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Local and Systemic T Cell Immunity in Fighting Pig Viral and Bacterial Infections

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Abstract

T cells are an essential component of the adaptive immune system. Over the last 15 years, a constantly growing toolbox with which to study T cell biology in pigs has allowed detailed investigations on these cells in various viral and bacterial infections. This review provides an overview on porcine CD4, CD8, and γδ T cells and the current knowledge on the differentiation of these cells following antigen encounter. Where available, the responses of these cells to viral infections like porcine reproductive and respiratory syndrome virus, classical swine fever virus, swine influenza A virus, and African swine fever virus are outlined. In addition, knowledge on the porcine T cell response to bacterial infections like *Actinobacillus pleuropneumoniae* and *Salmonella* Typhimurium is reviewed. For CD4 T cells, the response to the outlined infections is reflected toward the Th1/Th2/Th17/Tfh/Treg paradigm for functional differentiation.

1. INTRODUCTION

Detailed knowledge on the porcine immune system is highly relevant both for the use of this species as an animal model in biomedical research and because swine are a major source of animal protein (1–4). T cells together with B cells form the cellular components of the adaptive immune system (5). Whereas the major functional property of B cells is antibody production, T cells show a much higher degree of functional diversity. Based on the set of genes expressed to form the T cell receptor (TCR), αβ and γδ T cells can be differentiated. Within αβ T cells, the expression of TCR coreceptors is used to identify two major subsets: CD4 and CD8 T cells. Given the importance of the adaptive immune system in the control of many infectious diseases and its capacity for memory formation following vaccination or infection, this review outlines the current knowledge on T cells in the pig, with a focus on their role in bacterial and viral infections. Separate sections addressing CD4, CD8, and γδ T cells review what is known regarding the underlying biology of each respective subset, as well as its role in infection and vaccination.

2. CD4 T CELLS

2.1. Differentiation of CD4 T Cells Following Antigen Encounter

Similarities in human and porcine αβ T cell biology have been reviewed previously (6) and are updated here. One feature stands out when comparing human and porcine CD4 T cells: the upregulation of CD8αα homodimers in porcine extrathymic CD4 T cells following activation or antigen encounter. This peculiarity was first described in the second half of the 1980s (7, 8). Later on, numerous studies demonstrated that CD8α is upregulated on CD4 T cells following in vitro oligoclonal (9) or polyclonal (9–11) stimulation and that the frequency of CD8α-expressing CD4 T cells increases with age (12–19). Moreover, numerous studies showed that during in vitro recall experiments, most CD4 T cells responding via proliferation or cytokine production express CD8α (9, 11, 20–28). CD8α expression is therefore considered a marker for antigen-experienced porcine CD4 T cells; however, the functional relevance of CD8αα homodimer expression next to CD4 molecules is still unknown.

To further characterize porcine CD4 T cells, many other cell surface molecules have been investigated, including MHC-II (SLA-DR) (16, 27, 29), CD45RA or CD45RC (30–38), CD27 (16, 17, 22, 23, 39–42), CD28 (43), and CCR7 (11, 24–26, 32, 33). Studies on these molecules included ex vivo analyses of aging pigs (14, 16) and numerous investigations in infected and/or vaccinated animals (see Section 2.2.2 for details). Overall, it is currently assumed that phenotypes described in humans to identify central memory CD4 T cells (CD4 T_{CM}) and effector memory CD4 T cells (CD4 T_{EM}) also apply to pigs (6), with the major difference that CD8α (instead of CD45RA or CD45R0) expression can be used as a marker for CD4 T cells that have encountered their cognate antigen. With the help of functional experiments, Reutner et al. (11) concluded that $CD8\alpha^{-}CD27^{+}$ cells represent naïve $CD8\alpha^{+}CD27^{+}$ T_{CM} and $CD8\alpha^{+}CD27^{-}$ T_{EM} CD4 T cells. The authors also analyzed CCR7 (CD197) expression and found a largely overlapping expression pattern between CD27 and CCR7. Hence, more recently, the phenotypes $CD8\alpha^{-}CCR7^{+}$, $CD8\alpha^{+}CCR7^{+}$, and $CD8\alpha^{+}CCR7^{-}$ were used to identify naïve, central memory, and effector memory CD4 T cells, respectively. This was applied in porcine reproductive and respiratory syndrome virus (PRRSV) vaccination and infection studies (24, 25) but also in *Chlamydia suis* vaccination studies (26). One of these studies investigated the T cell response following PRRSV vaccination and infection. Next to other phenotypes, IFN-γ-producing CD4 T cells with a $CD8\alpha^{-}CCR7^{-}$ phenotype following in vitro restimulation with live PRRSV (25) were identified. The authors speculated that these $CD8\alpha^{-}CCR7^{-}$ CD4 T cells could be the porcine counterpart

of the rare human CD4 TEMRA (effector memory T cells that reexpress CD45RA) subset (44). This was further corroborated by ex vivo analyses showing the presence of this phenotype in CD4 T cells isolated from lung tissue and bronchoalveolar lavage (BAL) (3).

The lack of CD45RA or CD45RC has also frequently been used as a phenotype to identify antigen-experienced CD4 T cells in pigs (see above for references). However, CD45RA and CD45RC have rarely been investigated in combination with CD8α, CD27, CD28 and CCR7. Talker et al. (16) investigated phenotypic changes ex vivo in blood-derived T cells from birth to approximately six months of age. CD45RC$^-$ CD4 T cells were already found at the day of birth (~10–30% of total CD4 T cells). However, these cells had a CD8α$^-$CD27$^+$SLA-DR$^-$ phenotype, which indicates a naïve phenotype. This suggests that a CD4$^+$CD45RC$^-$ phenotype may not always indicate a memory or effector stage. SLA-DR is frequently expressed on antigen-experienced porcine CD4 T cells (see above for references), but not all CD8α-expressing CD4 T cells coexpress SLA-DR (16). Similar to that of CD8α, the functional relevance of SLA-DR expression on porcine CD4 T cells is not known. Recently, Milburn et al. (45) investigated CD9 expression on porcine leukocytes and found a positive correlation between CD9 expressing CD4 T cells and CD4 T_{CM} cell frequency. In addition, CD154 expression has been used to identify antigen-specific T cells in bacterial, fungal, and helminth infections (46).

In summary, several molecules have been studied to identify different stages of porcine CD4 T cell differentiation following antigen encounter, with the combination of CD8α and CD27 or CD8α and CCR7 being used most frequently to identify cells with T_{CM} and T_{EM} characteristics.

2.2. Functional Differentiation Following Antigen Encounter

From the 1980s toward the late 2000s, a series of seminal studies led to a paradigm regarding the functional differentiation of human and murine CD4 T cells that includes at least five different subsets: Th1, Th2, Th17, T-follicular helper (Tfh), and inducible regulatory T cells (iTregs) (reviewed in 47). However, a certain degree of plasticity between several subsets had already become clear; for example, Th2 cells can further differentiate into Th9 cells, iTreg into Th17 cells, and Th17 cells into Th1 cells (48, 49). More recently, Ruterbusch et al. (50) suggested that Tfh cell development bifurcates very early from the development of other effector Th subsets. In 2015, Gerner et al. (6) reviewed how these notions apply to porcine CD4 T cells, and we provide an update here.

2.2.1. General knowledge on Th1 cells. Th1 differentiation is driven by a cytokine milieu of IL-12, type I IFNs, and IFN-γ. This results in the expression of T-bet as the master regulator transcription factor and the production of the effector cytokines IFN-γ and TNF-α (reviewed in 51). Concerning the priming milieu for porcine Th1 CD4 T cells, Ebner et al. (52) investigated the influence of IL-12 in in vitro cultures and showed an increase in T-bet-expressing CD4 T cells. Many studies have investigated production of IFN-γ, and frequently coproduction of TNF-α, to characterize CD4 T cell responses in vaccinated or infected pigs (see below for details). In contrast, few studies have addressed T-bet expression (17, 52–54). Rodríguez-Gómez et al. (17) showed that ex vivo T-bet-expressing CD4 T cells have a CD8α^{high}CD27$^-$SLA-DR$^+$ phenotype, indicative of terminally differentiated effector or effector memory CD4 T cells. T-bet$^+$ CD4 T cells with this phenotype were present in blood, spleen, and lung tissue (5–30% of total CD4 T cells in ~6-month-old slaughterhouse pigs), but much lower frequencies were found in mediastinal lymph nodes. The analysis of CD4 T cells in pigs of different ages (2 weeks to approximately 4 years) showed a constant increase of CD8α-expressing cells with age, reminiscent of the accumulation of memory T cells also seen in human blood over the lifetime (55). At the same time,

T-bet-expressing CD4 T cells showed only a marginal increase in old pigs (4 years of age). This suggests that the increase in putative CD8α$^+$ CD4 memory T cells is also driven by other functional T cell subsets, which have not yet been elucidated. Other studies indicated that T-bet-expressing CD8α$^+$ CD4 T cells are already increased 14 days after birth in piglets with an intrauterine growth restriction (52) and after experimental infection with *Helicobacter pylori* (54). More recently, Schäfer et al. (53) investigated T-bet-expressing CD4 T cells in wild boars infected with the African swine fever virus (ASFV) strain Estonia2014. The authors found a decrease of T-bet-expressing CD4 T cells in the blood and an increase in the lung at 10 days post infection (dpi). Overall, these studies suggest that T-bet expression can be used to study changes in Th1 CD4 T cells in infections and metabolic disorders.

2.2.2. Porcine Th1 cells in viral infections. CD4 T cell responses have been studied in numerous viral infections in pigs, and IFN-γ production by these cells is considered here as the primary readout to identify bona fide Th1 cells.

2.2.2.1. Porcine circovirus type 2. Porcine circovirus type 2 (PCV2) is the smallest virus known to infect mammals (56). The elevated IL-10 levels widely observed in lymphoid tissues following PCV2 infection of pigs (reviewed in 57) are addressed below in the section on regulatory T cells. Concerning the potential involvement of Th1 responses, one early study indicated that higher levels of IFN-γ transcripts in peripheral blood mononuclear cells (PBMCs) correlate negatively with viral loads (58). Later on, IFN-γ ELISpot experiments with antibodies blocking CD4 or CD8α, as well as intracellular cytokine staining experiments for IFN-γ in combination with CD8α staining, suggested the presence of PCV2-specific, IFN-γ-producing CD4 T cells (59). Moreover, following both vaccination with open reading frame 2 (ORF2)-encoded capsid proteins and PCV2 infection, PCV2-capsid protein–specific, IFN-γ-producing, or IFN-γ/TNF-α-coproducing CD4 T cells are induced (28). The majority of these cells expressed CD8α and had a mixed phenotype for CD27, suggesting the generation of both T_{EM} and T_{CM} cells after both vaccination and infection. However, these cells' precise role in protecting pigs from clinical signs of PCV2 is unclear.

2.2.2.2. Foot-and-mouth disease virus. Another small RNA virus affecting pigs is foot-and-mouth disease virus (FMDV). In contrast to two other major host species, cattle and sheep, pigs do not become persistently infected with this virus but frequently show a high degree of morbidity following infection (reviewed in 60). Despite early efforts to identify T cell epitopes within the 8.5-kb genome of the virus (61), little work has been devoted to detailed investigations on the T cell response following infection. Early work indicated that CD4 T cells recognize overlapping peptides (27, 62), and a vaccine candidate based on a single B and a single T cell epitope arranged in dendrimers was effective in inducing IFN-γ/TNF-α-coproducing CD4 T cells (63). Previous work with this dendrimer vaccine approach did show protection in pigs challenged with FMDV. This may rely partially on T cell involvement, as indicated by proliferation and IFN-γ production of PBMC cultures restimulated with the peptide construct of the vaccine (64).

2.2.2.3. Classical swine fever virus. Classical swine fever virus (CSFV) has been studied intensely given its heavy economic impact, resulting from its status as a notifiable disease and related trade restrictions. Depending on the strain and host factors like age and genetic background, it causes high morbidity and mortality in infected pigs (reviewed in 65). Leukopenia and thymic atrophy, caused by lymphocyte apoptosis, are among the characteristics of the acute and peracute forms of the disease, resulting in substantial immunosuppression (66–68). Hence, studies on immune protection from CSFV have frequently focused on the live-attenuated C-strain, which

already shows protection if applied 5 days prior to challenge infection (69). Studies on the T cell response in C-strain-vaccinated and subsequently -infected animals showed a very moderate response of IFN-γ-producing CD4 T cells following in vitro restimulation with the challenge virus (70). Instead, IFN-γ-producing CD8 T cells dominated strongly among the IFN-γ-producing lymphocytes (41); see Section 3.2 for further details.

2.2.2.4. Porcine reproductive and respiratory syndrome virus. Another RNA virus that has been investigated intensely over the past ∼25 years is PRRSV. Two recent reviews address the immune response to PRRSV and provide a wider overview compared to what can be presented here (71, 72). Regarding Th1 responses to this infection, early work provided hints for a proliferation of CD4 T cells from PRRSV-infected pigs following in vitro restimulation with the virus (73). IFN-γ ELISpot assays have been employed frequently to study the cellular immune response to PRRSV (reviewed in 71), but they do not allow immediate conclusions on Th1 cells. Moreover, no positive or negative correlation between the frequency of IFN-γ-producing lymphocytes and viral loads in lung or lymphoid tissues was found (74). More recently, IFN-γ production was investigated in combination with CD4 expression (11, 24, 25, 75). In particular, Kick et al. (24) analyzed in detail the proliferation of CD4 T cells in combination with production of IFN-γ, TNF-α, and IL-2. Three different groups of pigs were either vaccinated with a modified live virus vaccine or infected with two different PRRSV-2 field isolates. The authors found a robust proliferation of CD4 T cells following in vitro restimulation with homologous and heterologous viral strains in the investigated time span from 14 to 56 dpi. Analysis of cytokine production at 28 dpi revealed PRRSV-reactive IFN-γ-producing CD4 T cells in the blood, which partially coproduced TNF-α but not IL-2. The majority of these PRRSV-reactive CD4 T cells were classified as effector memory cells based on a $CD8\alpha^{+}CCR7^{-}$ phenotype (24). This differs from a study that analyzed PRRSV-peptide-specific IFN-γ^{+} CD4 T cells, which mainly had a $CD27^{+}$ phenotype (75), indicative of CD4 T_{CM} cells. In Kick et al.'s (24) study, at 28 dpi, PRRSV viremia levels were still high in the investigated animals and started declining thereafter. A correlation analysis showed a positive correlation between the reduction of PRRSV viremia 21–35 dpi and the in vitro proliferation rate of CD4 T cells (excluding Tregs) isolated 28 dpi and restimulated with homologous PRRSV, suggesting some relevance of these cells in protection. However, no such correlation was found for cytokine-producing CD4 T cells and viremia levels. In contrast, a recent study involving authors from this review found a negative correlation between the frequency of IFN-γ-single and IFN-γ/TNF-α-coproducing blood-derived CD4 T cells and viral load in lung tissue 14 days post challenge infection. The pigs in that experiment had been vaccinated with a PRRSV-1 modified live virus vaccine 28 days prior to challenge infection (A. Pierron, E. Vatzia, M. Stadler, K.H. Mair, S. Schmidt, M. Stas, S. Dürlinger, H. Kreutzmann, C. Knecht, J. Lagler, M. Zaruba, T. Rümenapf, A. Saalmüller, A. Ladinig, E. Mayer & W. Gerner, manuscript in preparation). In summary, despite numerous studies on the CD4 T cell response following PRRSV vaccination or infection, their contribution to a protective immune response is unclear.

2.2.2.5. Swine influenza A virus. Swine influenza A virus (swIAV) is an RNA virus for which the T cell response in pigs has been studied in considerable detail over the past ∼10 years (reviewed also in 76). For CD4 T cells, an increase of $CD4^{+}CD8\alpha^{+}$ cells in BAL and tonsils 6 dpi with pandemic (pdm) H1N1 (combined intranasal and intratracheal inoculation) could be shown, although the increase of this phenotype in the lung tissue was only marginal (77). A later study, applying an intratracheal H1N2 infection, found an increase in Ki-$67^{+}CD8\alpha^{+}$ CD4 T cells in tracheobronchial lymph nodes (TBLN) at 4 dpi (23). This was accompanied by the appearance of cytokine-producing (IFN-γ, TNF-α, IL-2, and combinations thereof) CD4 T cells in blood, lung

tissue, and TBLN from 4 dpi onward, with the highest levels of cytokine-producing cells found 9–12 dpi. Edmans et al. (78) found very similar results for cytokine production in CD4 T cells, also showing the influx of such cells into BAL from 5 dpi onward, in both outbred and Babraham pigs following an intranasal pdmH1N1 infection. Moreover, several studies have focused on the T cell response following the immunization of pigs with live attenuated influenza vaccines. A non-structural protein 1 truncated H3N2 live attenuated influenza vaccine did elicit IFN-γ production in $CD8\alpha^+$ and $CD8\alpha^-$ blood-derived CD4 T cells 28 days post intranasal application (79). More recently, the S-FLU vaccine candidate, which is limited to a single cycle of replication through inactivation of the hemagglutinin signal sequence, was intensely tested in pigs (21, 80–83). This vaccine induces a very strong CD8 T cell response (see Section 3.2) but also induces cytokine-producing CD4 T cells, both locally and systemically, after intranasal, aerosol, or intratracheal application (20, 80, 82, 83). Hence, CD4 T cells are generated in response to swIAV infection and vaccination, but their contribution in protection from infection is not understood fully.

2.2.3. Knowledge on Th2 cells and their role in helminth infections. Although GATA-3 has been identified as a master regulator for Th2, this transcription factor is also involved in T cell development, proliferation, and maintenance (reviewed in 84). In this light, Rodríguez-Gómez et al. (17) identified low levels of GATA-3 expression in total porcine CD4 T cells isolated from thymus, blood, spleen, lymph nodes, and lung tissue of healthy pigs. Nevertheless, Ebner et al. (52) showed an increase of GATA-3 expression in a subset of CD4 T cells following in vitro stimulation with ConA+IL-4, which was absent when cells were stimulated with ConA alone or ConA+IL-12, suggesting that porcine Th2 cells develop under similar conditions as murine and human Th2 cells. In the context of helminth infections, the most prevalent pig helminths are *Ascaris suum* and *Trichuris suis*. Both worms have a direct life cycle and have been reported to stimulate porcine immunity toward a Th2 response (reviewed in 85). Indeed, real-time PCR (polymerase chain reaction) analysis of colon tissue and colon lymph nodes sampled from *T. suis*–inoculated pigs revealed a Th2-polarized gene expression pattern accompanied by a downregulation of Th1-related genes (86, 87). Similarly, a Th2-associated gene expression pattern was observed in various tissues in response to *A. suum* infection (88, 89). More directly related to bona fide Th2 cells, parasite-specific IL-4-producing cells have been identified by ELISpot following infection with *A. suum* and *T. suis*, respectively (90). Parasitic worms release so-called excretory secretory products into their microenvironment, which can modulate the host immune system (91). Splenocytes from *T. suis*–infected pigs were enriched for $GATA\text{-}3^+$ CD4 T cells after in vitro restimulation with *T. suis* excretory secretory products (52). For the pork tapeworm *Taenia solium*, a study with naturally infected swine showed that viable parasite stages in tissues coincided with high levels of IL-4 and IL-10 transcripts in the surrounding tissue (92). Recently, pigs infected with *Trichinella spiralis* were shown to exhibit elevated serum levels of IL-4 and IL-10, but also IL-17A (93). Taken together, available data on porcine helminth infections suggest that these parasites modulate the host immune response by evoking Th2 and regulatory immune responses. However, a more detailed phenotyping and functional investigation of porcine Th2 cells would be highly desirable.

2.2.4. General knowledge on porcine Th17 cells. Th17 cells are characterized by the production of IL-17A, IL-17F, IL-21, and IL-22 and expression of the transcription factor ROR-γt (94). The discovery of cross-reactive anti-IL-17A monoclonal antibodies (mAbs) has allowed the identification of porcine $CD4^+$ IL-17A-producing cells after phorbol-12-myristate-13-acetate (PMA)/ionomycin stimulation (37, 95). Via the use of a potentially cross-reactive antibody against ROR-γt, one study showed up to 15% of ROR-γt^+ cells within total CD4 T cells in the blood

of 4- to 10-week-old pigs (54). However, the authors of this review could not reproduce these findings (K.H. Mair, unpublished findings), as much lower frequencies were found in lymphatic and nonlymphatic organs. Similar to in mice, naïve porcine $CD4^+$ thymocytes seem to require TGF-β along with IL-6 and/or IL-1β to differentiate into Th17 cells (96). Recent years have seen a rise in published studies investigating the involvement of Th17 cells in bacterial infections in swine, as outlined below.

2.2.5. Porcine Th17 cells in bacterial infections. *Actinobacillus pleuropneumoniae* (APP) is the cause of the respiratory disease porcine pleuropneumoniae. Upregulation of IL-17A gene expression in response to APP was first described in the lung of APP-infected pigs during the acute phase of infection (97). A later study investigated blood- and organ-derived lymphocytes from pigs intranasally infected with APP serotype 2 that were restimulated in vitro with APP crude capsular extract (CCE) (98). In lung and blood of both acutely and chronically infected pigs, the authors found APP-CCE specific IL17A-producing $CD4^+CD8\alpha^{dim}$ T cells, the frequencies of which also correlated with lung lesions and antibody titers. Although this correlation was observed only in the chronic phase of infection, it suggests a possible role of Th17 cells in APP infection.

F4 fimbriae expressing enterotoxigenic *Escherichia coli* (ETEC) are associated with both postweaning diarrhea and diarrhea in neonatal pigs (99). Infection of piglets with $F4^+$ ETEC induced upregulation of IL-17A, IL-17F, IL-21, and IL-23p19 messenger RNA (mRNA) in the intestine and PBMC, whereas levels of Th1-associated genes remained largely unchanged (100). Another enteric bacterium, *Salmonella* Typhimurium (STM), has high zoonotic potential and leads to enterocolitis in humans and pigs while often persisting in subclinical carrier animals. A recent study observed a significant increase in IL-17 and IFN-γ gene expression in the ceca of STM-infected pigs at 2 dpi (101). This corresponds to data from other studies in which STM vaccination and/or infection in pigs induced $IL\text{-}17A^+$ multifunctional cytokine phenotypes such as IFN-γ/IL-17A, TNF-α/IL-17A, and IFN-γ/TNF-α/IL-17A coproducing $CD4^+$ T cells in various organs, but with highest frequencies in ileocolic lymph nodes and gut tissue (39, 102).

Another bacterial pathogen species that may induce multifunctional $CD4^+$ T cells is chlamydia, though reports on Th17 involvement are contradictory. One study restimulated porcine PBMC from inoculated pigs with *C. suis* and *Chlamydia trachomatis* antigen and found IL-17A-producing $CD4^+$ T cells to be largely absent (103). However, a significant IFN-γ and IL-17 response was measured in supernatants of PBMC and lymph node–derived lymphocytes after restimulation with vaccine antigen in a Göttingen minipig model in which pigs were immunized intramuscularly with a multisubunit vaccine with recombinant *C. trachomatis* antigen (104). However, because cytokine production was not measured on the single-cell level, IL-17A/IFN-γ-coproducing cells were not addressed in this study.

In summary, data show both the existence of Th17 cells within porcine CD4 T cells and multifunctional CD4 T cells that coproduce cytokines of the Th1 and Th17 lineage. In comparing APP and STM, this seems to correlate with the extracellular and facultative intracellular lifestyle of these pathogens, respectively.

2.2.6. Porcine Tfh cells. During the 2000s it became clear that Tfh cells form a separate functional CD4 T cell subset. In the following years a lot of research has focused on these cells (reviewed in 50, 105). However, porcine Tfh cells have been barely described. One study described the presence of Bcl-6-stained cells in germinal centers of porcine lymph nodes by immunohistochemistry (106). Another publication reports on these cells in the context of STM (107). The authors stimulated porcine splenocytes in vitro with live STM and ConA for 4 days, followed

by a final PMA/ionomycin stimulation. After this stimulation, up to 15% of $CD4^+$ splenocytes obtained a Bcl-6^+IL-21^+ phenotype. Lower frequencies of cells with this phenotype could be induced by TLR8 agonists and ConA. In the same study, this phenotype of Bcl-6^+IL-21^+ CD4 T cells was also enriched in the draining lymph nodes of pigs 30 days after subcutaneous application of a live STM vaccine. Recent work from the authors of this review showed the existence of Bcl-6^+CD79α^+ B cells and Bcl-$6^+$$ICOS^+$ CD4 T cells in the lymph nodes of healthy 6-month-old pigs (A. Hoog, S. Villanueva-Hernández, M. Adib Razavi, K. van Dongen, M. Stadler, K. de Luca, K.H. Mair & W. Gerner, manuscript in preparation). However, to the best of our knowledge, no further infection or vaccination studies addressing these cells have been published.

2.2.7. Porcine Treg cells. Tregs in humans that express the coreceptor CD4 are identified by a $CD25^{high}Foxp3^+$ phenotype (reviewed in 108). This phenotype, including its functional hallmarks of suppressing the proliferation of other T cells and IL-10 production, could also be confirmed in the pig (109–111). Two major subsets of CD4 Tregs can be distinguished. First, natural or thymic (t)Tregs have been identified. These cells typically show some degree of TCR reactivity with endogenous self-ligands and develop already in the thymus (reviewed in 112). Second, induced or peripheral (p)Tregs exist, which leave the thymus as naïve T cells and differentiate in the periphery into Tregs following encounter with their cognate antigen, which is typically nonself (reviewed in 113). One potential marker to separate pTregs from tTregs is the transcription factor Helios, which is expressed by tTregs (114). Helios expression was also investigated in porcine Tregs (115), and it could be confirmed that all thymic $Foxp3^+$ T cells coexpress Helios. However, blood-derived $Foxp3^+Helios^-$ CD4 Tregs, which should represent pTregs, partially had a $CD8\alpha^-CD27^+$ naïve phenotype, which conflicts with the notion that pTregs have encountered their cognate antigen and should therefore have an effector or memory phenotype. Further, in relation to the phenotype of porcine Tregs, one study showed that porcine $Foxp3^+$ CD4 T cells partially coexpress CCR4 (116). Based on studies with human Tregs, Wang et al. (116) postulated that $CCR4^+Foxp3^+$ Tregs represent effector Tregs.

2.2.8. Tregs in infection. Porcine Tregs have been investigated in various viral infections. As mentioned above, elevated IL-10 levels in the lymphoid tissue of PCV2-infected pigs were postulated to indicate aberrant Treg activity (57). However, the cellular source of the IL-10 is still not elucidated fully. One in situ study with infected pigs found IL-10 production mainly in T cell–rich areas of mandibular lymph nodes, spleens, and tonsils (117). In a similar study, an enrichment of IL-10-producing cells was found in the spleen of PCV2-infected pigs, and immunofluorescence histology indicated that both $CD163^+$ monocytes/macrophages and $CD4^+$ and $CD8\alpha^+$ cells could produce IL-10 (118). A subsequent in vitro study found high levels of IL-10 transcripts in monocyte-derived dendritic cells (moDCs) following infection with PCV2 or a combination of PCV2 and PRRSV, suggesting that elevated IL-10 production might result from myeloid cells (119).

The role of Tregs was also studied in PRRS. A 2012 report found an increase in $CD25^+Foxp3^+$ Tregs in the blood over time after infection with a PRRSV-2 strain (120). However, when this phenotype was studied over a similar time course in mediastinal lymph nodes and tonsils, the findings were inconsistent (120). A more recent study showed an accumulation of $CD4^+Foxp3^+$IL-10^+ cells in the lung tissue of PRRSV-infected pigs via immunofluorescence histology (121). Several studies also showed the induction of a Treg phenotype in CD4 T cells isolated from PRRSV-naïve pigs after cocultivation with PRRSV-infected moDCs (122, 123) or in lymphocyte bulk cultures from lung and TBLN (121). However, the in vitro induction of Tregs by infected moDCs could not always be reproduced (124). Kick et al. (24) investigated Treg proliferation after PRRSV

restimulation in vitro. Foxp3$^+$ CD4 Tregs showed similar proliferation rates as Foxp3$^-$ CD4 T cells, isolated and tested over a time course from 14 to 56 dpi. The same applied when the response to heterologous PRRSV-2 strains was compared at 28 dpi in the blood or at 63 dpi in TBLN. Of note, the authors did find a positive correlation between the reduction of PRRSV viremia from 21 to 35 dpi and the in vitro proliferation rate of Tregs isolated 28 dpi and restimulated with homologous PRRSV. This coincides with the same finding for proliferating non-Treg CD4 T cells, making Tregs' role in PRRSV infection unclear.

IL-10 production was also analyzed in the context of APP infection (98, 125). When cells isolated from the blood, TBLN, tonsils, and lung tissue of APP-infected pigs were restimulated in vitro with APP-CCE (see also Section 2.2.5 on Th17 cells), IL-10-producing CD4 T cells could be identified in the different organs. However, the frequency of IL-10-producing CD4 T cells showed substantial animal-to-animal variation and was rather low in tonsils (98). Of note, when tonsil tissue homogenates were investigated for IL-10 mRNA, higher IL-10 levels were found in tonsils of animals that also tested positive for APP at the time of necropsy (125). This suggests that putative pTregs, and possibly other cell types, show IL-10 production in APP-infected pigs.

In summary, despite considerable work on the function of porcine CD4 T cells in viral and bacterial infections, much remains unknown about their contribution to protection or even immunopathology, as well as immune evasion. **Figure 1** summarizes the current knowledge on porcine CD4 T cells and their cytokine production profile in the aforementioned infections.

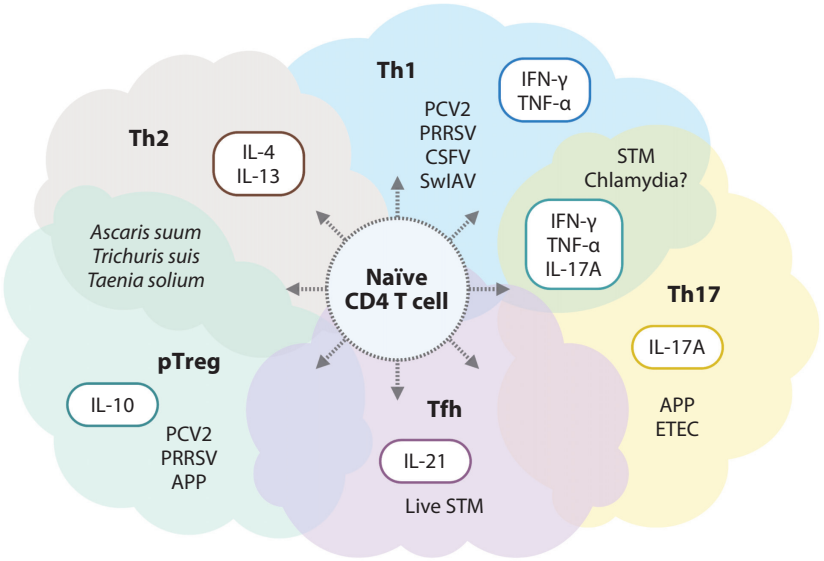

Figure 1

Functional differentiation of porcine CD4 T cells. Overview of functional CD4 T cells and their cytokine production profile based on published data. Pathogens for which the respective cytokine profiles have been identified are indicated. For chlamydia, conflicting data exist regarding IFN-γ and IL-17A coproduction. For IL-4 and IL-13, identification is based on messenger RNA in tissues or tissue homogenates. Direct identification of these cytokines on the single-cell level for the listed helminths is lacking. Abbreviations: APP, *Actinobacillus pleuropneumoniae*; CSFV, classical swine fever virus; ETEC, enterotoxigenic *Escherichia coli*; PCV2, porcine circovirus type 2; PRRSV, porcine reproductive and respiratory syndrome virus; pTreg, peripheral regulatory T cell; STM, *Salmonella* Typhimurium; SwIAV, swine influenza A virus; Tfh, T-follicular helper cell. Figure adapted from images created with BioRender.com.

3. CD8 T CELLS

3.1. Differentiation of CD8 T Cells Following Antigen Encounter

Phenotypic changes in porcine CD8 T cells following antigen encounter have been investigated less thoroughly compared to CD4 T cells. Talker et al.'s (16) study on phenotypic changes of blood-derived T cells from birth to 6 months suggested that naïve CD8 T cells (identified by expression of CD8αβ heterodimers) are $CD27^{+}$SLA-$DR^{-}$$perforin^{-}$, because this phenotype accounted for approximately 95% of CD8 T cells at the day of birth. The first change was then an increase of $CD27^{+}$SLA-$DR^{+}$$perforin^{-}$ CD8 T cells at approximately three weeks of age, which was still prior to weaning of the piglets. However, whether this phenotype is indicative of CD8 T cells that have encountered their cognate antigen is not clear. Instead, at around 7 weeks of age, higher numbers of $CD27^{dim}$SLA-$DR^{+}$$perforin^{+}$ cells were observed, which might represent early effector CD8 T cells, changing later into a $CD27^{-}$SLA-$DR^{+}$$perforin^{+}$ phenotype. In accordance with this, Moreno et al. (33) reported that blood-derived CD8 T cells could be subdivided into a $CD11a^{dim}$ and a $CD11a^{high}$ subset, of which the latter contained all $perforin^{+}$ CD8 T cells. Recent studies by the authors of this review also confirmed this correlation of $CD11a^{high}$ and perforin in CD8 T cells (J.V. Milburn & W. Gerner, unpublished findings). Moreno et al. (33) showed that $CD11a^{dim}$ CD8 T cells are primarily $CCR7^{+}CD45RA^{+}$, leading to the overall conclusion that naïve CD8 T cells in the pig are most probably $CD11a^{dim}CD27^{+}CCR7^{+}CD45RA^{+}$SLA-$DR^{-}perforin^{-}$. Recent work on this topic at The Pirbright Institute confirmed the $CCR7^{+}CD45RA^{+}$ phenotype for naïve CD8 T cells. Moreover, in lung tissue and BAL, $CCR7^{-}CD45RA^{-}$ CD8 T cells prevail (V. Martini, M. Edmans, S. Gubbins, S. Jayaraman, B. Paudyal, S. Morgan, A. McNee, T. Morin, P. Rija, W. Gerner, A.K. Sewell, R. Inoue, M. Bailey, T. Connelley, B. Charleston, A. Townsend, P. Beverley & E. Tchilian, manuscript submitted), reminiscent of the T_{EM} phenotype described for human CD8 T cells (126). In lung tissue, substantial proportions of $CCR7^{-}CD45RA^{+}$ CD8 T cells were also found, which in humans have been designated as TEMRA or terminally differentiated effector cells (127, 128). When the four different porcine CCR7/CD45RA CD8 T cell subsets were tested for IFN-γ and TNF-α production, the $CCR7^{-}CD45RA^{-}$ and $CCR7^{-}CD45RA^{+}$ had the highest capacity for cytokine production (V. Martini, M. Edmans, S. Gubbins, S. Jayaraman, B. Paudyal, S. Morgan, A. McNee, T. Morin, P. Rija, W. Gerner, A.K. Sewell, R. Inoue, M. Bailey, T. Connelley, B. Charleston, A. Townsend, P. Beverley & E. Tchilian, manuscript submitted), consistent with the concept that these phenotypes represent the most terminally differentiated CD8 T cells. **Figure 2** summarizes these postulated phenotypic changes that identify different stages of CD8 T cell differentiation.

Expression of the transcription factors T-bet and Eomesodermin (Eomes) was also studied in porcine CD8 T cells (17). $T\text{-}bet^{+}$ CD8 T cells had a $perforin^{+}CD27^{dim/-}$ phenotype, whereas $Eomes^{+}$ CD8 T cells were mostly $CD27^{high}perforin^{-}$. Although this suggests that T-bet expression correlates with an effector phenotype in porcine CD8 T cells, the study did not address T-bet/Eomes coexpression. This would have been important, because the current notion is that CD8 T cells can perform effector functions like IFN-γ production in the absence of T-bet but under the control of Eomes (reviewed in 129).

3.2. Porcine CD8 T Cells in Viral Infections

Owing to space restrictions, only CSFV, PRRSV, swIAV, and ASFV are addressed in the following, for which published data are most abundant.

For CSFV, vaccination with the live attenuated C-strain results in the priming of IFN-γ-producing CD8 T cells, which appear to contribute to protection based on their appearance prior to neutralizing antibodies and a negative correlation to viral load (69). A follow-up study showed

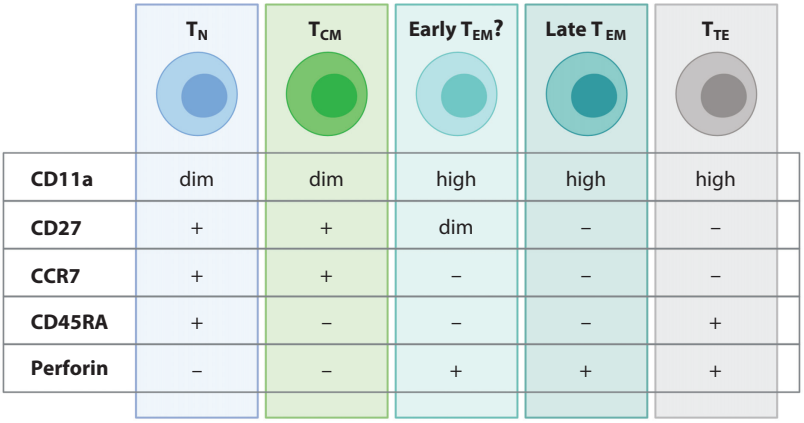

Figure 2

Changes in cell surface molecules of porcine CD8 T cells following antigen encounter. Changes in the expression of selected cell surface markers are indicated. The early T_{EM} phenotype is hypothesized based on results with subsets of the given molecules; a final experimental proof for its existence with all given markers is lacking. Abbreviations: T_N, naïve T cell; T_{CM}, central memory T cell; T_{EM}, effector memory T cell; T_{TE}, terminal effector T cell. Figure adapted from images created with BioRender.com.

that some of these IFN-γ-producing CD8 T cells are multifunctional; i.e., they produce in addition TNF-α and IL-2 (41). Moreover, some of the IFN-γ-producing cells also expressed CD107a, which might indicate their capacity for cytotoxic activity. The same group also showed that overlapping pentadecapeptides, spanning the entire C-strain polyprotein sequence, were recognized primarily by CD8 T cells, using IFN-γ production as a read-out (130). This coincides with earlier findings showing in in vitro killing assays the cytotoxic activity of CD8 T cells in PBMC derived from $SLA^{d/d}$-haplotype miniature pigs that had been infected repeatedly with CSFV strains of different virulence (131).

Following in vitro stimulation with PRRSV, IFN-γ-producing CD8 T cells were identified in several studies, in blood (75, 132) but also in TBLNs, lung tissue, and BAL (24, 25). However, one early study performed an in vivo depletion of CD8α-expressing cells and found no increase in PRRSV viral loads in depleted pigs in the early phase of infection (until 8 dpi), when the depletion was effective (133). Considering the late reactivity of CD4 and CD8 T cells following PRRSV infection, this might have been too early to draw conclusions on the importance of CD8 T cells in fighting PRRSV infections.

Several studies have investigated CD8 T cell responses to swIAV. Khatri et al. (77) showed an increase of total CD8 T cells and $CD3^+CD8^+CD25^+$ T cells in the BAL 6 dpi with pdmH1N1 (combined intranasal and intratracheal inoculation). Talker et al. (23) later described the appearance of Ki-67^+perforin$^+CD27^+$ CD8 T cells in lung tissue from 4 dpi, with a peak of this phenotype at 6 dpi, in intratracheally H1N2-infected pigs. The authors also analyzed IFN-γ-, IL-2-, and TNF-α-producing CD8 T cells and found cytokine-producing (IFN-γ-single, TNF-α-single, and IFN-γ/TNF-α-coproducing) CD8 T cells in lung tissue in considerable numbers from 6 dpi onward. SwIAV-reactive CD8 T cell numbers in TBLN and blood were much lower in this study, suggesting a strong homing potential of effector CD8 T cells to the lung. Edmans et al. (78) performed an even more detailed study on this, analyzing the CD8 T cell response in intranasally pdmH1N1-infected Babraham and outbred pigs. The authors found similar kinetics of IFN-γ- and TNF-α-producing CD8 T cells in lung tissue, TBLN, and blood as Talker et al. (23), but they also analyzed the BAL and could show an even higher frequency of swIAV-reactive CD8 T cells

in the lavage. Of note, this was also the only sample type in which a considerable number of IL-2-producing CD8 T cells were found, and nearly all of these cells coproduced IFN-γ and TNF-α.

Next to some data on adenovirus-vector vaccination and FMDV challenge infection (134), swIAV is the only viral infection for which tetramers for the labeling of antigen-specific porcine CD8 T cells have been used to a large extent. Pedersen et al. (135) used the NetMHCpan algorithm for epitope prediction in the context of the SLA-1*04:01 allele; they successfully identified four peptides from different viral proteins that bound to SLA-1*04:01 tetramers and could be used to identify peptide-specific CD8 T cells. Using the Babraham inbred pig, Tungatt et al. (81) tested overlapping peptides of the influenza virus nucleoprotein (NP) for epitope identification. Two NP epitopes that bound to SLA-1*14:02 and two that bound to SLA-2*11:04 were identified and could be used to label CD8 T cells in S-FLU-immunized or pdmH1N1-infected Babraham pigs. Moreover, HA-, neuraminidase-, and M1-derived epitopes that bound to SLA-1*07:02 could be used for tetramer labeling (136).

The NP tetramers suitable for the Babraham pigs were deployed to study epitope-specific CD8 T cell response after pdmH1N1 infection and S-FLU vaccination. In the pdmH1N1-infected pigs (78), a tetramer loaded with $NP_{290–298}$ was used. The highest numbers of tetramer$^+$ CD8 T cells were found in BAL 9 dpi, with tetramer-labeled CD8 T cells accounting for ∼4% of total CD8 T cells. Frequencies in lung tissue were lower (∼1–2%) but at later time points (14 dpi onward), owing to a decrease of tetramer$^+$ cells in BAL, tetramer-labeled cells in BAL and lung tissue reached similar levels. Of note, tetramer-stained CD8 T cells in blood were much lower, ∼0.1–0.2%, again demonstrating these swIAV-specific CD8 T cells' strong propensity for lung homing and capacity to acquire tissue residency (see below). In a related study, Martini et al. (82) used $NP_{217–225}$ tetramers in intranasally infected Babraham pigs to investigate CD8 T cells from BAL, lung tissue, trachea, and nasal turbinate. At 21 dpi, the highest frequencies of tetramer$^+$ CD8 T cells were again found in the BAL (82). The authors also investigated tetramer$^+$ CD8 T cells for their CCR7/CD27 phenotype, and the vast majority of cells were CCR7$^-$, although a mixture of CD27$^+$ and CD27$^-$ cells was found. This confirms previous observations in which effector CD8 T cells, identified by perforin expression, were either CD27^{dim} or CD27$^-$ (16). Immunization and challenge studies with the S-FLU vaccine hinted that these CD8 T cells (potentially in combination with CD4 T cells) are relevant in protection from swIAV infection (21, 80). When this vaccine was applied via aerosol or intratracheally, no neutralizing antibodies were generated in the serum. Nevertheless, when the vaccine was applied via these routes, it induced cytokine-producing (IFN-γ, TNF-α, and a combination of both) CD4 and CD8 T cells in BAL, TBLN, and lung tissue. Challenged pigs had reduced viral loads in lung and nasal swabs (21) or reduced pathology (80) to heterosubtypic challenge virus. This was confirmed more recently for aerosol application of the vaccine, whereas a combination of S-FLU aerosol and intramuscular application also induced neutralizing antibodies and stronger reductions in pathology and viral loads compared to aerosol application alone (83). Moreover, this study showed again the S-FLU vaccine's potential to induce CD8 effector cells in BAL, lung tissue, trachea, and nasal turbinates, identified by NP-tetramer staining and the capacity for IFN-γ/TNF-α/IL-2 production (see the sidebar titled Porcine T Cells as Correlates of Protection?).

For ASFV, a large double-stranded DNA virus with a genome ∼170–193 kb, it was concluded in the 1990s and 2000s that CD8 T cells seem to be involved in protective immune responses (reviewed in 137). Takamatsu et al. (137) also provide evidence that in the majority of pigs immunized with the nonvirulent OURT88/3 isolate, a peculiar CD4$^+$CD8α$^+$perforin$^+$ phenotype dominates within proliferating T cells following in vitro restimulation with the virus. The presence of this phenotype in the in vitro cultures correlated with protection from a challenge infection

PORCINE T CELLS AS CORRELATES OF PROTECTION?

Correlates of protection are an essential parameter for the prediction of vaccine efficacy. They also serve as a guideline in the development of novel vaccines and for vaccine improvement. From the studies outlined in this review, it can be seen that for many infections in pigs the ultimate contribution of T cells to protective immunity is still not clear. However, some conclusions can be drawn from the studies using the influenza A vaccine candidate S-FLU (21, 80–83). When this vaccine was applied via mucosal surfaces, no or very low neutralizing antibody titers were induced (21, 80, 83). However, the induction of local effector CD4 and CD8 T cells was shown, in particular in the BAL (see Sections 2.2.2 and 3.2 for details). Following challenge infection, S-FLU could reduce lung pathology (76, 82) or viral titers in nasal swabs and lung (21), demonstrating T cells' capacity to fight the virus.

Other possibilities to identify the role of T cells in protection would be via in vivo depletion of T cell subsets, but in large animals such as pigs these experiments are expensive and have rarely been performed (133, 153). Another possibility would be adoptive transfer studies using, for example, the inbred Babraham pig (154).

with the virulent OURT88/1 strain. However, recent ex vivo analyses of perforin-expressing $CD4^{-}CD8\alpha^{+}CD8\beta^{-}$ and $CD4^{-}CD8\alpha^{+}CD8\beta^{+}$ αβ T cells from domestic pigs and wild boars infected with the moderately virulent ASFV strain Estonia2014 showed no increase of perforin-expressing cells with these phenotypes (53). Instead, there was a decrease of $perforin^{+}$ cells with these phenotypes during the time the animals showed clinical signs of disease (4 and 5 dpi) in all investigated organs, i.e., blood, spleen, liver, and gastrohepatic lymph nodes. Perforin-expressing CD4 T cells were not addressed in this study. Similarly, in an infection study by the same group using the highly virulent Armenia08 ASFV strain, perforin-expressing T cells were diminished 5 dpi in spleen, liver, and gastrohepatic lymph nodes but returned to preinfection levels 7 dpi (138). Hence, T cells' role in infections with ASFV strains of different virulence is not understood fully.

4. TISSUE-RESIDENT αβ T CELLS AND THEIR ROLE IN INFECTIONS

Tissue residency was first described for herpes simplex virus–specific CD8 T cells in the skin and for lymphocytic choriomeningitis virus–specific CD8 T cells in the small intestine of mice (reviewed in 139, 140). It is now clear that most leukocyte lineages have the capacity for tissue residency (reviewed in 141). However, research on tissue-resident (Trm) T cells in pigs is still very limited. This may result from a lack of antibodies to identify these cells. The combined expression of CD69 and CD103 has been used frequently to identify Trm cells, although not all Trm cells have this phenotype (reviewed in 140). Data on the generation of an antibody against porcine CD69 have been published (142), but to our knowledge this antibody has not been commercialized. For the αE integrin CD103, a cross-reactive antibody has been reported (143), but it was not cross-reactive in our work on porcine T cells (K.H. Mair & W. Gerner, unpublished findings).

Tchilian and colleagues (80, 83) took a different approach to identify Trm in the pig. They infused anti-CD3 mAbs intravenously 10 min prior to euthanasia of the pigs and isolated cells from various tissues and BAL. After isolation, cells were labeled with a second anti-CD3 mAb, conjugated to a different fluorochrome. In this way, single-labeled Trm cells could be visualized, whereas circulating T cells showed a colabeling by both CD3 mAbs. More than 80% of cells in the BAL and approximately 38% of cells in the trachea were Trm cells (83). In contrast, lung tissue preparations were dominated by circulating T cells (>90%). When these cells were checked for swIAV specificity by tetramers (see above for details), the vast majority of tetramer-labeled cells were identified as Trm cells according to the CD3 labeling pattern (V. Martini, M. Edmans,

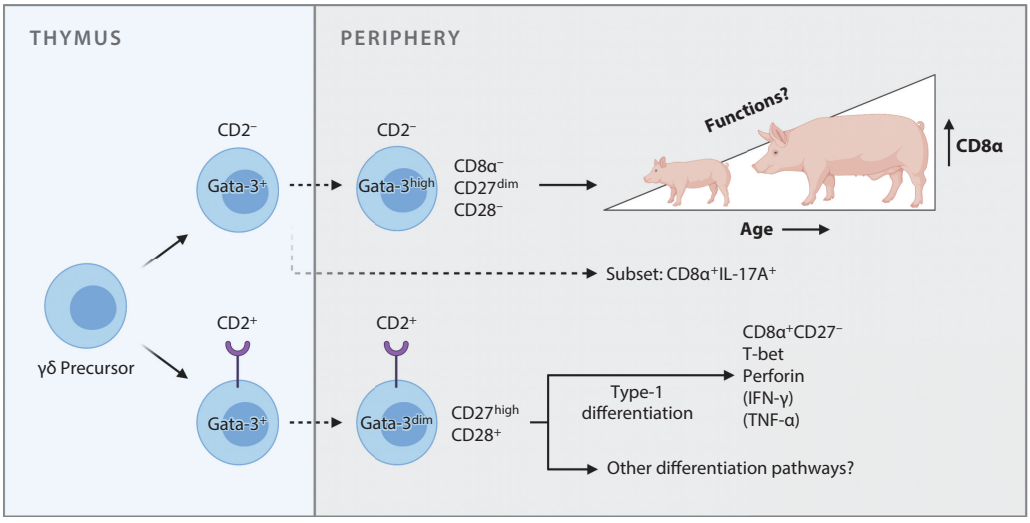

Figure 3

Summary of phenotypic and functional development of porcine γδ T cells. $CD2^+$ and $CD2^-$ develop as separate lineages in the thymus. A subset of $CD2^-$ IL-17A-producing γδ T cells may develop already in the thymus, but experimental evidence for this is lacking. For $CD2^-$ γδ T cells, phenotypic changes in the blood during aging are described. There are currently no data on the function of this γδ T cell subset. Within $CD2^+$ γδ T cells, a substantial subset seems to undergo a type-1 differentiation. Other functional differentiation pathways for $CD2^+$ γδ T cells may exist but have not yet been described. Figure adapted from images created with BioRender.com.

S. Gubbins, S. Jayaraman, B. Paudyal, S. Morgan, A. McNee, T. Morin, P. Rija, W. Gerner, A.K. Sewell, R. Inoue, M. Bailey, T. Connelley, B. Charleston, A. Townsend, P. Beverley & E. Tchilian, manuscript submitted). These data strongly suggest that Trm cells also contribute to local immunity in pigs.

5. γδ T CELLS

5.1. Knowledge on γδ T Cell Subsets

Swine, together with various other farm animal species like cattle, sheep, and chickens, belong to the so-called γδ-high species because of their large number of γδ T cells in peripheral blood (reviewed in 144). In secondary lymphatic organs like the spleen, up to 30% of γδ T cells within total lymphocytes were also reported for pigs (95). The knowledge on porcine γδ T cells was summarized in a 2014 review (145), and owing to space restrictions, we focus here on more recent findings.

The separation of porcine γδ T cells into $CD2^+$ and $CD2^-$ subsets was described from early on, and in 2013, Stepanova & Sinkora (146) suggested that $CD2^+$ and $CD2^-$ γδ T cells are separate lineages of porcine γδ T cells that are generated in the thymus. This was corroborated further by the finding that IL-17A production is an exclusive feature of a subset of $CD2^-$ γδ T cells (95), whereas $CD2^+$ γδ T cells harbor perforin and T-bet-expressing as well as IFN-γ-producing subsets (95, 147). Data from Rodríguez-Gómez et al. (147) showed that perforin$^+$T-bet$^+$ $CD2^+$ γδ T cells were in their majority $CD8\alpha^+CD27^-$ (**Figure 3**), suggesting that $CD2^+$ γδ T cells show some changes in phenotype that resemble the differentiation described for porcine CD4 and CD8 T cells (see Sections 2.1 and 3.1). **Figure 3** summarizes these phenotypic changes. Hints that $CD2^+$ and $CD2^-$ γδ T cells diverge in their way of antigen recognition come from a recent

observation showing that $CD2^+$, but not $CD2^-$, γδ T cells express the costimulatory molecule CD28 (43).

Also, data are accumulating that $CD2^-$ but not $CD2^+$ porcine γδ T cells express mRNA transcripts orthologous to the WC1 family found in cattle (148; S. Schmidt, G. Freimanis, J. Schwartz, W. Mwangi, L. Endler, A. Hoog, K. van Dongen, K.H. Mair, J. Hammond, W. Gerner, manuscript in preparation), confirming earlier findings showing that the anti-bovine WC1 mAb CC101 cross-reacts with a subset of porcine $CD2^-$ γδ T cells (149). Another difference is the expression of GATA-3: $CD2^+$ and $CD2^-$ GATA-3-expressing γδ T cells were found in the thymus, whereas in the periphery, $CD2^-$ γδ T cells showed a much higher expression of this transcription factor (147). Of note, these GATA-3 expression levels in $CD2^-$ γδ T cells seem to vary between organs, with higher levels, for example, in the blood than in lung tissue (147). This was also observed for $CD2^-$ γδ T cells isolated from the maternal endometrium, fetal placenta, and fetal spleen, where the lowest levels of GATA-3 expression were found in the maternal endometrium and highest levels in the fetal spleen (42). Of note, the same pattern, albeit at much lower expression levels, was found for $CD2^+$ γδ T cells in these locations. The phenotype of γδ T cells in blood also seems to change substantially with age: Whereas in younger pigs $CD2^-$ γδ T cells dominate up to an age of approximately 6 months (up to 90% of total γδ T cells), later in life $CD2^+$ cells start to prevail, accounting for more than 50% in 4-year-old sows (147). Age-related changes have also been observed for CD8α. Whereas in younger pigs $CD2^-$ γδ T cells are mainly $CD8\alpha^-$, in 4-year-old sows more than 80% of $CD2^-$ γδ T cells are $CD8\alpha^{dim}$ (147). Another recent study showed that within intraepithelial T cells of the gut mucosa, γδ T cells are a prominent subset, and that $CD2^+CD8\alpha^+$ is the dominating phenotype within γδ T cells (40). Finally, the methodology to study the TCR repertoire of different γδ T cells has now been established (150). This should allow study of the TCR diversity of γδ T cells in various contexts, such as tissue distribution, aging, and response to vaccination/infection.

Despite this accumulating knowledge on substantial phenotypic differences between $CD2^+$ and $CD2^-$ γδ T cells in the pig, little is known about their contribution in immune responses to pathogens or immune homeostasis. However, given the recent findings of WC1-orthologous transcripts in porcine $CD2^-$ γδ T cells, it is tempting to speculate that this subset shares some functional similarities with the $WC1^+$ γδ T cell subset in cattle (reviewed in 151).

5.2. Porcine γδ T Cells in Viral and Bacterial Infections

As reviewed by Mair et al. (145), there are hints that porcine γδ T cells respond to *Mycobacterium bovis* Bacillus Calmette-Guérin antigen, FMDV antigen, and human rotavirus (the latter in gnotobiotic piglets). More recent studies investigated γδ T cells in the context of ASFV infections (53, 138). For the moderately virulent Estonia2014 strain, an increase in $CD2^+CD8\alpha^+$ γδ T cells was found in the spleen and liver of infected wild boars around 5 to 7 dpi, but this was not observed in domestic pigs (53). Similarly, an increase of this phenotype in the spleen was also found following infection with the highly virulent Armenia08 strain in wild boars (138). However, the study of further molecules like perforin, T-bet, or Ki-67 did not lead to further insights on the role of γδ T cells in response to ASFV infection.

Data also show that γδ T cells respond to PRRSV (24, 152). Kick et al. (24) showed that γδ T cells, isolated from blood 28 dpi with a high-pathogenic PRRSV-2 strain, respond via proliferation and IFN-γ production to in vitro restimulation with the homologous virus, or heterologous PRRSV-2 strains. Hence, γδ T cells may contribute to the T cell immune response against PRRSV. For swIAV, Edmans et al. (78) showed that $CD2^+$ γδ T cells isolated from the BAL of pdmH1N1-infected pigs already have ex vivo the capacity for IFN-γ or TNF-α production 2 to 5 dpi. When the same cytokines were analyzed after in vitro restimulation, a response was found between 7

and 13 dpi, with the highest frequencies of cytokine-producing $CD2^+$ γδ T cells in BAL and lung tissue. So, at least for swIAV, data now show that $CD2^+$ porcine γδ T cells can respond by the production of Th1-related cytokines and have the capacity to recall antigen. However, more detailed studies are required to elucidate how such γδ T cells recognize their antigen, whether they really have the capacity for memory formation, and if they contribute to protective immunity.

SUMMARY POINTS

1. For CD4 T cells, a selection of markers (CD8α, CD27, CCR7, CD45RA, or CD45RC) can be used to identify different stages of effector and memory CD4 T cells.
2. Th1 cells identified by IFN-γ and or T-bet expression were identified in various viral infections but also *Salmonella* Typhimurium (STM) and chlamydia infections. These T cells can be multifunctional (producing IFN-γ, TNF-α, IL-2, e.g., in response to swIAV).
3. Th17 cells were identified in response to *Actinobacillus pleuropneumoniae* (APP), and mixed Th1/Th17 phenotypes were identified in response to STM and chlamydia (**Figure 1**).
4. IL-10-producing regulatory T cells (Tregs) have been identified or postulated in the context of porcine circovirus type 2, porcine reproductive and respiratory syndrome virus, and APP infections, but their contribution to a potential immune escape of the respective pathogens is not clear.
5. Following antigen encounter, for CD8 T cells, a similar set of markers as in human T cells can be used to identify different stages of differentiation (**Figure 2**).
6. Available data show that CD8 T cells contribute to protection against classical swine fever virus and swine influenza A virus (swIAV).
7. Porcine Trm have been identified in the context of swIAV infections.
8. γδ T cells can be separated into the two major lineages of $CD2^+$ and $CD2^-$ γδ T cells (**Figure 3**), but their contribution to protective immune responses or immune homeostasis is unclear.

FUTURE ISSUES

1. Despite constant advances, the toolbox to study porcine T cells is still limited. However, novel technologies like single-cell RNAseq will partially overcome these limitations and will further broaden our knowledge on porcine T cells in health and disease. Moreover, recent developments in technology to identify gene expression in situ should allow a better understanding of the T cell response within the tissue environment.
2. Novel sequencing and amplification technologies will also allow study of the T cell receptor (TCR) repertoire, which should aid in understanding the contribution of TCR diversity.
3. Little research has focused on porcine T-follicular helper cells. Their detailed study should elucidate the contribution of T cells to humoral immunity in more detail and may help identify correlates of protection.

4. Despite their high numbers in blood circulation and secondary lymphatic organs, the role of γδ T cells and their different subsets remains enigmatic. In particular, their mechanisms of antigen recognition and subsequent response in infectious disease are understudied.

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