamma\delta^{+}$ T cells and analysis of γδ T cell percentage in PBL among Control, W/D and WO/D groups ($n = 3$, 6 and 22). **b**, Representative contour plot of CD27 expression in γδ T cells and graphic statistical analysis of CD27 expression in γδ T cells. **c**, Representative pseudo-colour plot of $CD3^{-}CD21^{+}$ B cells, graphic statistical analysis of B cell percentage and MFI of CD21 on B cells. **d**, Representative contour plot indicative of $CD3^{+}CD4^{+}$ T cells and graphic statistical analysis of IFN-γ and CD27 expression in CD4 T cells among Control, W/D and WO/D groups (mean ± s.e.m., one-way ANOVA, $^{*}P < 0.05$; $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, $n = 3, 6, 22$)

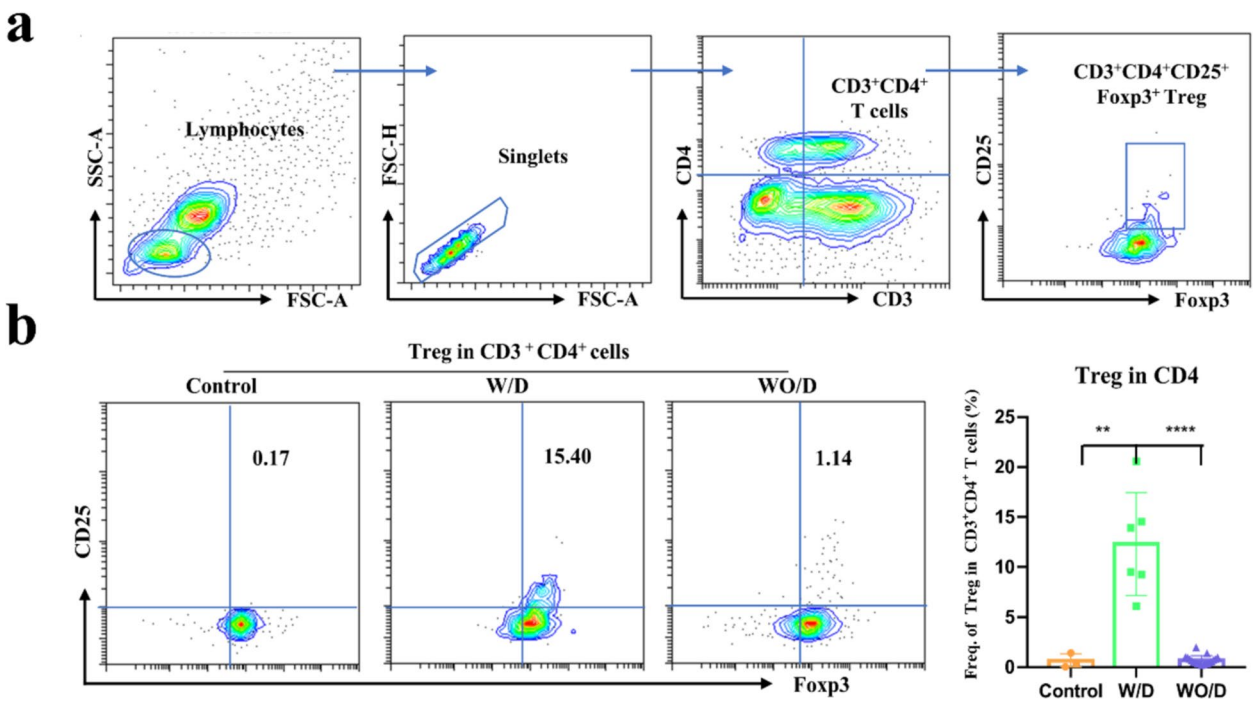

Fig. 6 The proportion of Treg tends to increase in ASFV infection. **a**, Gating strategy of $CD3^+CD4^+CD25^+Foxp3^+$ Treg in PBL during peripheral blood detection. **b**, Representative contour plot by four-colour flow cytometry indicative of $CD25^+Foxp3^+$ Treg in gated $CD3^+CD4^+$ T cells and graphic statistical analysis of Treg among Control, W/D and WO/D groups (mean ± s.e.m., one-way ANOVA, $^*P < 0.05$; $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, $n = 3, 6, 22$)

Additionally, our in vitro model further monitored the percentage of Tregs at 3 d.p.i., 5 d.p.i. and 7 d.p.i. The proportion of Tregs continuously increased from 3 d.p.i. to 7 d.p.i., confirming the phenomenon observed in swine PBLs (Fig. 7d, e), with upregulated RNA levels of TGF-β in the ASFV group, which was consistent with the accumulation of suppressive Tregs (Fig. 7f). Both in vitro and in vivo data suggested that ASFV could induce immunosuppression by Tregs, which further led to failure of the immune response against ASFV.

Discussion

Since the first outbreak of ASFV in 2018, attenuated or less virulent strains of ASFV have emerged in China with continuous viral adaptation and evolution (Wang et al. 2022). The animals that were infected with attenuated or less virulent ASFV in the field presented chronic or atypical clinical symptoms and survived longer. However, pathological changes, especially changes in immune cells, have not been fully investigated. Here, we studied the diverse immune profiles and changes during early acute infection with the field-isolated strain ASFV HB-2208 and developed an in vitro coculture system to mimic ASFV-triggered lymphopenia.

ASFV targets the mononuclear phagocyte system (MPS) for replication, enabling its encoded proteins to interfere more efficiently with the antigen presentation process. MPS is composed of macrophages, monocytes and dendritic cells, whose origins are both myeloid progenitors. Our study revealed that during ASFV HB-2208 infection, the numbers of $CD172a^+CD14^{low}$ macrophages and $CD172a^+CD14^{high}$ monocytes increased to varying degrees in the diseased group compared with those in the control group or asymptomatic individuals. Moreover, as professional antigen-presenting cells (APCs), the expression of MHC I molecules and CD80 costimulatory molecules on the MPS distinctly decreased, suggesting that the antigen-presenting capacity of antigen-presenting cells in the diseased group was inhibited after ASFV HB-2208 infection. In support of viral replication, ASFV can functionally promote the survival of host cells during viral infection, especially in the early stage. For example, in a study of the moderately virulent ASFV isolate Netherlands'86, despite the age and dose, $CD14^{low}CD163^+$ representative macrophages in all infected pigs presented a peak cell number at 10 d.p.i. but decreased afterwards, considering the migration of macrophages to diseased tissue or excessive viral burden (Zsak et al. 2001). Although the subpopulation of the MPS is skewed, its antigen presentation ability might also be impaired, as evidenced by the expression of MHC I molecules in the MPS and $CD172a^+CD16^+$ DCs. It has been reported

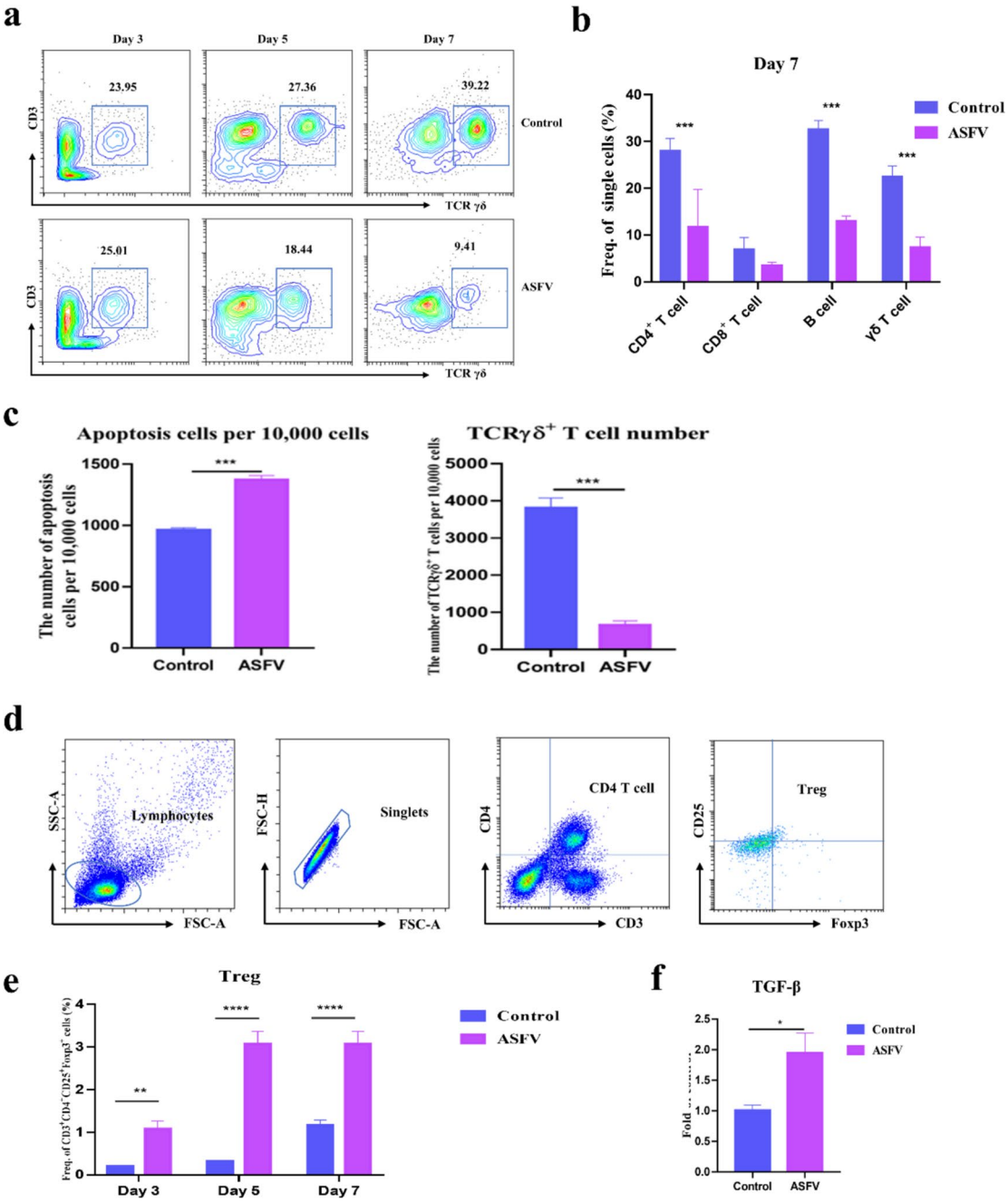

Fig. 7 ASFV-induced lymphopenia and Treg increase in the coculture system. Density gradient-separated peripheral blood mononuclear cells (PBMCs) were co-cultured with primary pulmonary alveolar macrophages (PAMs) infected with/without 0.1 MOI ASFV (HB-2208 strain) in medium with porcine IL-2, IL-4 added for 7 days. **a**, Representative contour plot of $CD3^+$TCR $\gamma\delta^+$T cells in co-culture system at 3, 5, 7 d.p.i.. **b**, Analysis of cell population subsets in PMBC using five-colour flow cytometry (mean ± s.e.m., Two-way ANNOVA, $^{**}P < 0.01$, $^{***}P < 0.001$, $n = 3$). **c**, Absolute count of $CD3^+$TCR$\gamma\delta^+$T and apoptosis cells in PBMCs per 10^4cells (mean ± s.e.m., Student's *t*-test, $^{***}P < 0.001$, $n = 3$) using FITC/Annexin V, PI apoptosis assay kit at 7 d.p.i.. **d**, Gating strategy of $CD3^+CD4^+CD25^+Foxp3^+$Treg in co-culture system. **e**, Analysis of Treg percentage in co-culture system at 3, 5, 7 d.p.i.. **f**, TGF-β mRNA expression in Control and ASFV groups at 7 d.p.i (mean ± s.e.m., Student's *t*-test, $^{*}P < 0.05$; $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, $n = 3$)

that MHC II expression is negligibly different in multiple types of original macrophages during ASFV infection (Lithgow et al. 2014), whereas MHC I expression is downregulated in moMΦs (unactivated macrophages) and moM2s (activated monocyte-derived macrophages) (Franzoni et al. 2017). Franzoni reported that MHC I downregulation on moDCs occurs only in attenuated ASFV infection, not in the virulent strain (Franzoni et al. 2018a, b). Moreover, single-cell transcriptome analysis revealed that macrophages infected with ASFV exhibit dramatic downregulation of MHC II (Zhu et al. 2019).

Anti-ASFV antibody alone is insufficient to protect swine from ASFV (OUR/T88/3) challenge, and the cellular immune response is indispensable for complete antiviral clearance of ASFV (Oura et al. 2005). In acute ASFV HB-2208 infection, the proportion of $CD8\alpha^{high}$ T cells remained unchanged, whereas perforin expression in $CD8\alpha^{high}$ T cells was strongly increased according to our analysis. Previous research inoculated a highly virulent strain, "Armenia08", in domestic pigs and wild boars. $CD8\alpha^{high}$ effector T cells ($CD3^{+}CD4^{-}CD8\alpha^{high}$) decreased at 5 and 7 d.p.i. in domestic pigs, whereas a temporary increase occurred at 5 d.p.i. in wild boars. However, almost total loss of perforin occurred in domestic pigs at 5 d.p.i. and then recovered to normal levels, but perforin levels in wild boars tended to increase over time (Hühr et al. 2020). For the moderate strain "Estonia2014", the number of $CD8\alpha^{+}$ T cells in the peripheral blood and spleen tends to increase (Schäfer et al. 2021). Double-positive cells usually exist in the early stage of thymocytes and coexpress CD4 and CD8. Surprisingly, domestic pigs possess a relatively high number of DP cells in the spleen, mesenteric lymph nodes and even peripheral blood (Saalmüller et al. 1987). During "Armenia08" strain infection, the total number of DP cells continuously increased and peaked at 7 d.p.i., whereas the number of DP cells remained constant in wild boars. Nevertheless, our data revealed that, compared with the asymptomatic group, the W/D group had a greater percentage of DP cells, which might be attributed to the mass loss of $CD4^{+}$ T cells. However, in line with our study, another study on a moderately virulent strain "Estonia2014" revealed a similar result in DP cells, as the percentage of $CD3^{+}CD4^{+}CD8\alpha^{+}$ cells increased continuously after Estonia2014 infection at 4, 5, 7, and 10 days (Schäfer et al. 2021).

ASFV pathogenesis is also characterized by massive apoptosis of B cells (Takamatsu et al. 1999). In vivo and in vitro studies demonstrated that ASFV isolates can stimulate porcine Ig secretion and B-cell proliferation in the presence of virus-infected macrophages, but costimulatory molecules on B cells were diminished (Takamatsu et al. 1999). Our data also confirmed that B cells in diseased individuals underwent massive loss and that the level of CD21 on B cells, which is a surface marker and a costimulatory molecule, was almost reduced to minimal levels during acute ASFV infection. Concurrently, the $CD3^{+}CD4^{+}$ T-cell ratio decreased in the diseased group. Although B cells can be indirectly activated by ASFV-infected macrophages, the number of B cells is decreased due to the depletion of T cells, especially T helper cells, in the relatively early stage of ASFV infection. There was a drastic decrease in the number of B cells ($CD3^{-}CD21^{+}$) and T helper cells ($CD3^{+}CD4^{+}CD8\alpha^{-}$) in the peripheral blood of ASFV-inoculated pigs at 5 d.p.i., regardless of the age of the pigs and the doses of ASFV (Post et al. 2017). Similarly, the number of $CD4^{+}CD8\alpha^{-}$ T cells in peripheral blood decreased at 5 and 7 d.p.i. in the highly virulent "Armenia" strain, implying that $CD4^{+}$ T-cell loss is a common feature of ASFV infection (Hühr et al. 2020). However, the expression of the CD27 activation marker on $CD3^{+}CD4^{+}$ T cells was strongly upregulated, suggesting that activation likely occurred in the remaining $CD4^{+}$ T cells during ASFV HB-2208 infection.

In contrast to humans and mice, pigs feature a high proportion of γδ T cells in circulating blood, bridging the gap between innate immunity and adaptive immunity, as they express both innate-like and adaptive-like markers. Owing to the large individual differences in pigs, the frequency of γδ T cells varies from 15 to 50% in an age-dependent decreasing manner within peripheral blood lymphocytes (Le Page et al. 2022). In the present study, ASFV-infected pigs with signs of classical ASF symptoms, in which γδ T cells were almost depleted by ASFV HB-2208 infection, were on the verge of death. In contrast, a high proportion of γδ T cells remain in asymptomatic pigs. In the in vitro model in which PAMs were cocultured with ASFV-infected PAMs, a decreased frequency of γδ T cells was observed in porcine PBMCs. A drastic decrease in γδ T cells occurred except at low doses of ASFV infection in adult pigs. More specifically, regardless of the age or doses of inoculated ASFV, the number of γδ T cells significantly decreased at 5 or 7 d.p.i. Notably, among those that survived ASFV infection, there was a greater proportion of γδ T cells, which highlights the importance of γδ T cells in preventing ASFV infection (Post et al. 2017).

The accumulation of Tregs ($CD4^{+}CD25^{+}Foxp3^{+}$) could impair the capacity of the adaptive immune response. Our data demonstrated that the frequency of Tregs dramatically increased in the peripheral blood of pigs with disease symptoms and in in vitro coculture systems and that the expression of the regulatory cytokine TGF-β also increased, suggesting that Tregs could also contribute to the pathogenesis of ASF. The existing 7-gene-deleted vaccine application is also accompanied by an increasing

amount of Tregs during the immune process, resulting in nonideal protection of domestic pigs (Chen et al. 2020a, b), which further suggests that ASFV infection could induce strong immune suppression.

Overall, ASFV HB-2208 is a moderately virulent strain. In diseased individuals, infection predominantly impairs the antigen-presenting abilities of the MPS but in turn promotes their expansion, which might favor viral replication in these target cells. The depletion of bystander lymphocytes, including γδ T cells, B cells and CD4+T helper cells, destroyed the immune system, and not surprisingly, the expansion of Tregs further deteriorated the failure of the immune response against ASFV. Our study is the first to conduct a systematic analysis of the domestic pig immune response upon current naturally attenuated ASFV (HB-2208) infection, contributing to the rational design of new therapeutics and providing insights for further evaluation of newly developed ASFV vaccines. However, as the specific mechanism and checkpoint of ASFV-induced lymphopenia remain elusive, further research needs to be done in the future.

Conclusions

Our study elucidates the dynamic alterations observed in peripheral blood mononuclear cells following the natural infection of domestic pigs with ASFV. An analysis of these changes underscores the pivotal role of the divergent cell fates exhibited by distinct cell populations in ASFV pathogenesis. In particular, the reduction caused by apoptosis in both B cells and T cells emerges as one of the predominant mechanisms underlying lymphopenia, which impedes the effectiveness of the adaptive immune response. Moreover, increased numbers of myeloid cells exhibit diminished functions. These findings have implications for understanding the intricate interplay between ASFV pathogenesis and immune evasion, specifically shedding light on the perturbations elicited in the adaptive immune system by ASFV infection in swine.

Methods

Animal samples, viruses, and cell isolation

In August 2022, the Centre for Emerging Infectious Diseases received heparinized peripheral blood samples from a large pig farm in central China, which experienced an ASF outbreak with an estimated loss rate greater than 20%. Twenty-six ASFV serum-positive samples were identified, of which 6 animals in the W/D group presented typical ASF symptoms, and the other 22 animals in the WO/D group were asymptomatic. The timepoint of the disease process in W/D samples was estimated to be 7 ~ 10 d.p.i. according to the observations of pig farm staff. All samples from asymptomatic pigs were collected from the same pigs as symptomatic pigs. Three healthy control samples were acquired from ASFV-free pigs at separate breeding farms; these samples were tested for ASFV and other common virus-negative strains, such as PRRSV. The virus was separated and amplified from PAMs in complete culture medium for 5 days, after which it was isolated from clinically healthy ASFV-free pigs. The virus DNA was sequenced (attached in the supplementary data), which was characterized as genotype II ASFV and named ASFV HB-2208.

Red blood cells were lysed with 1 mL ammonium chloride (ACK lysis buffer, TBDscience, Tianjin, China) per 80 μL sample for 30 min in the P3 laboratory of the Wuhan Institute of Virology, Chinese Academy of Sciences. In accordance with biosafety guidelines, all samples suspected to contain live virus were analyzed in the P3 laboratory of the Wuhan Institute of Virology, Chinese Academy of Sciences and Huazhong Agricultural University.

Ex vivo FCM (Flow cytometry) staining

PBMCs or blood samples were evaluated for cell viability via trypan blue and then fixed and resuspended in 0.2% bovine serum albumin (BSA) solution. During the staining process, the samples were incubated at 4°C for more than 40 min. All commercial antibodies used in this experiment were as follows: CD3ε-PerCP-Cy5.5 (Mouse IgG2a, clone BB23–8E6–8C8; BD Bioscience, Franklin Lakes, NJ, USA), CD4a-PE (Mouse IgG2b, clone 74–12–4; BD Bioscience), CD8α-FITC (Mouse IgG2a, clone 76–2–11; BD Bioscience), TCR-γδ-APC (Rat IgG2a, clone MAC320; BD Bioscience), CD4a-FITC (Mouse IgG2b, clone 74–12–4; BD Bioscience), CD27-FITC (Mouse IgG1, clone B30C7; Bio-Rad antibodies, Shanghai, China), CD16-PE (Mouse IgG1, clone G7; Bio-Rad antibodies), CD172a-FITC (Mouse IgG1, clone BL1H7; Bio-Rad antibodies), The samples were incubated with the above directly conjugated antibodies, and single-color-stained samples were prepared for automatic compensation. Then, every sample subjected to flow cytometry was set up with a flow absolute count to record the events with a Beckman Cytoflex LX cytometer.

Intracellular and intranuclear staining

After erythrocyte lysis of the whole blood samples, the cells were washed and resuspended in complete cell culture medium, and then a leukocyte activation cocktail (BD Bioscience, catalog number: 550583) was added to all the intracellular staining groups (IFN-γ and perforin) rather than the virus restimulation. After 4 h, the stimulated cells were harvested for cell surface antigen staining as described above. A Cyto-Fast Fix/Perm Buffer Set (Biolegend, San Diego, CA) was used to fix and permeabilize the cells according to the manufacturer's

instructions, and the cells were subsequently incubated with the following mAbs: IFN-γ-Alexa Fluor 647 (mouse IgG1, clone P2G10; BD Bioscience) and perforin-APC (mouse IgG2b, clone dG9; eBioscience). Isotype controls, including an isotype control, Alexa Fluor 647 (mouse IgG1κ, clone MOPC-21; BD Bioscience), and an isotype control, APC (mouse IgG2b, clone eBMG2b; eBioscience), were used. The level of the transcription factor Foxp3 was determined via an anti-Foxp3-APC antibody (rat IgG2a, clone FJK-16 s; eBioscience) via a commercial intranuclear staining kit (Foxp3-Kit; eBioscience), and isotype control-APC (rat IgG2a, clone eBR2a; eBioscience) served as a negative control.

In vitro model establishment

The PAMs were allowed to recover to stable conditions the night before, and then the autologous PBMCs were separated from the peripheral blood of ASFV-free, clinically healthy pigs via porcine lymphocyte separation medium (TBDscience) through a SepMate™ tube (STEMCELL Technologies). Additional porcine IL-2 and IL-4 were added to the culture medium (10% FBS, 4% penicillin–streptomycin, and RPMI 1640) to maintain normal lymphocyte function in vitro (IL-2 and IL-4 stably expressing the PK15 cell line established by our laboratory, patent No. ZL 2021 1 1,364,146.0). Autologously isolated PBMCs were evenly seeded into adherent PAMs in 24-well plates at a density of 5×10^5/mL 48 h after the wells were treated with or without 0.01/0.1 multiplicity of infection (MOI) of the ASFV HB-2208 strain as the ASFV group or control group.

Data analysis

All the cell experiments were repeated at least three times. The data are presented as the means ± SEMs and were analyzed by Student's t test, one-way ANOVA or two-way ANOVA (analysis of variance) (means ± s.e.m.s, Student's t test, one-way ANOVA or two-way ANOVA, $^{*}P<0.05$; $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$). Graphs were plotted and analyzed via GraphPad Prism software (Prism 8, San Diego, CA, USA), and flow cytometry data were analyzed via CytExpert V3.0 (Beckman Coulter, Indianapolis, IN, USA) and FlowJo v10.8.1 software (Tree Star) (BD Biosciences, Franklin Lakes, NJ, USA).

Supplementary Information

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Supplementary Material 1.

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Authors' contributions
W.X. and M.C. wrote the manuscript with the support of J.Y. M.C. designed the experiments. W.X. performed the experiments with the help of H.C., Y.C., K.W., T.L., W.Z., Q.Y., X.G., and J.S. X.W. and Q.H. kindly provided the samples. M.C. and J.Y. revised the manuscript. All the authors read and approved the final manuscript.

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Data availability
The data will be shared upon reasonable request by the readers.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The author declares that he/she has no competing interests. Author Min Cui was not involved in the journal's review or decisions related to this manuscript.

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