Expanded characterisation of porcine lymphocytes: CD9 expression on T cells and IL-10 production in B cells

Thesis submitted by

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Declaration

I hereby declare that I have written this thesis independently and I have followed the rules of good scientific practice in all aspects to the best of my knowledge.

— Jemma Victoria Milburn

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1 List of publications included and author contributions

Publication 1:

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List of author contributions to publication 1:

Mouse immunisation and cDNA library construction was done by previous members of the Institute of Immunology, University of Veterinary Medicine, Vienna, prior to the start of this work.

Jemma V. Milburn conducted the literature review, wrote the manuscript, generated data for figures: 1 B-E, 2, 3, 4, 5, 6, 7 and 8. and supplementary figures 1, 2, and 6, did the data analysis and designed and presented all figures.

Anna M. Hoog assisted in lymphocyte isolation and generated data for figure 8.

Simona Winkler conducted the tissue staining and fluorescence microscopy for suppl. figure 4.

Katinka A. van Dongen assisted in lymphocyte isolation and generated data for figure 1 E and suppl. figure 2 and 3.

Judith Leitner and Peter Steinberger provided advice on screening of the cDNA library and cloning of pCD9 into the expression vector and with provision of the vector, pMIGII, a gift from Dario Vignali. Both served as supervisors in the establishment of the cDNA library which was screened in this study.

Peter Steinberger also approved the conceptualisation of the study and critically read the manuscript.

Martina Patzl conducted the immunoprecipitation and western blot for figure 1a.

Armin Saalmüller approved the conceptualisation of the study and the manuscript. He was principal investigator of the mouse immunisation experiments laying the foundations for the current work.

Karelle De Luca represented the industry partner, Boehringer Ingelheim, who collaborated with the CD Laboratory for Optimized Prediction of Vaccination Success in Pigs on the broader aims of the research proposal and who approved the conceptualisation of the study and the manuscript.

Kerstin H. Mair was involved in conceptualisation of the project, established the hybridoma cell lines that express monoclonal antibodies, shortlisted monoclonal antibody candidates for screening on the cDNA library, conducted all flow cytometry cell sorts of cDNA library cells and lymphocytes for figures 1 and 4, advised on data interpretation and critical reading of the manuscript.

Wilhelm Gerner conceptualised the project and research overview, assisted in data analysis, designed the figures and critically assisted with writing of the manuscript.

List of author contributions to publication 2:

Jemma V. Milburn co-conceptualised the project, conducted the literature review, wrote the manuscript, generated data for all figures and supplementary figures, did the data analysis and co-designed and presented all figures.

Anna Hoog assisted in optimisation of flow cytometry staining panels, and generated data for figures 4, 5, 6, 7 and suppl. figures.

Sonia Villanueva-Hernández provided advice on identifying porcine plasma cells, conducted a preliminary experiment on CD9 expression on B cells and contributed to figure 2.

Kerstin H. Mair co-designed and optimised flow cytometry staining panels, assisted in project conceptualisation, conducted unpublished exploratory flow cytometry cell sorting experiments, and critically read the manuscript.

Wilhelm Gerner co-conceptualised the project, designed the manuscript, co-designed flow cytometry staining panels, assisted in data interpretation and co-designed the figures, and critically assisted in writing of the manuscript.

2 Summary

A comprehensive study of the porcine immune system will contribute to our understanding of immune memory in swine and enable us to develop better vaccines; to this end, we endeavour to expand the repertoire of monoclonal antibodies (mAbs) available for characterising the cellular components of it. In this work we phenotype porcine immune cells using novel monoclonal antibodies specific for the CD9 molecule, alongside established differentiation markers. CD9 is ubiquitously expressed on monocytes and variably expressed on lymphocytes. We found that CD9 can be expressed *de novo* on T cells following *in vitro* activation using concanavalin A, linking CD9 to activation status. Moreover, we found that CD9 is expressed on the majority of CD4 T cells that respond to virus antigen, in an *in vitro* antigen specific recall assay, by expressing intracellular interferon gamma (IFNγ) and tumour necrosis factor alpha (TNFα) cytokines, we therefore propose that $CD9^+$ T cells play a role in immune memory. I discuss these findings in comparison with the expression of the human and mouse paralogue, to consider the possible functional roles of CD9 on immune cells. Whether CD9 is a marker of long-lived immune memory warrants further investigation. Next, we questioned whether CD9 is a marker of interleukin-10 (IL-10) competent B cells, based on reported observations in mice. Our data indicated that this is not the case in pigs, so we interrogated the phenotype of IL-10 competent B cells in swine further. To do this, we created flow cytometry panels suitable for identifying porcine B cell subsets based on recent advances for discerning plasma cells and B1 cells, as well as isotype switched B cells. Porcine B cells could be induced to express IL-10 *in vitro* following 18 h culture with CpG containing oligodeoxynucleotides (CpG-ODN) and 4 h phorbol 12-myristate 13-acetate (PMA) and ionomycin stimulation, similar to methods used for mouse and human B10 cells. Our data indicates that porcine IL-10 competent B cells belong to multiple B cell subsets in pigs. Both the characterisation of CD9

expressing T cells and identification of IL-10 competent B cells lays a foundation for future study of these immune cells when evaluating porcine immune responses following vaccination.

3 General Introduction

3.1 The need for vaccine research in swine

Hailed as one of the greatest achievements of modern medicine, vaccines are among the most effective methods for preventing infectious disease.[1,2] Comprehensive immunological studies are progressing our ability dissect the mechanisms of vaccine mediated protection from disease and to develop rational vaccine design strategies. Outbreaks of zoonotic diseases threaten both pigs and humans, a notable example being the 2009 H1N1 influenza epidemic which circulated in humans after circulating in swine for over 10 years following historical triple reassortants from avian and human influenza viruses.[3] Meanwhile, epidemics of porcine viruses pose considerable threat to pig health and economic risks to the livestock industry, and as such are subjects of intensive research. Notable examples are porcine reproductive and respiratory syndrome virus (PRRSV), African swine fever virus (ASFV), classical swine fever virus (CSFV), and foot and mouth disease virus (FMDV), among others.[4] There is currently no licensed vaccine for ASFV, and existing vaccines for the other three diseases have limitations.[5] Additionally, pigs are susceptible to various pathogenic parasites, and bacterial infections.[5]

Future risks to food security may be mitigated by more efficient farming, which is afforded by optimal livestock health; adjusted farming practices and biosecurity measures should reduce infectious disease risk.[6] However, intensive farming practices and a globalised livestock industry contribute to increased spread of pathogens, particularly air-borne viruses such as PRRSV and influenza, which are not as well controlled by biosecurity measures as bacterial and helminth outbreaks,[6] and as such are subjects for novel or improved vaccine research.[5] Additionally, the food industry must monitor and mitigate against pathogens which contaminate food, posing a risk to consumer health; *Salmonella* Typhimurium is one such

example of a bacterium against which an effective vaccine for swine is deployed.[7] It follows that development of better vaccination strategies should be a primary focus of veterinary immunology research. Even acknowledging economic risks posed by livestock infection and changing food demand, it should be appreciated that any animal life which is incepted and expended for the sake of humans should receive the highest quality provision of wellbeing possible. It is therefore important to strengthen our ability to protect livestock animals from disease through developing rational vaccination strategies,[5] and to do so requires a more detailed characterisation of their immune systems.

This thesis explores two characteristics of porcine immune cells — the variable expression of the cell surface molecule tetraspanin 29 (CD9) on lymphocyte subsets and whether this correlates with immune functions, and secondly the ability of porcine B cells to produce IL-10 and possible implications that this has for regulation of the immune response in pigs. A somewhat comparative approach has been taken to allow for consideration of established knowledge on the function of CD9, and IL-10 producing B cells, attained from more commonly studied species. Ultimately, this work will lead to a more detailed characterisation of the porcine immune system, to facilitate research into improving vaccines for pigs.

3.2 Towards the discovery of novel correlates of protection

Traditionally, vaccine efficacy is evaluated by quantifying the incidence of infection, or signs of disease, of vaccinated vs unvaccinated individuals within or between populations. Simultaneously, tests for serum antibody reactivity against the pathogen are often used to verify the establishment of a humoral immune response within individuals.[1] However, an immune response is multifaceted, and appropriate activation of the multiple arms of the system can be necessary for long term memory. The development of rational vaccination strategies depends

on a thorough understanding of the mechanisms of immune protection, which must be appropriate to the disease aetiology. Such strategies involve the selection of antigens in various forms from whole particles to immunogenic sub-units, consideration of the route of administration and use of appropriate adjuvantation.[8] A successful vaccine will activate innate crosstalk with adaptive responses, polarise the immune response to employ mechanisms specialised in combating the type and location of a pathogen, activate both humoral and cellular components and ultimately generate highly adapted cells with the ability to survive in a quiescent state until re-challenge initiates their rapid expansion and re-initiation of effector functions.[9] Immune cells bear markers of activation and differentiation in the form of molecules that can be detected using techniques such as flow cytometry and fluorescence microscopy, which utilise monoclonal antibodies – which bind an epitope of a target molecule in a highly specific manner and have therefore become indispensable tools for immunologists.[10] As such, in addition to traditional correlates of protection, we seek to predict appropriate activation of the cellular response. This can be done by phenotyping cells, e.g. detecting molecules that are expressed by activated lymphocytes following primary challenge or immunisation and correlate such phenotypes with cellular function and with protection from disease. As such, immunologists now aim to be in a position wherein we have the information to predict vaccination outcome by identifying and using correlates of protection beyond measurement of serum antibodies.[1]

3.3 Expanding the toolbox for immunophenotyping in swine

There is a severe deficiency of monoclonal antibodies that are available for studying the porcine immune system. Here, we seek to expand upon the toolbox available by introducing two new antibodies specific for porcine CD9. We also identify B cells that express the cytokine

interleukin 10 (IL-10) by using an available anti-IL-10 mAb for intracellular cytokine staining. Within this project, two mAbs, designated 2E12 and 3B3 were identified as being reactive against porcine CD9. We utilised a previously established cDNA expression library, in which mouse Bw5147 cells were transduced with cDNA derived from PMA & ionomycin stimulated and Concanavalin A activated porcine peripheral blood mononuclear cells (PBMC).[11] cDNA library screening using the novel mAbs was performed as described elsewhere.[11] Partial sequencing of a section of PCR amplified cDNA was achieved and basic local alignment search (using NCBI BLAST) returned an alignment with porcine CD9, so primers were designed to amplify the entire CD9 cDNA from PBMC. Reactivity of the mAbs against CD9 was thereafter confirmed using porcine CD9 transduced mouse Bw5147 cells. These novel mAbs were then used for the phenotypic characterisation of porcine lymphocytes alongside markers for established porcine immune cell subsets, and functional assays. CD9 was found to be variably expressed across immune cell subsets, staining a distinct subset of B cells and exhibiting variable expression on T cells. We focussed on elucidating whether the variable expression of CD9 on T cells correlated with their functional capacity. We then considered existing literature concerning $CD9^+$ B cells in mouse and human and questioned whether $CD9^+$ B cells in pigs possess regulatory function. To this end, we identified porcine B cells that have the capacity for IL-10 production for the first time in pigs and go on to discuss the implications of this.

3.4 A brief introduction to porcine CD4 T cell subsets

T cells mature in the thymus, where they generate unique T cell receptors (TCR) by somatic recombination of VDJ gene segments and undergo a process of selection for appropriate (TCR) reactivity.[12] TCRαβ T cells then move through stages of $CD8^+CD4^+$ co-expression and gain expression of the co-stimulatory molecule CD27,[13] before differentiating into mature $CD8^+$ or

$CD4^+$ T cell subsets. Concomitantly, T cells gradually acquire CD3 expression as they mature.[12] It is also in the thymus that a subset is fated to differentiate into natural regulatory T cells (nTreg) which are critical for prevention of autoimmunity.[14,15] Following thymic egress, T cells enter the peripheral blood, loose CD1 expression, and the majority express CD45RC.[12,16]

T cells possess one of two types of TCR, which differ according to the chains of the T cell receptor α and β or γ and δ. αβ T cells in pigs may be identified by detection of CD3 and exclusion of γδ T cells.[17] Porcine TCRαβ T cells are further subdivided according to expression of the co-receptor molecules CD4 and CD8, defining T cells that are restricted to respond to peptides presented on swine leukocyte antigen (SLA) class II and SLA class I molecules respectively. The majority of CD8 T cells are cytotoxic lymphocytes (CTL) [17] and are not a focus of this thesis. Porcine CD4 T cells are $CD5^{high}$ and parallel that of other species by responding to cognate antigen with cytokine release and probably by providing help during the priming of other lymphocytes.[18,19]

3.4.1 Naive CD4 T cells

Following thymic egress, the majority of T cells are naive and circulate through the blood and secondary lymphoid organs to maximise the likelihood of encountering cognate antigen presented by professional antigen presenting cells. Naive, porcine CD4 T cells are negative for the activation induced CD8αα homodimer.[18] Naive cells highly express the CD45RA splice variant in humans and mice, and there is a prominent $CD45RA^+$ population also in swine,[20] which likewise predominantly consists of naive cells and also a minor subset of $CD8\beta^+$ differentiated effector cells.[21] CD45RA expression is also a feature of naive porcine CD4 T cells, however its distribution across activated subsets of CD4 T cells is less defined, so a

conclusive characterisation of CD45 splice variant expression on porcine CD4 T cells is yet to be established.[22,23] Expression of the chemokine receptor CCR7 and adhesion molecule CD62L (L-selectin) enable circulation of T cells through the blood and secondary lymphoid organs. As such, these molecules, and the costimulatory molecules CD27 and CD28 are expressed by all naive cells as well as populations of memory cells, and this has also been demonstrated for pigs.[13,16,24,25] Naive T cells also express the porcine 2E3 antigen, for which the orthologous human molecule could not yet be identified.[23] When stimulated, peripheral naive T cells produce high amounts of IL-2 and proliferate.[16]

3.4.2 Activated CD4 T cells

T cells are primed following sufficiently avid encounter of the TCR with cognate antigen presented on the surface of professional antigen presenting cells (APC) along with co-stimulatory signals, at which point the T cell proliferates and differentiates into effector cells, which will rapidly produce cytokines, and a small population of memory cells which are long lived. To enable the characterisation of activated cells, *in vitro* stimulation methods are employed. T cells can be induced to proliferate using mitogens such as concanavalin A (ConA),[16] or polyclonal primary activation can be demonstrated using superantigens such as staphylococcus enterotoxin B (SEB)[18] and cytokine expression is induced using PMA. Activation induced proliferation can be assayed directly using various methods.[16,18,26,27]

Expression of the CD8αα homodimer represents an activation marker of CD4 T cells in swine and this has been thoroughly demonstrated: CD8α is upregulated following *in vitro* antigenic stimulation, stimulation with SEB, or ConA + IL-2 [16,18], and only CD4 T cells that express CD8α exhibit an antigen (Ag) specific recall response to virus antigen *in vitro*, by proliferation and IFNγ release.[28]

Another interesting trait of porcine CD4 T cells is the expression of SLA-II (which includes SLA-DR and SLA-DQ) on CD8α$^+$ cells.[18] SLA-II is expressed *de novo*, by CD4 T cells, following *in vitro* stimulation, using SEB or primary antigen stimulation, and cells which proliferate in response to recall antigen belong to the SLA-II population.[18] Furthermore, as the frequency of activated and memory lymphocytes increases with age, this is reflected by an increase in CD8α$^+$CD4 T cells,[29,30] as well as upregulation of SLA-DR within this population.[30]

CD25, the α-chain component of the high affinity IL-2 receptor, is also used as a marker of activation, as it is absent on the majority of *ex vivo* PBMC but is expressed on all CD4 T cells following *in vitro* stimulation using superantigen.[18] CD4$^+$CD8α$^+$ T cells therefore contain CD25$^+$ cells, with a low to medium antigen density, this subset corresponds with upregulated SLA-DR and downregulated CD45RC.[18] In contrast, CD4$^+$CD25^{high} T cells mostly express the Treg transcription factor Fox-P3.[31] Saalmüller et al., (2002) proposed that CD25 is a marker of T cell activation but is downregulated on resting memory T cells.[18]

3.4.3 Memory CD4 T cells

Whereas markers of general T cell activation may be detected following non-specific or polyclonal stimulation methods, memory T cells are specifically characterised by phenotyping T cells which have been generated in a primary response *in vivo* and then respond to antigen in an *in vitro* antigen specific recall response. In this way, virus specific CD4 T cells can be indirectly identified by their Ag-induced activation. Saalmüller et al.[18] identified proliferative CSFV specific CD4 T cells as CD8α$^+$CD25$^+$CD45RC$^-$SLA-DR$^+$. Similarly, memory cells may be identified by their expression of effector molecules, such as perforin or cytokine, in response to recall antigen, for example CD4 T cells that respond to swine influenza A virus express TNFα and IFNγ which can be detected by intracellular cytokine staining using flow

cytometry.[16] Antibodies specific for chemokine receptors expressed by memory T cells are not yet available, however detection of mRNA for CCR4 and CX3CR1 has aided in defining memory populations.[16,32]

In humans and mice, central memory T cells (T_{CM}) are identified using mAbs specific for CCR7 and CD62L, in combination with CD45RO.[33] Functionally, they are poised to proliferate in response to activation, replenishing the pool of effector cells available. Conversely, effector memory T cells (T_{EM}), which do not express CD62L or CCR7 and home to sites of inflammation, are less proliferative but respond by rapid release of high amounts of cytokine, such as IFNγ and TNFα in the case of virus specific CD4 T cells.[33] Downregulation of costimulatory molecules such as CD27 and CD28 on memory T cells is also a phenotype that is used to divide human T cells, with $CD27^-$ cells being associated with increased effector functions, and $CD28^-$ cells are closer to terminal differentiation and senescence.[34,35] Such functional characteristics of memory CD4 T cell subsets have been similarly identified in swine, where co-staining for CD8α and CD27 yields four subsets, three of significant size in the peripheral blood, which exhibit features and tissue distribution of naive ($CD27^+CD8\alpha^-$), central memory ($CD8\alpha^+CD27^+$) and effector memory ($CD8\alpha^+CD27^-$) cells. This model was based on the observations that $CD8\alpha^+CD27^-$ cells had reduced proliferative capacity in response to ConA stimulation than $CD8\alpha^+CD27^+$ cells, which had similar proliferative potential to $CD8\alpha^-CD27^+$ naive cells. Sorted $CD8\alpha^+CD27^-$ cells secreted substantially more IFNγ and TNFα at earlier time points than sorted naive and $CD8\alpha^+CD27^-$ cells following up to 4 days stimulation using ConA and rhIL-2.[16] This model was corroborated by the observation that naive cells, and the vast majority of T_{CM} co-expressed CCR7, the distribution of which closely matched that of CD27, and more CD62L mRNA was detected in naive and T_{CM},

whereas T_{EM} sorted cells had increased CX3CR1 mRNA.[16] Further, the porcine T_{EM} population also contains the $CD28^-$ population, suggestive of the later stage of differentiation as seen in mice and humans.[24]

In this work we analyse T cells from tissues without using specialist isolation methods to separate tissue resident and circulating lymphocytes. Tissue resident T cells (T_{RM}) are distinguished by expression of the molecules which retain them in the tissue: CD69, which inhibits sphingoseine-1-phosphate receptor signalling on immune cells to re-enter circulation,[36] and CD103 (integrin αE) which is mainly restricted to $CD8^+$ $T_{RM,}$ however an antibody reactive for porcine CD69 has only recently been used to identify CD8β T cell $T_{RM,}$ and tissue resident CD4 T cells are not yet defined.[21]

3.4.4 CD4 T cell polarisation

The presence of polarising cytokine results in initiation of transcription profiles which enable the implementation of specialist T cell functions, which should be appropriate to the disease aetiology, resulting in polarisation of CD4 T cells, that are defined on the basis of master transcription factors and signature cytokine profiles. The transcription factor T-bet drives expression of the signature T-helper 1 (Th1) cytokine IFNγ, in CD4 T cells.[29] T cell polarisation is less well defined in the pig than in mouse and human, but a pragmatic focus of porcine immunology has been to detect cytokine, either intracellularly, using flow cytometry, or cytokine encoding mRNA, as an indication of activated effector functions and polarisation.[29,37] In this work, we detect intracellular IFNγ and TNFα in cells which respond to swine influenza A antigen, these cytokines are typical of a Th1 driven antiviral response.

3.4.5 Regulatory T cells

Discrimination between natural and peripherally induced Treg remains unclear within the field of porcine immunology. Generally, porcine natural Treg (nTreg) which differentiate in the thymus are $CD4^+CD25^+FoxP3^+$, whereas peripherally induced Treg (iTreg) don't have a common phenotype and are rather defined by their immunomodulatory function and cytokine profile.[14] Similar to their murine counterparts, *ex vivo* porcine $FoxP3^+$ Treg are $CD4^+CD25^{high}$,[31,38] these cells suppress proliferation of activated T helper cells,[31] CTL ($CD4^-CD8a^{high}$ T cells), and γδ T cells.[39] Only $FoxP3^+CD25^{high}$ cells can mediate direct cell contact mediated suppression, whereas both $CD25^{hi}$ and $CD25^{dim}$ subsets secrete IL-10.[39] Also, $CD25^{dim}$ cells contain a low frequency of $FoxP3^+$ cells but a higher frequency of $IL\text{-}10^+$ cells.[31,40] The authors suggest that $CD25^{dim}IL\text{-}10^+$ subset may be indicative of porcine TR1 cells.[17] In line with findings in other species, a subset of peripheral $CD4^+FoxP3^-$ T cells can be induced to upregulate FoxP3 expression *in vitro*, by non-specific TCR stimulation using anti-CD3 mAbs in the presence of transforming growth factor-β (TGF-β) and IL-2 and these cells also inhibit T cell proliferative responses but not IFNγ production and secrete more IL-10 than the *ex vivo* $CD25^{high}$ population containing nTreg.[15] There is also a minor population of $CD4^-CD8\alpha^+FoxP3^+CD25^+$ Treg.[31] In terms of activation status, the porcine $FoxP3^+$ Treg population contains various populations of cells with different activation associated phenotypes.[31]

3.4.6 T cells are heterogeneous

Despite the general approach to phenotyping major T cell subsets outlined above, porcine T cells are heterogeneous. Boolean analysis of CD8α, CD27, SLA-DR and CD45RC reveals 16 different subsets.[16] An earlier model described naive cells as $CD8\alpha^-CD45RC^+SLA\text{-}DR^-$ and

memory cells as $CD8\alpha^{+}CD45RC^{-}SLA\text{-}DR^{+}$,[18] which possibly represents two extremes along a differentiation spectrum.

In pigs the antigen 2E3 can also be used as a marker of naive cells,[23] because only sorted $CD4^{+}2E3^{-}$ cells proliferate in response to antigen specific stimulation and these cells express more IFNγ and IL-4 than $2E3^{+}$ cells, accordingly $2E3^{+}$ and $2E3^{-}$ cells express the same amount of IL-2 mRNA. Moreover, sorted $CD4^{+}2E3^{+}$ cells contain CCR7 mRNA, whereas $CD4^{+}2E3^{-}$ cells contain mRNA for the CCR4 and CXCR3 chemokine receptors expressed by some memory subsets.[32] However the vast majority of the $CD8\alpha^{+}CD27^{+}$ defined T_{CM} population expresses CCR7 [16] meaning sorting on the basis of a $CD4^{+}2E3^{-}$ phenotype excludes T_{CM}. All $2E3^{-}$ cells express the activation marker CD95 (Fas) and the vast majority are $SLA\text{-}DR^{+}$, however over 10 % of $2E3^{+}$ cells also express SLA-DR.[32] What's more, CD8α expression was detected on both $2E3^{+}$ and $2E3^{-}$ cells.[23] Sorted $2E3^{-}SLA\text{-}DR^{-}$ cells which responded to lysozyme upregulate SLA-DR. The authors of this study also suggest that the phenotype of naive cells as $CD4^{+}2E3^{+}CD8\alpha^{-}CD45RA^{+}SLA\text{-}DR^{-}CD29^{low}$ is the extreme opposite of memory $CD4^{+}2E3^{-}CD8\alpha^{+}CD45RA^{-}SLA\text{-}DR^{+}CD29^{high}$ cells on a differentiation spectrum.[22]

Prior to the availability of a cross reactive monoclonal antibody (clone 3D12) specific for porcine CCR7,[16] the specificity of its natural ligand (CCL19) was exploited, achieving detection of porcine CCR7 through the use of recombinant porcine CCL19 fused to the human IgG1 Fc fragment.[41] In this study, CCR7 was detected on the majority of 2E3 defined naive cells and a smaller subset of $2E3^{-}$ cells.[41] In this work we define naive cells according to the absence of CD8α expression [18] alongside expression of CD27, which closely follows that of CCR7.[16]

Table 1 Markers used for studying CD4 T cell populations in swine

CD4 T cell population $CD3^+CD4^+$	**Phenotype used in this study**	**Expanded phenotype used in the wider field***
Naive cells	$CD8\alpha^-$ $CD27^+$	$CD1^-$ $CD5^{high}$ $2E3^+$ $CCR7^+$ $CD25^-$ $CD28^+$ $CD29^{low}$ $CD45RC^+$ $CD45RA^+$ $SLA\text{-}II^-$
Activated effector and memory cells	$CD8\alpha^+$ $CD25^+$ $CD95^+$ $CD27^{+/-}$	$2E3^-$ $SLA\text{-}II^+$ $CD45RC^{loss}$ $CD28^{+/-}$
Central memory cells	$CD8\alpha^+$ $CD27^+$	$CCR7^+$ $CD29^{high}$
Effector memory cells	$CD8\alpha^+$ $CD27^-$	$CCR7^-$ $CD29^{high}$ $CD28^{+/-}$
Regulatory T cells	$CD4^+FoxP3^+CD25^+IL\text{-}10^+$	Various activation associated phenotypes

*These markers were not detected in the enclosed work, for simplicity, but are relevant when considering our results alongside other published work, referenced in the main text.

3.5 Identification of porcine B cells

The most prominent function of B cells, that receives attention in the field of vaccinology, is their contribution to adaptive immunity and immune memory, ultimately resulting in antibody production. This stems from an almost limitless receptor variation, as a result of both homologous recombination and somatic hypermutation.[42] B cell contribution to immune memory is afforded by the survival of long lived memory cells that participate in an anamnestic response, upon re-exposure to antigen, that is more specific and of a greater magnitude than a primary response, and by secretion of both natural and affinity matured antibody by subsets of antibody secreting cells, including long lived plasma cells.[9] However, there is mounting attention to other features of B cell responses in which B cells fulfil innate-like functions, and

other roles.[43] One such role is their potential for immune regulation, by virtue of cytokine release and a capacity for antigen specific cross-talk with T cells; among other cognate interactions and release of tolerogenic Ab isotypes.[44] Here, I introduce the current status of discriminating innate and memory B cell subsets in pigs, so that we may contextualise our efforts to phenotype IL-10 producing B cells.

In pigs B cells constitute between a quarter and a third of lymphocytes in blood.[30] However, studies on porcine B cells have been hindered by technical limitations. Antibodies reactive against the B cell co-receptor CD19 are not available, therefore detection of porcine CD79α is currently the standard method for discriminating total B cells in swine.[10,45] CD79α is the immunoglobulin α component of the B cell receptor complex (BCR). The BCR complex is downregulated upon B cell differentiation into plasma cells and this is probably also the case in pigs, since a population of $Ig^{+}CD79\alpha^{-}$ cells exist [46] and plasma cells are $CD79\alpha^{dim/-}$ in the bone marrow.[47] The epitope for cross reactive anti-human CD79α mAb clones HM57 and HM47 is intracellular, so positive selection strategies for sorting live total B cells based on CD79α expression are not possible and this, alongside the absence of marker specific mAbs for discrimination of porcine memory B cells and plasma cells, has somewhat hindered research into functional aspects of porcine B cell subsets.

Interestingly, CD52, which is broadly expressed across lymphocytes, monocytes and dendritic cells in humans, is absent from the majority of porcine B cells, but present on monocytes and T cells.[11] Likewise, CD27, which is a marker of human memory B cells, is absent on porcine B cells.[13] However, porcine B cells, are $CD3^{-}CD8\alpha^{-}CD16^{-}CD52^{-}CD172a^{-}$ lymphocytes [10] and negative sorting strategies have been attempted accordingly on $CD3^{-}CD16^{-}CD172a^{-}$ cells.[47]

3.5.1 Identifying naive, activated and memory B cells in swine

Porcine B cells develop in the bone marrow throughout life, where they gradually loose the expression of CD172α upon differentiation [48] and may be profiled into developmental stages on the basis of IgM, CD172α and SWC7, expression patterns.[12] The bone marrow is currently considered to be the primary site of B cell lymphogenesis [42,48] though there has been some debate about the importance of the Peyer's patches to the development of B cells in pigs.[49] Naive B cells may enter the blood following expression of the authentic BCR and B cells isolated from the blood of new-born piglets, which are typically naive to foreign antigen, are in their vast majority $CD2^{+/low}CD21^{+}CD25^{+}CD45RC^{+}SLA\text{-}DR^{+}$.[48]

Activation of B cells *in vitro* results in proliferation,[50] upregulation of CD25 and CCR7,[51] SLA-DR and CD80[50] and CD5 on a fraction of B cells[52] and ultimately differentiation into Ab secreting cells.[50] Furthermore, a model for activated B cell differentiation is proposed by Šinkora and Butler, based on CD2 and CD21 expression, described below.[42] In this work we use expression of FAS (CD95) as an indicator of lymphocyte activation, using a cross reactive anti-human antibody.[32] CD95 is also upregulated following activation of human and mouse B cells.[53]

The vast majority of porcine peripheral blood B cells express SLA-DR.[30,47] *In vitro* activation of $CD21^{+}$ splenocytes using hCD40L (human CD40 ligand) results in upregulation of SLA-DR, as well as upregulation of CD80 and proliferation.[50] However, SLA-DR is downregulated on $Blimp1^{+}IRF4^{+}$ defined plasma cells, consistent with the situation in humans and mice.[47] Due to this almost universal expression of SLA-DR on porcine B cells we did not choose it as a marker for discriminating B cell subsets in this work. Little is known regarding the role of porcine B cells in Ag presentation. B cell-T cell cross talk is proposed to be important for Ag

specific immune regulation of T cells by B cells in mice [54,55] and the generally established model from other species is that germinal centre B cells internalise Ag following BCR engagement and present it to T cells which provide B cell selection signals.

Immunoglobulin (Ig) variability is increased by somatic hypermutation of B cell immunoglobulin genes, resulting in more diverse BCR on progeny cells, which are then clonally selected on the basis of reactivity against target antigen. This occurrence is most common for B2 cells, which are able to enter the germinal centres of secondary lymphoid organs, where they undergo intense proliferation, in the presence of T-follicular helper cells, which have now also been identified in pigs.[19] Although CD27 is a convenient marker for human cells that have undergone somatic hypermutation [56] porcine B cells do not express CD27.[13]

Specialisation of the B cell response to diverse pathogens and sites of immune challenge is achieved by the capacity of B cells to undergo Ig isotype class switching, which ultimately results in the generation of antibodies with Fc regions that mediate different functions. As in other species, isotypes dominate at different anatomical sites, for example, IgA^+ plasma cells dominate in lung mucosa.[47] Since isotype switching occurs following engagement of the BCR with antigen it is an indicator of Ag-experienced B cells. Although the capacity for isotype class switching in the newly defined B1-like cells [51] has not yet been addressed in pigs, since the efficiency of B1 cell class switching to IgG is much lower than that of B2 cells, in other species,[57] it is assumed that porcine IgG^+ B cell populations represent, in their majority, memory B2 cells.[58] In pigs, these immunoglobulin isotypes are detected using specific antibodies and in this work we analyse IgG expression (publication 2 fig. 6) to detect subsets of B2 cells and IgM expression alongside CD21 to distinguish naive and B1 cells (publication 2 fig. 7).

3.5.2 The use of CD21 as a marker to discriminate porcine B cell subsets

CD21 (complement receptor 2) is a receptor for activated CR3 and associates with CD19 and CD81 to enhance BCR signalling, so is involved in the ability of B cells to respond to complement coated antigens.[46] As such, it is expressed on the vast majority of B2 cells, and has been used as a surrogate B cell marker in some studies.[50] However porcine innate like B cells are CD21 negative.[51] Furthermore, there is a decrease in the frequency of $CD21^+$ cells, detected using mAb clone IAH-CC51, following *in vitro* stimulation (using pokeweed mitogen, PMA & ionomycin or CpG-ODN), and isotype switched cells are more frequently $CD21^-$ [46]. Therefore Šinkora et al., developed a differentiation scheme according to B cell CD21 and CD2 expression, the latter also being variably expressed on porcine B cells [59]. B cells from germ-free piglets and from foetal piglets are all $CD2^+CD21^+$, but conventional piglets develop a population of $CD2^-CD21^-$ cells as they age,[46,60] suggesting this population arises following antigen exposure.[59] However, CD2 can be re-expressed on mature B cells [42] and is therefore not a reliable marker of B cell maturity alone. Although loss of CD21 on B2 cells has been associated with later stage differentiation, there remains a distinct population of $CD21^+$ cells within $Blimp1^+IRF4^+$ defined plasma cells, particularly in the secondary lymphoid organs, however CD21 expression was markedly downregulated in this population compared with $CD79\alpha^+IRF4^{dim}$ cells, the vast majority of which are $CD21^+$ in the lymph node.[47]

In this work, we used anti-CD21 mAb clone BB6-11C9.6, which recognises the CD21a isoform, designated by Šinkora and colleagues.[46] This antibody stains the vast majority of IgM^+ cells in adult swine, however there remains a distinct subset of CD21 low / negative cells. Although other mAbs (B-ly4 and IAH-CC51) have been reported to stain only subsets of IgM^+ or $CD21a^+$ cells, these findings were not reproduced by our group (Kerstin Mair, personal

communication). It is not known whether clone BB6-11C9.6 reacts with an isoform of CD21 that is co-expressed alongside alternative isoforms which the other mAb clones react with, or whether BB6-11C9.6 is a pan-specific mAb that reacts with multiple isoforms of CD21. Nevertheless, for simplicity and consistency we chose to use mAb BB6-11C9.6 for our study, as this was also the clone used by Braun et al.[51] for their study of porcine B1 cells.

3.5.3 Porcine innate-like B cells

Innate-like B cells provide a first response to pathogens by the production of polyreactive natural antibodies*. Reviewed in [43] Among these, B1 cells are a functionally defined subset, though identification can be based on certain characteristics. They develop earlier during ontogeny and are selected against self-antigens.[61] The Ig repertoire of B1 B cells is more restricted than that of B2 cells, possibly due to events which evolutionarily selected for specificities that would counter common pathogens.[62] B1 cells also have a capacity for self-renewal, in part due to heightened tonic signalling.[51] B1 cells were first identified in mice as a subset of $CD5^+$ B cells which are enriched in the spleen and peritoneal cavity but are now subdivided into $CD5^+$ B1a cells and $CD5^-$ B1b cells. CD5 is also expressed on human innate-like B cells, however CD5 is more broadly expressed on human B cells, including activated B2 cells and anergic cells.[43,62] Murine and human cells with a B1a phenotype have received particular attention for containing cells with a capacity for IL-10 production and resultant role in regulation.[63–65] Since CD5 is absent on B1b cells, murine B1 cells are commonly identified by expression of CD11b. However, CD11b is absent on splenic B1 cells in mice, and some peripheral B1 cells, which

* Natural antibodies derive from the original gene sequence that arises from VDJ heavy and VD light chain recombination during the ontogenic selection of the B cell i.e. without antigen driven somatic hypermutation and are constitutively produced.[42,62]

are thought to be less differentiated [66] or primarily responsible for production of natural antibody.[64]

Porcine innate like B cells have been identified as potential counterparts to B1 cells of other species, by virtue of their increased cell size, IgM cell surface density, heightened tonic signalling and secretion of high levels of IgM in response to toll-like receptor (TLR) agonists.[51] Porcine B1 cells are $CD79\alpha^{+}CD21^{-}IgM^{high}$ and are phenotypically subdivided on the basis of CD11R1† into two major subsets.[51] Among total B cells, the $IgM^{+}CD21^{-}CD11R1^{+}$ subset distinctly has the highest capacity for proliferation in response to TLR2/1 TLR7/8 and TLR9 agonists, this was also the only subset not to upregulate CD25 expression in response to CpG-ODN. Further phenotyping indicated that porcine B1 cells also express CD80/86, are in their majority $CD11c^{+}$, and a sizeable population of $CD14^{+}$ cells is contained within the CD11R1 population.[51]

CD5 is expressed on 10-30 % of porcine blood B cells and is upregulated following 25 h of PMA stimulation.[52] It was therefore initially suggested that CD5 might also be a marker for B1 cells in pigs. However, the same group concluded otherwise, because they found no differences in mRNA expression of IgM and IgG or differences in the V D and J gene elements or CDR3 length of IgM, in $CD5^{+}$ and $CD5^{-}$ B cells. This along with no difference in IL-10 mRNA expression lead the authors to conclude that CD5 is not a marker of B1 cells in swine.[58] Due to the undefined significance of CD5 expression on porcine B cells, we also chose not to analyse CD5 expression in this work.

† Detected using cross-reactive anti-human mAb MIL4, specific for human CD11b, however the distribution of CD11 detected by this mAb differs between humans and pigs so the Ag is designated CD11R1.[182]

3.5.4 Antibody secreting cells and plasma cells

Plasma cells are a subset of differentiated B cells which actively secrete antibody and long lived plasma cells can be maintained for long durations of time, therefore contributing to constitutive humoral immunity. Until recently porcine plasma cells were identified based on their increased scatter properties in flow cytometry and mRNA expression of transcription factors.[67] Now, porcine plasma cells can also be identified based on co-expression of Blimp-1 and IRF4, detected using cross reactive antibodies.[47] Blimp-1^{+}IRF4^{+} cells isolated from the blood and lung are also CD49d^{high}, have the morphological properties of antibody secreting cells and spontaneously release IgG.[47] Similar to the downregulation of the BCR complex seen in antibody producing cells of other species, porcine CD79α expression is lost on bone marrow IRF4^{+}Blimp$^{-}1^{+}$ cells.[47] Cell surface markers CD9 [68] and CD28 which are associated with murine plasma cells were not detected on the vast majority of porcine plasma cells, and are even further downregulated in the bone marrow.[47] The vast majority of plasma cells in the blood express the mitotic marker Ki-67, in contrast to the majority of B cells, which are Ki-67 negative (~90%), however a proportion of plasma cells which enter secondary lymphoid organs and lung mucosa appear to have exited the cell cycle.[47]

3.5.5 IL-10 secreting B cells

To my knowledge the work herein is the first comprehensive study of IL-10 competent B cells in swine. IL-10 is a cytokine that is well known for its role in down-regulating immune responses.[69] IL-10 production can be a feature of multiple populations of B cells, that have been particularly studied in mice and humans; including Breg [55,70,71] (see this thesis chapter 6.2.2) and B10 cells [55,71] (this thesis chapter 6.2.3), which can share overlapping phenotypes with innate like B cells [63,65,72] (this thesis chapter 6.2.4), and a diversity of other subsets

including plasmablasts and transitional B cells.[73–77] More recently it has emerged that the capacity for IL-10 production may be a cell state that is acquired by cells belonging to various populations of B cells.[78,79] Some B cells have also been shown to respond to autocrine IL-10 signalling as a survival factor.[80,81] Therefore B cell derived IL-10 may fulfil different signalling functions and be a product of various activation states across B cell subsets, the complexity of which is not clear.

Because IL-10 producing B cells belong to various subsets, I chose to strictly refer to the porcine B cells that we characterise as "IL-10 competent B cells", for the sake of precision and to avoid confusing and exclusive nomenclature which was designated to populations of B cells and Breg prior to a consensus over what constitutes these 'subsets'. A brief introduction to studies examining CD9 expression on IL-10 competent B cells is offered in chapter 3.7.2. Further discussion on the identity of IL-10 competent B cells is covered in the discussion of this thesis (chapter 6.2).

3.6 Introduction to tetraspanins

The CD9 molecule is tetraspanin 29, a 22 kDa glycoprotein located at the cell surface membrane. Tetraspanins are a family of type III transmembrane proteins (i.e. both terminal peptides are intracellular) and are so named for their four alpha-helical membrane spanning domains.[82] Tetraspanins are a superfamily of proteins which can be subdivided into 4 major phylogenic clades, within which CD9 belongs to the 'CD' family and is most closely related to CD81 and tetraspanin 2, the three of which form a cluster from which CD9 diverged with the emergence of vertebrates.[83,84] Tetraspanins have two extracellular loops: one small, glycosylated loop, and a second large loop with a fold formed by 2-4 disulphide bonds at conserved cysteine residues, this large extracellular loop (LEL) is divided into a conserved region and a region which contains the highest sequence variability, and is the subunit against which most tetraspanin specific antibodies bind.[82,84] Each domain is responsible for mediating interactions with various partner proteins on the cell membrane, which usually occur *in cis*.[84] The LEL laterally interacts with extracellular domains of membrane proteins.[85] Whereas the membrane spanning helices form close interactions with other membrane spanning proteins, resulting in dimers and tetrameric complexes.[86] Palmitoylation of internal juxta-membrane cysteine residues contribute to tetraspanin stability, localisation and association of tetraspanins with each other [87] to form both homodimers and heterodimers, with the former being more common.[82] Finally, the C-terminal tail interacts with intracellular signalling molecules,[88–90] and in some cases, cytoskeleton associated protein.[91] This ability of the various domains of tetraspanins to engage in different interactions is key to their function as adaptor molecules, linking signalling molecules in the membrane to intracellular components and organising complexes of signalling molecules within tetraspanin enriched microdomains (TEMs).[84]

TEMs are detergent insoluble glycolipid enriched membrane fractions that are also enriched in cholesterol and contain tetraspanins and their interaction partners and are now widely believed to be distinct from lipid rafts.[82,92–94] Primary interactions of tetraspanins with molecular partners and dynamic, transient, tetraspanin-tetraspanin interactions, occur either in platforms, or small diffuse clusters on the plasma membrane.[92,95] These small subunits of less than 150 nm in diameter, are fluidly distributed across the membrane but coalesce into caps to modulate various cell-cell interactions‡.[92] Accordingly, single molecule tracking of CD9 has shown its localisation both within larger complexes and smaller membrane units, with the location of each molecule being in constant flux.[93] The mechanistic function of TEMs is dualistic,[96] because the organisation of molecules within microdomains can both partition them away from the complexes required for signal initiation and disrupt the optimal distribution of molecules, or facilitate signalling by the colocalization molecules into signalling complexes.[97] Due to the diversity of surface molecules which can interact with TEMs, their fluid redistribution upon the plasma membrane regulates a huge variety of cell surface events, including signal initiation and enhancement, adhesion, migration, membrane fusion, and formation of exosomes.[82] Specific examples of these interactions in the case of how CD9 modulates integrin mediated signalling are given below (chapter 3.7 and illustrated in figure 2).

‡ This model supersedes the paradigm of a more static 'tetraspanin web', widely referred to in earlier literature.

3.7 Tetraspanin CD9: current knowledge based on mouse and human literature

3.7.1 CD9 structure and molecular interactions

The crystal structure of human CD9 has recently been attained.[86,98] The four transmembrane helices form a cone shape, with their intracellular ends being more tightly packed, so that a hydrophilic cavity is formed which is more open towards the extracellular side of the molecule.[86,98] This asymmetric shape is proposed to contribute to membrane curvature, potentially explaining the contribution of CD9 to membrane remodelling.[86] The extracellular loops cover the cavity by weakly interacting with each other in a closed conformation, but computational simulation suggests they spontaneously alternate between a closed and an open conformation with the LEL positioned upwards.[86] The LEL of CD9 interacts directly with the integrin: lymphocyte function antigen 1 (LFA-1) [99] whereas close intramembrane interactions are formed via the four transmembrane alpha helices of CD9 with EWI (E: glutamine W: tryptophan I: isoleucine) family proteins EWI-2 or EWI-F.[86,98] In such a way, CD9 and EWI-2 form a hetero-tetramer.[86] It is proposed that other interactions form in a similar manner between tetraspanin family proteins and single pass membrane proteins, such as TGF-β family receptors and other EWI family proteins.[86] CD9 has 6 acetylation sites spread across the four internal juxta-membrane regions, the palmitoylation of which contributes to the stability of CD9-CD81 interactions,[87] and probably also to the stability of CD9 within the membrane.[86] The 8 residue C-terminal tail of CD9 (amino acid sequence: IRRNREMV) is highly conserved across species, including pigs, chickens, mice, and humans and also contributes to functional complex formation[100] and is a candidate for protein kinase interactions.[88,90]

Figure 1 The CD9 molecule

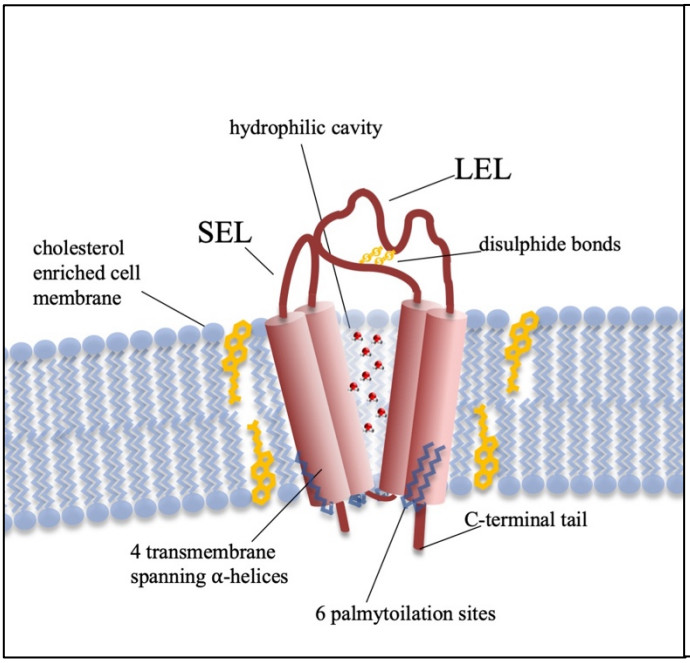


Illustration of the CD9 molecule based on literature referenced within the main text. Domains and key features are labelled.

SEL: small extracellular loop

LEL: large extracellular loop

Not drawn to scale. Illustration drawn in Microsoft PowerPoint.

3.7.2 Distribution of CD9 on T and B cell subsets

Though the expression of some tetraspanins is restricted to only certain cells, for example tetraspanins CD37 and CD53 that are restricted to human immune cells,[101,102] many tetraspanins, including CD9, are broadly expressed across tissues. CD9 is highly expressed on endothelial cells and variably expressed across the immune systems of mice, humans and pigs. In addition to their variable tissue distribution, the tetraspanin profiles of cell subsets varies between species.[103] Here, I provide a focussed introduction to the distribution of CD9 on lymphocyte subsets.

Whether CD9 can be regarded as a marker of cell state, or lymphocyte subsets in combination with other phenotypes, is not well established for any species. Reports of CD9 expression across human and murine lymphocytes are inconsistent and vary depending on the anti-CD9

Ab clones used, which is suggested to be explained by their differing affinities and epitope specificity.[104] Anti-CD9 clone 9D3 reacts with the vast majority of CD3-sorted murine splenic and lymph node T cells.[105] In contrast, a small proportion (between 1 and 3 %) of *ex vivo* human B and T cells express CD9, with a higher expression level on B cells.[106,107] The majority of human T cells which stained with anti-CD9 clone 5H9 are $CD45RA^{+}CD62L^{+}$.[104] In order to consider the implications of CD9 expression on porcine T cells, I have chosen to introduce the molecular functions of CD9 below (chapters 3.7.3-3.7.6) so that we may consider the potential impact these may have on the functionality of cells expressing this molecule.

CD9 is reported to be a marker of B1 cells, marginal zone B cells,[68,108] and plasma cells in mice,[68] including thymus independent splenic innate-like B cells responding to FMDV in the lung.[109] It has also been reported to be a marker for germinal centre plasma cell precursors in humans.[110] However CD9 is not required for normal B cell function in CD9 knock out mice.[108] The pattern and function of CD9 expression across B cell subsets, and between species is therefore not fully elucidated. Nevertheless work from two groups suggest that CD9 could be used as a marker of IL-10 competent regulatory B cells. Transcriptomic analysis indicates that CD9 mRNA is increased in populations of B cells that express IL-10[111] and a population of $CD1d^{hi}CD5^{+}$ cells that are enriched in $IL\text{-}10^{+}$ cells.[112] In mice, adoptive transfer of $CD9^{+}$ B cells controls Th1 mediated contact hypersensitivity[112] and TH2/TH17 driven allergic airway inflammation.[111] In humans, the frequency of $CD9^{+}$ Breg are reduced in severely asthmatic patients compared with healthy controls.[113] *In vitro* assays of lymphocytes from these patients and mice indicates that $CD9^{+}$ B cell induction of T cell cycle arrest and death is dependent on IL-10.[113]

3.7.3 CD9 involvement in *in vitro* T cell activation

CD9 was initially proposed to be a T cell co-stimulatory molecule in mouse cells, when anti-CD9 (clone 9D3) was immobilised on an *in vitro* cell culture plate alongside sub-mitogenic concentrations of anti-CD3.[105] The mechanism of CD9 mediated co-stimulation was deduced to be independent of CD28 because CD9 mediated co-stimulation was not diminished in T cells isolated from CD28 knock out mice.[105] In support of this, a synergistic effect on co-stimulation was demonstrated when both CD28 and CD9 immobilised along with anti-CD3 resulted in enhanced T cell proliferation.[114] However, proliferation was followed by apoptosis of mouse T cells [115], this was originally thought to be a form of FAS-independent activation induced cell death,[116] or due to absent NFκB activation and subsequent insufficient autocrine IL-2.[115] Likewise, co-immobilised anti-CD9 alongside anti-CD3 or anti-CD28 induced proliferation in human T cells,[104,117] however this was not followed by cell death.[104] Importantly, unlike anti-CD28 mediated co-stimulation, anti-murine CD9 antibody 9D3 could not induce a co-stimulatory effect when in solution, and had to be immobilised.[105] Subsequent work on the function of CD9 within the cell surface membrane somewhat clarified the stimulatory effect of *in vitro* immobilised antibodies on CD9 expressing cells. Because TEMs are located within the detergent insoluble fraction, studies which considered CD9 to be localised within lipid rafts shed light on the role of this molecule in signalling events.[118] This membrane fraction is rich in T cell signalling molecules (but does not contain the CD3/TCR complex itself) but antibody mediated cross linking causes passive raft redistribution into caps. Therefore concomitant engagement of the CD3/TCR complex with immobilised anti-CD3 and anti-CD9, results in co-localisation of intracellular molecules within TEMs which activate signals downstream of the TCR which result in proliferation of T cells.[118] In support of this model, treatment of T cells with anti-CD9 and PMA (without anti-CD3) did not induce activation.[114] Moreover, unlike co-

stimulation through CD28, anti-CD9 mediated co-stimulation may not be considered as 'signal two' for complete T cell activation because it does not induce IL-2 expression, due to insufficient NFκB activation.[119] Some reviews still describe CD9 as a T cell costimulatory molecule, referencing these earlier papers, despite the mechanism which since explained these *in vitro* effects being one that highlights the difficulty of translating *in vitro* experiments using immobilised antibodies with *in vivo* scenarios which involve complex cell-cell interactions. We now know that CD9 forms close interactions with integrins (see chapter 3.7.4 and 3.7.5). Nevertheless, these studies at least showed that CD9 co-localisation with the TCR promotes T cell activation *in vitro* regardless of if the resultant signal was indirectly produced. Moreover, anti-CD9 clone 5H9 inhibited the proliferation of primary human naive T cells in response to β-2-glycoprotein 1 (β-2-GP1) autoantigen and tetanus toxoid loaded macrophages in a dose dependant manner,[104] indicating that CD9 plays a physical role in the T cell antigen recognition complex and this is consistent with its role in recruiting integrins to the immune synapse, introduced below. In addition to this, CD9 may modulate cell activation and other signalling events via various other direct and indirect interactions with signalling molecules, since a huge variety of intracellular signalling interactions occur, at least indirectly, via CD81 and EWI2 [91] (see figure 2).

3.7.4 CD9 involvement in integrin mediated signalling events

Integrin mediated signalling is necessary for multiple cell surface events, including cell migration and the complex multistep processes of cell polarisation and activation which occurs during the formation of an immune synapse. The role of CD9 and its associated molecules during immune cell adhesion and migration have been reviewed.[99,120] LFA-1, which is integrin $\alpha_L\beta_2$ (CD11a and CD18 respectively) and with which CD9 associates via its LEL,[121] is

expressed on various leukocytes including monocytic cells, B cells and T cells.[121] Activated LFA-1 is instrumental in the transition of leukocyte rolling on endothelium to firm adhesion, by binding receptors such as ICAM-1. Stimulation of T cells using PMA induces the intermediate affinity conformation of LFA-1 and its ligand dependant clustering. Super resolution microscopy of the adhesion contact site of Jurkat T cells interacting with ICAM-1 showed that in the presence of CD9 there was an increased number of LFA-1 clusters, however they were reduced in size and more diffuse, resulting in less avid cellular interactions.[121] Therefore, a model for how CD9 modulates LFA-1 avidity has been proposed, whereby organisation of LFA-1 clustering results in altered spatial distribution of integrin-receptor interactions, so that clustered integrins have a lower total valency, whereas evenly distributed integrins establish higher valency interactions, enabling cell adhesion and spreading.[121] The CD9 partner protein EWI-2 has similar clustering effects on VLA-4 (Very Late Antigen 4, $\alpha_4\beta_1$) resulting in decreased cell spreading and ruffling of a T cell line, but not decreased adhesion.[122] Another pertinent case for the direct involvement of CD9 with integrin mediated signalling events is made by Zhang, Bontrager and Hemler, 2001.[88] The extracellular domain of integrin α3 (of VLA-3) is required for the recruitment of protein kinase C (PKC) to its intracellular tail which is subsequently phosphorylated, however CD9 association is necessary for this to occur. Therefore, the authors propose that CD9 can serve as a linker molecule that participates in a CD9-integrin-PKC complex.[88] Similarly, the association of CD9 at β1 integrin complexes with phosphoinositide 4-kinase (PI4K) can activate Ras GTPases upstream of kinase pathways that lead to proliferation.[90] Interestingly, CD9 positively regulates ICAM-1 and VCAM-1 function on endothelial cells,[123] whereas it negatively modulates LFA-1 ($\alpha_L\beta_2$) integrin adhesion to ICAM-1.[121] Therefore, CD9 is implicated in both positive and negative,

direct and indirect, modulation of integrin function on various cells across the immune system, including B and T cells.

3.7.5 CD9 involvement in the immune synapse in T cells

CD9 plays a role in the immune synapse formation on both antigen presenting cells and T cells. RNAi silencing of CD9 expression in human T cells by Rocha-Perugini et al., 2014 [124] has advanced our understanding of the role of CD9 at the T cell immune synapse, where it facilitates VLA-4 integrin signalling, resulting in somewhat increased expression of the T cell activation marker CD69 and IL-2 production.[124] VLA-4 is the $\alpha_4\beta_1$ integrin (CD49d and CD29, respectively) and is located in the peripheral ring of the T cell immune synapse where it stabilises cell-cell interactions and enhances signalling. CD9 partially colocalises with CD3 within the central-SMAC (supramolecular activation cluster) but does not modulate CD3 dependant signalling; silencing of CD9 does not affect the localisation of CD3 to the immune synapse, the duration and frequency of immune synapse formation, nor the re-localisation of the cytoskeletal elements F-actin or the microtubule-organising centre, neither does CD9 affect talin localisation which mediates inside-out signalling.[124] However, they found that CD9 promotes α4β1 integrin accumulation at the peripheral-SMAC in an activated, high-affinity form.[124] Moreover, CD9 expression was required for phosphorylation and accumulation of the integrin associated signalling molecule FAK (focal adhesion kinase) at the immune synapse, therefore enabling subsequent downstream signalling via ERK (extracellular signal-regulated kinase) activation.[124] CD9 knock down results in decreased phosphorylation and colocalization of FAK and ERK1/2 in Jurkat T cells, during engagement with B cell presented cognate antigen.[124]

3.7.6 Further functions of CD9 on immune cells

CD9 mediated regulation of proteases adds another layer of complexity to how it modulates the function of integrins as well as other receptors. CD9 interacts with a disintegrin and metalloproteinase (ADAM) 10 and to a higher extent ADAM17 via the LEL.[125] These 'shedases' cleave other cell surface molecules including adhesion molecules and growth factor receptors, allowing cells to quickly change their surface phenotype and consequently their capacity for various functions. Binding of CD9 by anti-CD9 antibodies inhibits ADAM17 mediated shedding of TNFα and ICAM-1 on the surface of the Raji B cell line but induced ADAM10 mediated shedding of TNFα.[123] Also, the expression of CD9 on human lymphoblastic cell lines inhibits both ADAM17 and $\alpha_5\beta_1$ integrin by enhancing their colocalisation.[125]

B cell development and activation appears to be normal in CD9 null mice, leading to suggestions that CD9 plays a redundant role on murine B cells.[108] The same group report in a review that "The distribution of lymphocyte subsets in [CD9] deficient mice . . . is generally normal" and "The immune response of mice that lack CD9 . . . to T cell dependant and T cell independent antigens are normal." [126] Though a thorough evaluation of immune cell functions, including T cell functions, have not been published in an experimental paper, to my knowledge.

CD9 and CD81 double null mice develop multinucleated giant cells as these tetraspanins together inhibit the fusion of mononuclear phagocytes.[127] This is in contrast to other cell types in which CD9 is required for cell fusion, notably gametes, myoblasts and some virus infected cells,[127,128] further exemplifying the variable effects of tetraspanins on different cell types.

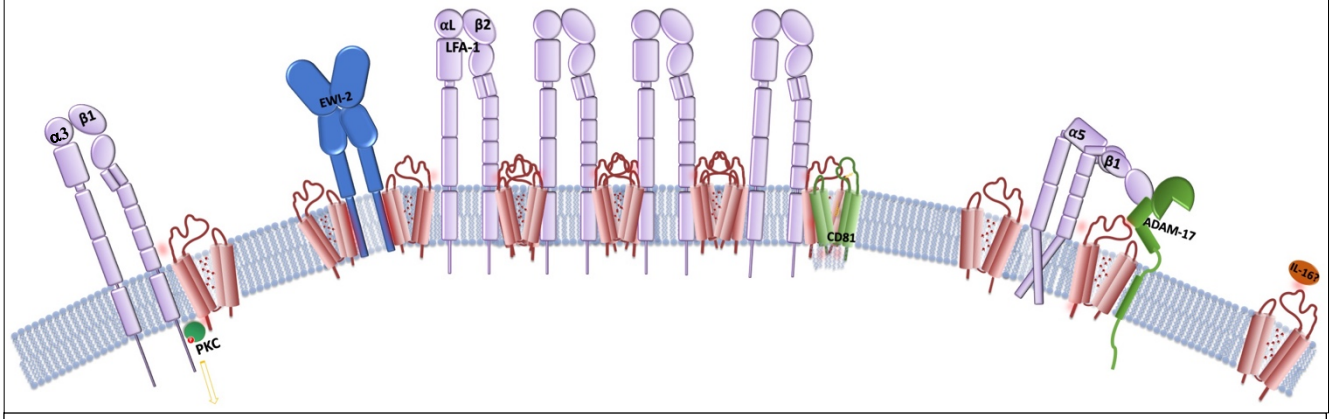
CD9 expression on macrophages downregulates lipopolysaccharide (LPS) signalling via its interaction with both CD14 and TLR4, resulting in decreased inflammatory cytokine

production.[129] CD9 is in turn downregulated upon inflammatory macrophage activation via IFNγ and STAT-1 signalling,[130] it therefore appears to play a regulatory function in macrophages.[131] On antigen presenting cells, TEM are reported to have a role in stabilising MHC-II clusters at the immune synapse and regulation of degranulation and cytokine production in myeloid cells.[131] Trafficking of MHC-II to and from the cell surface is reduced in CD9 knock out murine monocyte derived dendritic cells, resulting in reduced antigen presentation to T cells.[132]

The vast majority of known Tspan-partner protein interactions occur *in cis*, hence they are not generally considered to function as receptors to ligands on opposing cells or to soluble ligands. However, CD9 was shown to be a direct receptor for soluble Pregnancy Specific Glycoprotein 17 (PSG17) on mouse oocytes, the function of which inhibits gamete fusion in the absence of CD9.[85,133] On immune cells, CD9 is involved in PSG17 induced IL-10, IL-6 and TGF-β1 production in murine, but not human, macrophages.[134] In addition, CD9 is an IL-16 receptor in both mouse and human mast cells.[135] In T cells, IL-16 induces chemotaxes and promotes Th1 polarisation among other functions,[reviewed in 136] and whilst CD4 is the primary IL-16 receptor on T cells the contribution of CD9 to this shared signalling pathway in this cell subset is still in question.[135] Likewise, whether porcine CD9 can also function as a receptor for IL-16 on other immune cells subsets would require investigation.

In sum CD9 is involved in a plethora of molecular interactions at the cell surface, its functional roles vary according to its respective interaction partners and different mechanisms of modulating them, the outcome of which may also vary depending on the cell type.

Figure 2 Example CD9 interactions at the cell surface membrane



Illustrations of CD9 interacting with molecules in the cell surface membrane. From left to right: CD9 links integrin α3β1 with PKC, CD9 forms a complex with EWI-2 via close interactions in the transmembrane domains, CD9 promotes clustering of LFA-1, CD9 forms dimers with CD81, CD9 colocalises integrin α5β1 with ADAM-17 in their inactive states, CD9 is a putative cytokine receptor. Interactions are based on models that are described and referenced in the main text. Interaction sites are indicated by red blush. Not drawn to scale. Illustrations were made in Microsoft PowerPoint.

PKC: protein kinase C, EWI: glutamine tryptophan isoleucine containing immunoglobulin-like family, LFA: leukocyte function antigen, ADAM: a disintegrin and metalloproteinase.

4 Aims and Hypotheses

We aim to expand on the range of monoclonal antibodies that are available for phenotyping the porcine immune system, in particular for studying adaptive immunity. We hypothesise that mAbs that result in variable staining patterns across porcine B and T cells may be used to identify cell subsets or differentiation stages. To this end, we co-stain for CD9 alongside established markers of T cell differentiation, identify whether CD9 expression can be induced on activated T cells and interrogate CD9 expression on T cells which exhibit an antigen specific memory response *in vitro*. Based on indications in the literature, we also hypothesise that CD9 is a marker of IL-10^{+} regulatory B cells. We therefore aim to provide a basic characterisation of porcine IL-10 competent B cells according to the latest B cell characterisation methods we have available. Because we ultimately we aim to expand on our ability to characterise the immune cells which respond to infectious challenge or vaccination, our discussion of CD9^{+} T cells and IL-10 competent B cells focuses on this context.

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Expression of CD9 on porcine lymphocytes and its relation to T cell differentiation and cytokine production

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ABSTRACT

In this work, we report on two novel monoclonal antibodies, specific for porcine CD9. CD9 is a tetraspanin that is expressed on a wide variety of cells. We phenotyped porcine immune cell subsets and found that CD9 was expressed on all monocytes as well as a subset of B cells. CD9 was variably expressed on T cells, with CD4 T cells containing the highest frequency of $CD9^{+}$ cells. CD9 expression positively correlated with the frequency of central memory CD4 T cells in *ex vivo* PBMC. Therefore, we proceeded to explore CD9 as a marker of T cell function. Here we observed that CD9 was expressed on the vast majority of long-lived influenza A virus-specific effector cells that retained the capacity for cytokine production in response to *in vitro* recall antigen. Therefore, the new antibodies enable the detection of a cell surface molecule with functional relevance to T cells. Considering the importance of CD9 in membrane remodelling across many cell types, they will also benefit the wider field of swine biomedical research.

1. Introduction

Methods for investigating long-term protective immunity are still insufficient for swine, because there is a lack of reagents to do so. In particular, monoclonal antibodies (mAbs) are required that are specific for immune molecules, and enable the identification of cell phenotypic subsets. A more detailed characterisation of the immune systems of livestock animals will enable the development of new vaccination strategies, which will both improve animal welfare and help protect pigs from infectious disease. Here, two novel anti-porcine mAbs were investigated that showed staining patterns on lymphocytes which indicated an antigen that is variably expressed across subsets of T and B cells. To identify the antigen recognized by these two mAbs, they were screened on cells expressing a porcine cDNA library and it was determined that they bind to CD9. We then explored whether the CD9 antigen could serve as a marker of immune cell subsets and T cell function in swine.

CD9 (TSPAN-29) is a member of the tetraspanin family of proteins, which coordinate lateral interactions with other membrane proteins, particularly integrins, on the cell surface. Tetraspanins share the basic structure of four alpha helical membrane spanning domains, connected by two extracellular loops, and a short intracellular C-terminal tail (Umeda et al., 2019). The large extracellular loop of CD9 enables the formation of homodimers and heterodimers with other tetraspanins in tetraspanin-enriched-microdomains (TEMs) (Charrin et al., 2001; Oosterheert et al., 2020). The impact of tetraspanin expression on cell function depends on the cell type because TEMs coordinate a wide variety of cell surface events. Indeed, tetraspanins are a large family of proteins which emerged during the evolution of multicellular organisms. As such they have diverse functional roles in cell-cell interactions

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and have been widely studied across many biological disciplines (Toyo-oka et al., 1997). The role of CD9 in various cellular functions is recognised, from enabling fertilisation (Kaji et al., 2000; Le Naour et al., 2000; Miyado et al., 2000), to allowing the formation of extracellular vesicles and their subsequent contribution to cell signalling (Böker et al., 2018; Li et al., 2020). CD9 is also widely studied in infection biology, particularly virology. For example, human CD9 (hCD9) facilitates Middle East respiratory syndrome coronavirus entry by linking its receptor dipeptidyl peptidase-4 to a membrane fusion activating protease (Earnest et al., 2017). Yet CD9 decreases human papilloma virus-16 infection through modulation of sheddase activity (Mikuličić et al., 2020). Similarly, CD9 is mechanistically involved in fusion events during viral infection (Singethan et al., 2008) for example, anti-CD9 antibodies inhibit cell-cell fusion during to canine distemper virus infection (Singethan et al., 2006). It has also recently been shown to be essential to the formation of multinucleated giant cells during infection with *Burkholderia thailandensis* bacteria (Elgawidi et al., 2020; Singethan et al., 2008). CD9 is also studied within the field of cancer biology, where aberrant expression contributes to neoplasia and metastasis, depending on the malignancy (Huang et al., 2004; Kwon et al., 2017; Leung et al., 2020; Schaper and van Spriel, 2018).

Within the field of immunology CD9 expression is most commonly studied in the context of innate leukocytes and antigen presentation (Orinska et al., 2020; Rocha-Perugini et al., 2017; Wang et al., 2002; Yu et al., 2017). Although some early research into CD9 explored its impact on human T cell activation (Kobayashi et al., 2004; Lagaudrière-Gesbert et al., 1997; Tai et al., 1997), such function is now ascribed to the tetraspanin control of cell signalling in association with other stimulatory molecules (Rocha-Perugini et al., 2014). For example, CD9 associates with the β1 integrin chain (CD29) of VLA-4, on both human and mouse cell lines (Rubinstein et al., 1994) and CD9 negatively regulates the function of LFA-1 by controlling its aggregation on the cell surface via its interaction with the β2 integrin (CD18) (Reyes et al., 2015). In addition to the role of CD9 in the orchestration of cell surface events via TEMs (Yunta and Lazo, 2003), it is implicated in directly facilitating downstream signalling and initiating cytoskeleton rearrangement (Wang et al., 2011). The expression of CD9 can therefore impact a variety of immune processes, from immune cell homing and transmigration of the endothelium, to the formation of a stable immune synapse (Jiang et al., 2015; Murru et al., 2018; Reyes et al., 2018; Roth et al., 1995; Yeung et al., 2018).

CD9 divergently evolved with the emergence of vertebrates (Garcia-España et al., 2008). The molecular functions of CD9 in cell-cell interactions has therefore resulted in it being a molecule of interest to shed light on the evolutionary development of immunity in vertebrate biology. CD9 expression has been analysed in various fish species (Fujiki et al., 2002; Uinuk-Ool et al., 2002; Wu et al., 2012), wherein a widespread tissue distribution of CD9 is also seen, particularly in the spleen, with highest CD9 expression in innate immune cells (Zhu et al., 2006). An approach of comparing CD9 mRNA expression in tissues during bacterial infection and non-infectious conditions has lead authors to speculate that CD9 is upregulated during the immune response also in turtle (Zhou et al., 2010). A most recent study in Japanese flounder comprehensively evidences, through use of genetic knockdown, the mechanistic causality of CD9 in host defence to pathogenic bacteria (He et al., 2021).

There has also been increasing interest in CD9 as a functional marker upon lymphocytes in mouse and human, particularly as a marker for innate-like B cell subsets (Matsushita et al., 2016) and also as a functional marker of IL-10 competent B cells (Brosseau et al., 2018a; Matsushita et al., 2016; Ostrowski et al., 2007; Sun et al., 2015). However, it must be considered that the tetraspanin profiles of cell subsets differ between species (Balise et al., 2020). In this study, CD9 expression was related to porcine immune cell phenotype and function. It was observed that CD9 was expressed *de novo* following mitogenic T cell activation. We go on to suggest that $CD9^+$ T cells may have particular functional relevance during long term immune memory.

2. Materials & methods

2.1. Animal work

2.1.1. Blood and organ collection from pigs

For experiments using porcine peripheral blood mononuclear cells (PBMC) unless otherwise indicated, heparinized blood was obtained from healthy six-month-old fattening pigs from a local slaughterhouse. Animals were exsanguinated following high voltage electric anaethesia, in accordance with the Austrian Animal Welfare Slaughter Regulation. For experiments requiring lymphocyte isolation from organs alongside the blood, 14-week-old pigs, which had been bred on a conventional farm in Lower Austria and housed at the University of Veterinary Medicine, Vienna, were used. Organs were collected for lymphocyte isolation after anesthesia by intramuscular injection of Ketaminhydrochlorid (Narketan®, 10 mg/kg body weight, Vétoquinol, Lure Cedex, France) and Azaperon (Stresnil®, 1.3 mg/kg body weight, Elanco, Greenfield, USA) followed by euthanasia via intracardial injection of T61® (tetracaine hydrochloride, mebezonium iodide and embutramide, 1 mL/10 kg body weight, MSD Animal Health). Since organ and blood collection were done on dead animals, no federal animal ethics approval was required, in accordance with Austrian law. For some experiments PBMC from animals experimentally infected with swine influenza A virus were used. Details on the experimental procedures of these animals have been published previously (Talker et al., 2015; Talker et al., 2016). Infection and blood sampling of these animals was approved by the institutional ethics committee. Final approvals were obtained from Advisory Committee for Animal Experiments (§12 of Law for Animal Experiments, Tierversuchsgesetz – TVG) and the Federal Ministry for Science and Research (reference numbers BMWF-68.205/0180-II/3b/2011 and BMWF-68.205/0103-II/3b/2013).

2.1.2. Mouse immunization

Porcine $CD21^-$ B cells and NK cells were enriched from PBMC by magnetic depletion of $CD3^+$ T cells, $CD172a^+$ myeloid cells, and $CD21^+$ B cells. The obtained fraction of enriched B and NK cells were lysed and used to generate mAbs as follows. Female, six-week-old BALB/c mice were immunized subcutaneously four times in intervals of three weeks, using 1×10^7 cells along with 50 μg lipopeptide adjuvant (EMC microcollections, Tübingen, Germany) per immunization. Then, cell lysates were injected intraperitoneally, at a dose of 1×10^7 cells, without adjuvant on three consecutive days, during the week prior to splenocyte isolation. Generation of spleen cell derived hybridoma cells was then achieved as previously described (Köhler and Milstein, 1975). These experiments were approved by the institutional ethics committee. Final approvals were obtained from the Advisory Committee for Animal Experiments (§12 of Law for Animals Experiments, Tierversuchsgesetz—TVG) and the Federal Ministry for Science and Research (BMBWK GZ 68.205/0115-BrGT/2005). Hybridoma supernatants were screened for reactivity against porcine PBMC. Accordingly, two monoclonal Ab clones were identified for further study in this work, 3B3 (full designation: 3A1 3B3, for envisaged commercialisation: VIV-3B3), and 2E12 (full designation: 3H6 2E12, for envisaged commercialisation: VIV-2E12).

2.2. Lymphocyte isolation

PBMC were isolated from heparinized whole blood by density centrifugation on lymphocyte separation medium (Pancoll®, human; density, 1.077 g/mL; PAN Biotech, Aidenbach, Germany) at 920×*g*, for 30 min, at room temperature. PBMC were either used immediately where indicated, or cryopreserved at −150 °C according to the protocol described in (Leitner et al., 2012).

Lymphocytes were isolated from spleen and mediastinal lymph

nodes by mechanically disrupting the tissue by pressing it through a sieve. The cells were then filtered through cotton wool and separated by density centrifugation under the conditions described above. Lymphocytes from the lung were isolated by first subjecting small pieces of tissue to enzymatic degradation before mechanical disruption, filtration, and density centrifugation, as described in detail previously (Talker et al., 2016). Cells isolated from organs were immediately stained for flow cytometry.

2.3. Immunoprecipitation

PBMC from slaughterhouse pigs were biotinylated using Sulfo–NHS–LC-Biotin (Pierce™; Thermo Fisher, Waltham, MA, USA) and lysed afterwards. Immunoprecipitation was performed using Pierce™ Direct IP Kit (Thermo Fisher). In brief, the mAb clone 2E12 in hybridoma supernatant, or a mouse IgG1 isotype control mAb (Thermo Fisher), were covalently bound to AminoLink Plus Coupling Resin (Thermo Fisher), following manufacturer's instructions, and used for immunoprecipitation of the antigen in the cell lysates, as described previously (Reutner et al., 2012). Following elution from the mAb 2E12 coupled resin, the precipitated proteins were separated by 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis and semidry-blotted onto a polyvinylidene fluoride membrane, upon which they were detected using streptavidin-HRP (Roche, Vienna, Austria).

2.4. Screening of a porcine cDNA library to identify the target antigen of mAb clone 3B3

A previously established cDNA library was used, whereby mouse Bw5147 cells had been transduced with cDNA isolated from activated porcine PBMC, using retroviral expression vector pBMN and retrovirus plasmid pEAK12-MLV-env-gag-pol, detailed in Leitner et al. (2012). The cDNA library screening procedure was followed as detailed in Leitner et al. (2012). In brief, hybridoma supernatant containing mAb clone 3B3 was incubated with 2×10^7 Bw5147 cells expressing the cDNA library, for 20 min, at 4 °C, then washed twice in sort buffer (PBS [PAN Biotech] with 5% foetal calf serum [FCS, Sigma-Aldrich, St Louis, MO, USA] and 2 mM EDTA). Library cells against which mAb 3B3 reacted were detected using a goat anti-mouse-IgG1 secondary antibody conjugated to PE (Southern Biotech, Birmingham AL, USA). Cells showing a staining with mAb 3B3 were sorted using a FACSAria (BD Biosciences, San Jose, CA, USA) and expanded *in vitro* for nine days; then sorted a second time. Single cell clones were established through limiting dilution of the cells. Samples to control for specific staining consisted of the isotype-specific PE-conjugated secondary antibody only, and mouse IgG1κ isotype control (clone P36281, Thermo Fisher) followed by the secondary antibody. Bw5147 cells, which had not been transduced with the cDNA library, were treated likewise for comparison. Single cell clones were expanded and reactivity of 3B3 on colonies was verified by flow cytometry. Genomic DNA was isolated from the colonies, and retroviral inserts were amplified using primers (Eurofins, Munich, Germany) specific for pBMN flanking the porcine cDNA inserts:

Ban1b: 5′-GAC CAT CCT CTA GAC TGC CGG ATC-3'
Ban2b: 5′-CAT TCC CCC CTT TTT CTG GAG ACTAAA TAA AAT C-3'

A touchdown PCR was performed with the following cycles: 98 °C 2 min, 11 cycles 98 °C 20 s, 68 °C (−1 °C each cycle) 30 s, 68 °C 3 min, followed by 35 cycles with 94 °C 20 s, 57 °C 30 s, 68 °C 3 min and finally 68 °C 10 min. Phusion® High-Fidelity DNA Polymerase (NEB, Frankfurt, Germany) was used. PCR products were separated according to size by electrophoresis on a 1.2% agarose gel. DNA bands of a size common across clones were sent for sequencing (Eurofins Genomics Ebersberg, Germany). Due to an over-representation of certain cDNA in the PCR, and not all cDNA fragments being favoured in the reaction, all bands from a single clone were sequenced, as a second confirmatory approach.

2.5. Confirmation of mAb specific reactivity against porcine CD9

RNA was extracted from porcine PBMC using Direct-Zol RNA MiniPrep (Zymo Research, Irvine, CA, USA) and cDNA was synthesized using oligo dT (Promega, Madison, WI, USA) and superscript II reverse transcriptase (ThermoFisher). Primers were designed against porcine CD9 (Eurofins Genomics Ebersberg, Germany) containing Sfi-I restriction sites, which enable unidirectional cloning:

CD9-Sfi-F 5′-CGAGGTGGCCATTACGGCCACCATGCCGGTCAAAGGA -3'
CD9-Sfi-R 5' -CAGGGCGGCCGAGGCGGCCCTAGACCATCTCTCGG CT-3'.

These primers were used for PCR with the following conditions: 98 °C 2 min, followed by 25 cycles with 94 °C 20 s, 60 °C 30 s, 68 °C 1.5 min, and finally 72 °C 5 min. The product was directly cloned into the retroviral expression vector pMIGII (a gift from Dario Vignali: (Holst, 2006), which also contains enhanced green fluorescent protein (eGFP) downstream of an internal ribosome entry site. CD9-pMIGII was used to retrovirally transduce mouse Bw5417 cells, which were then sorted on the basis of eGFP expression and expanded *in vitro*. Monoclonal antibody clones 3B3 and 2E12, and an anti-human CD9 clone, MM2/57 (Bio-Rad, Hercules, CA, USA), reported to be cross reactive with porcine CD9 (Saalmüller et al., 2005; Saalmüller and Aasted, 2007), were tested for reactivity against porcine CD9 transduced cells, by flow cytometry using a FACSCanto II (BD Biosciences).

2.6. Fluorescence microscopy of porcine lymph nodes

Inguinal lymph nodes were covered in Tissue-Tek® O.C.T. Compound (Sakura Finetek, Alphen aan den Rijn, The Netherlands) and frozen in liquid isopentane, on dry ice, then stored at −80 °C until sectioning using a microtome (Leica CM1950). Six μm thick sections were dried onto slides for 1 h then fixed for 10 min in methanol/acetone (1:1), at −20 °C. Slides were washed in PBS three times for 5 min. To block unspecific binding, slides were incubated with 5% goat serum (Vector Laboratories, Burlingame) in PBS, for 40 min. Primary antibodies were applied sequentially overnight: SLA-DR, MSA3, mouse IgG2a, in PBS (in house preparation); CD9, 3B3, mouse IgG1 in PBS (in house preparation); CD3, BB23-8E6, mouse IgG2b, in 5% goat serum/PBS (Southern Biotech). Slides were washed in PBS three times for 5 min, and a secondary antibody panel was applied for 40 min: goat F(ab') 2 anti-mouse IgG2a Alexa 555, (Thermo Fisher); goat-anti-mouse IgG1 Alexa Fluor 647, goat-anti-mouse IgG2b Alexa Fluor 488, (both Jackson ImmunoResearch, PA, USA). Following antibody staining, slides were counterstained with DAPI for 3 min, washed in PBS and dH_2O, and mounted with a cover slide with Fluoromount™ Aqueous Mounting Medium (Sigma Aldrich, F4680). Images were scanned using an Axioimager Z.1 microscope (Zeiss, Germany), with TissueFAXS hardware and software (TissueGnostics GmbH, Austria).

2.7. Analyses by flow cytometry

Samples generated for experiments using the methodology detailed below were analysed using a FACSCanto II flow cytometer (3 lasers with 407, 488 and 633 nm, respectively, BD Biosciences) equipped with FACS Diva software version 6.1.3 (BD Biosciences). Results were processed with FlowJo version 10.6.2 (BD Biosciences). Where an indirect detection strategy for flow cytometry labelling was used, specificity was controlled for using appropriate isotype matched antibodies (Supplementary Table 1) preceding the secondary reagent. Secondary reagents are also listed in Supplementary Table 1. Fluorescence-minus-one controls aided with the determination of positive populations of less definite phenotypes. Single stain controls were used for compensation, which was calculated using FACS Diva software version 6.1.3 (BD Biosciences)

automatic compensation, and where necessary manually adjusted in FlowJo.

2.7.1. Reciprocal epitope blocking assay using anti-CD9 clones 3B3 and 2E12

Thawed PBMC were treated with either mAb clone 3B3 or 2E12 in four parallel samples, using a $\log_{10}$ dilution series. Cells were then washed and incubated in a second step with the other clone. When mAb 2E12 was used in the second step, an Alexa 647-conjugate of this mAb was used (in house conjugation). When mAb 3B3 was used in the second step, a biotinylated mAb was used (in house biotinylation) and detected using streptavidin-Alexa647. The median fluorescence intensity of positively stained cells was analysed by flow cytometry. All incubations were performed in flow cytometry (FCM) buffer (PBS, PAN-Biotech, with 10% porcine plasma, in-house preparation) for 20 min at 4 °C.

2.7.2. Staining procedure for CD9 expression on PBMC alongside established phenotypic leukocyte markers

Cryopreserved PBMC were thawed and washed twice by centrifugation in FCM buffer. Cells were plated at $0.5 - 2 \times 10^6$ cells per well in a round bottom microtitre plate. All incubation steps lasted 20 min at 4 °C. Cells were incubated with primary antibodies and washed twice, prior to addition of secondary reagents as required. Where necessary, free binding sites of isotype specific secondary reagents were blocked using mouse total IgG (2 μg per sample; Jackson ImmunoResearch), enabling a further incubation using primary reagents of the same isotype. For exclusion of dead cells, cells were treated with Fixable Viability Dye eFluor780 (Thermo Fisher), diluted and incubated in PBS. Cytoplasmic epitopes were made accessible using BD Cytofix/Cytoperm and BD Perm/Wash (BD Biosciences), according to the manufacturer's instructions. Nuclear and vesicular epitopes were made accessible using eBioscience FoxP3 staining buffer set (Thermo Fisher), according to manufacturer's instructions. Details of the antibodies used in these experiments are listed in Supplementary Tables 2 and 3.

2.7.3. Intracellular cytokine analysis of polyclonally stimulated cells

Cryopreserved PBMC were thawed and cultured in RPMI 1640 (PAN-Biotech) containing 10% (v/v) FCS (Sigma-Aldrich), 100 IU/mL penicillin and 0.1 mg/mL streptomycin (PAN-Biotech). Cells were plated at 5×10^6 cells per well, in a round bottom 96 well plate (Greiner Bio-One, Kremsmünster, Austria) and incubated overnight, at 37 °C, with 5% CO_2. Cells were then treated for 4 h using 50 ng/mL phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich) and 500 ng/mL Ionomycin (Sigma-Aldrich), in the presence of 1 μg/mL Brefeldin A (BD Biosciences GolgiPlug™).

Cultured PBMC were washed and re-suspended in PBS with 3% FCS (Sigma-Aldrich) which served as staining buffer. The staining procedure for cell surface markers was as described above (chapter 2.7.2), however, prior to intracellular cytokine staining, cells were fixed and permeabilised using BD Cytofix/Cytoperm and BD Perm/Wash (BD Biosciences), according to the manufacturer's instructions. Details on the antibodies used in these experiments are listed in Supplementary Table 4.

2.7.4. Analysis of CD9 expression on sorted T cells

Freshly isolated PBMC were stained using antibodies against T cell subsets and CD9, outlined in Supplementary Table 5. Cells were sorted using a FACS Aria, into CD9 negative T cell populations. After sorting, cells were washed and diluted to 1.5×10^6 cells/mL of RPMI1640 (supplemented with FCS and antibiotics as above) containing 3 μg/mL Concanavalin A (Amersham Biosciences, Uppsala, Sweden) and 50 ng/mL recombinant porcine IL-2 (R&D Systems, Minneapolis, MN, USA). Cells were plated at 200 μl per well of a 96 well round bottom cell culture plate, and incubated at 37 °C for two days. Cells were then analysed by flow cytometry using the same staining procedure as prior to sorting.

2.7.5. Analysis of CD9 expression during in vitro antigen-specific recall response

For experiments with PBMC from swine influenza A virus-infected pigs the details on selection and infection of the animals have been published previously (Talker et al., 2015; Talker et al., 2016). In brief, cross-bred piglets, which had tested negative for swine influenza A virus (FLUAVsw) nucleoprotein antibodies prior to the study (ID Screen Influenza A Antibody Competition Multispecies ELISA, IDvet, Grabels, France), were infected with FLUAVsw isolate A/swine/Kitzen/IDT6142/2007 (H1N2) with $10^{7.25}$ $TCID_{50}$/mL into the trachea. One group of pigs was infected at ten weeks of age and blood samples were taken 12 and 42 days post infection, as detailed previously (Talker et al., 2016). Another group of pigs was infected at both four and eight weeks of age, as detailed previously (Talker et al., 2015). Blood samples from these pigs were taken 12, 28, 35, 49, 56 and 62 days post primary infection. PBMC were isolated and cryopreserved at −150 °C, prior to thawing and culture as outlined above. PBMC were plated at 5×10^5 cells per well, and rested at 37 °C, for 4 h. Then, supernatant of Madin-Darby bovine kidney cells (MDBK [NBL-1], available from ATCC with the code ATCC® CCL-22™) producing FLUAVsw isolate A/swine/Kitzen/IDT6142/2007 (H1N2) was added to the cells, at a multiplicity of infection of 1, and incubated overnight. Control cells received a mock stimulation of supernatant from the MDBK cell line. Cytokine release was inhibited by adding Brefeldin A, at a final concentration of 1 μg/mL, during the final 4 h of incubation. Cells were stained for flow cytometry using the antibody panels listed in Supplementary Table 6.

2.8. Statistical analyses

Data was copied into GraphPad Prism 8 for graphical presentation, with the mean of the values displayed (GraphPad Software, San Diego, CA, USA). For analysing the correlation between CD9 expression and T cell differentiation status, data was tested for normality and Spearman's correlation test was chosen, and done using GraphPad Prism.

3. Results

3.1. Two novel anti-porcine monoclonal antibodies are specific for porcine CD9

Two monoclonal antibody (mAb) clones designated 2E12 and 3B3 were identified that bound to peripheral blood mononuclear cells (PBMC) with identical staining patterns (data not shown), when analysed alongside established phenotypic markers. Monoclonal Ab clone 2E12 precipitated a protein of approximately 20kD from biotinylated PBL (Fig. 1A). The specificity of these mAbs was determined using Bw5417 cells expressing a cDNA library generated from porcine lymphocytes. A small population, 0.1% of the library cells, were stained by mAb 3B3. These were sorted twice to a final purity of 91.5% (Fig. 1B). Single cell clones of the positively sorted cDNA library population were established and the expanded clonal populations were completely stained by mAb 3B3 upon re-analysis (Fig. 1C). Next, retroviral cDNA was retrieved from 3B3 reactive cDNA library clones using PCR specific for retroviral DNA flanking the insert. A BLAST search identified a cDNA, which was common to multiple 3B3 selected clones, as *Sus scrofa* CD9 (Suppl. Fig. 1). Proof of the reactivity of mAbs 3B3 and 2E12 with CD9 was achieved through the specific detection of the porcine CD9 (pCD9) molecule expressed on mouse Bw5417 cells. Primers specific for pCD9 were designed and used for RT-PCR to amplify the CD9 ORF directly from porcine PBMC. Mouse Bw5417 cells were then retrovirally transduced with the porcine CD9 cDNA using a retroviral expression vector, which also encoded green fluorescent protein on the cassette downstream of an internal ribosomal entry site. Ectopic expression of pCD9 was specifically detected on the cell surface of $eGFP^+$ cells using both mAb clones 3B3 and 2E12 (Fig. 1 D).

Next, it was determined whether both anti-CD9 mAb clones

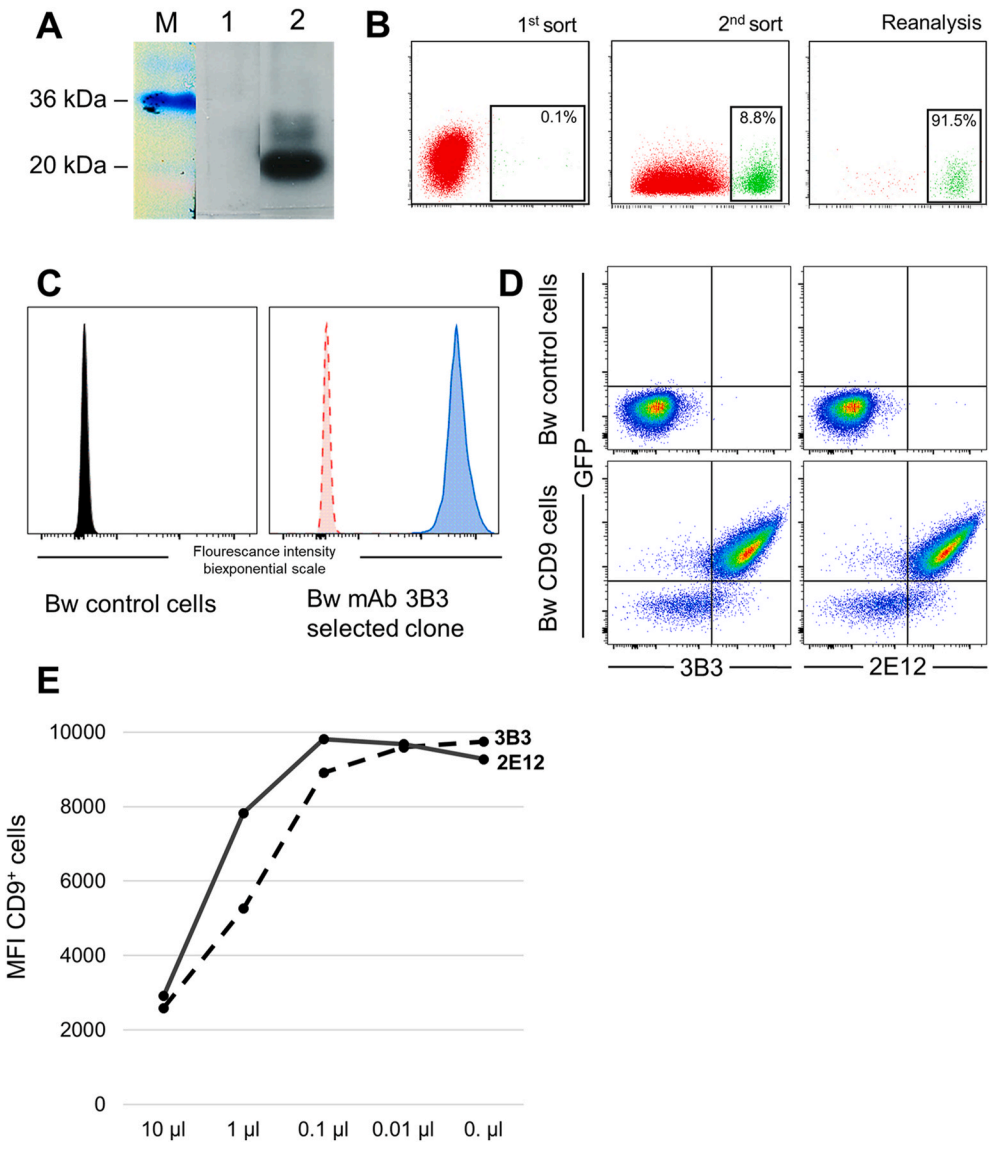

Fig. 1. Monoclonal antibodies 3B3 and 2E12 recognise porcine CD9. (A) Immunoprecipitation of mAb 2E12 antigen from biotinylated PBMC lysates by mAb 2E12. M: biotinylated mass standards, Lane 1: isotype control, Lane 2: mAb 2E12 antigen. (B) Bw5147 cDNA library cells were stained using mAB 3B3, detected using anti-mouse-IgG1 conjugated to PE and sorted (left). Following a seven-day expansion, cells were re-analysed and sorted again (middle). Final purity of 3B3 selected cells was analysed (right) prior to establishing single cell clones. (C) Bw5147 control cells (left) versus a 3B3 selected Bw5147 cDNA library colony (right). Stained with mAb 3B3 followed by goat anti-mouse IgG1-1A647 (blue solid line) or goat anti-mouse IgG1-A647 only (red, dashed outline). (D) Mouse Bw5147 cells expressing porcine CD9 upstream of eGFP were stained with mAb 2E12 and mAb 3B3. (E) Competition between the anti-porcine CD9 mAb 2E12 and mAb 3B3. The dashed line indicates staining with labelled clone 3B3 following pre-treatment with unlabelled clone 2E12. The solid line indicates staining with labelled 2E12 following pre-treatment with unlabelled clone 3B3. Median fluorescence intensity of positive cells is shown. The amount of unlabelled antibodies used for pre-incubation is shown on the x-axis.

recognise different epitopes or not. An increase in epitope accessibility of the detection Ab, as determined by median fluorescence intensity (MFI), corresponded with decreasing volumes of the clone used for initial binding (Fig. 1E). This indicates that there is competition between the two mAbs for antigen binding. Such stearic hindrance suggests the mAbs 2E12 and 3B3 recognise the same or overlapping epitopes. One other monoclonal antibody (anti-human CD9 clone MM2/57) was also tested, which has been reported to be weakly cross reactive with porcine CD9 (Bio-Rad) (Saalmüller et al., 2005; Saalmüller and Aasted, 2007). A weak co-stain with CD172a^{+} cells was confirmed (Suppl. Fig. 2A). CD172a^{high} cells highly express pCD9, as demonstrated using clones 2E12 and 3B3 but this co-staining pattern was not adequately reproducible when using MM2/57 even at high concentrations. Therefore MM2/57 was tested on the porcine CD9 transduced Bw5417 cells, and only a partial co-stain of eGFP positive cells was observed (Suppl. Fig. 2B). We therefore propose that the affinity of this clone is low.

3.2. *Porcine CD9 is variably expressed upon major leukocyte subsets*

To determine the utility of the anti-CD9 specific mAbs as tools for immune cell characterisation the potential for pCD9 to be a phenotypic marker of populations within the major leukocyte subsets of the peripheral blood was investigated.

PBMC were isolated from the blood of healthy pigs and characterised using the novel mAb clones 3B3 and 2E12, alongside markers for major PBMC subsets, and analysed using flow cytometry (Fig. 2A). We collected data from multiple phenotyping experiments of PBMC from a large number of pigs, to determine a median frequency of CD9^{+} cells for each subset (Fig. 2B). CD9 and CD172a were uniformly co-expressed, the vast majority of which were monocytes, as determined by a CD14^{+}CD172a^{high} phenotype (Suppl. Fig. 3). Natural killer cells, identified according to a CD3^{-}CD8α$^{+/dim}$CD16^{+} phenotype, did not express CD9 *ex vivo* in healthy animals, as indicated by the absence of a staining signal. B cells could be divided into two distinct CD9^{+}CD79α^{+} or CD9^{-}CD79α^{+} populations (Fig. 2A). Frequency of CD9^{+} cells within CD79^{+} B cells ranged from 5% to 44% (Fig. 2B). Within T cells, there were variable cell surface expression levels of CD9, as a continuum, with the highest and most frequent expression being on CD4 T cells. Within γδ T cells, CD8β T cells and CD4 T cells, the frequency of CD9^{+} cells ranged from 1.4% to 21%, 0.3 – 13.4% and 13.7% – 62%, respectively (Fig. 2B). To further characterise CD9 expression, we decided to test the suitability of mAb 2E12 for immunofluorescence microscopy, in combination with mAbs against CD3 and SLA-DR, on tissue sections from inguinal lymph nodes (Suppl. Fig. 4). CD9 brightly stained endothelial cells, clearly

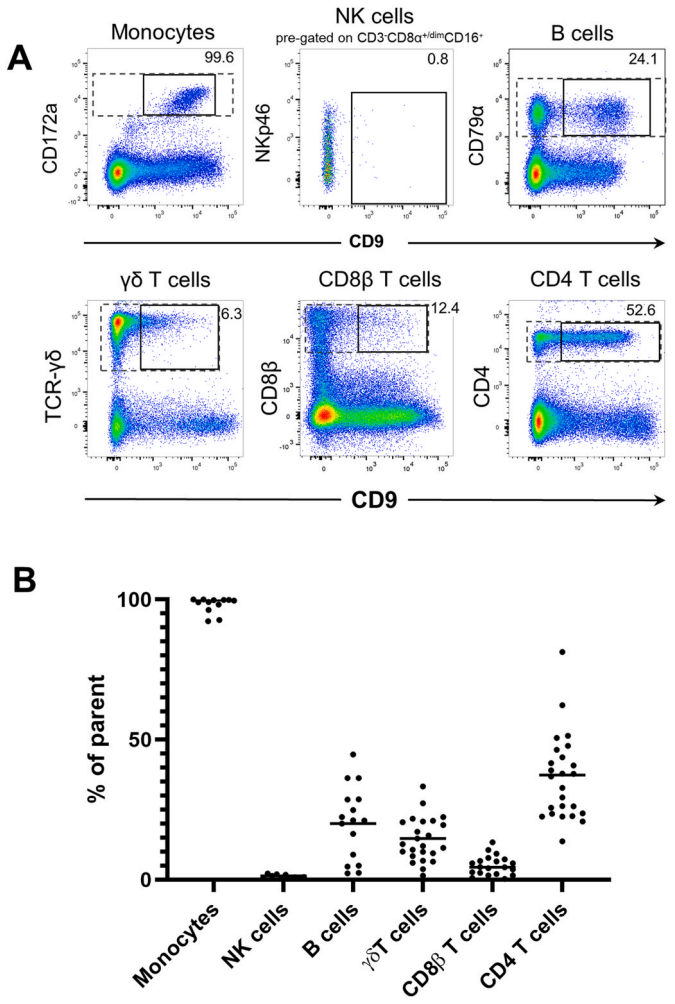

Fig. 2. Frequency of CD9$^+$ cells within major leukocyte subsets of the blood. (A) Phenotyping of blood leukocytes using anti-CD9 mAb 2E12 alongside established phenotypic markers. Representative FCM data from one animal. Top Left: Within total PBMC (large FSC-A/SSC-A gate, not shown) CD172a^{high} cells, representing mainly monocytes, are gated (dashed line) and the percentage of CD9$^+$ cells (solid line) within this subset is shown. Top Middle: CD3$^-$CD8α$^{+/dim}$CD16$^+$ cells are shown and analysed for CD9 against NKp46. Top Right: total lymphocytes are shown. The frequency of CD9$^+$CD79α$^+$ cells (solid line) within total CD79α$^+$ B cells (dashed line) is given. Bottom panels: total lymphocytes are displayed. From left to right: the solid line gate indicates CD9$^+$TCRγδ$^+$ cells within total TCRγδ$^+$ cells (dashed line), CD9$^+$CD8β$^+$ cells within total CD8β$^+$ cells and CD9$^+$CD4$^+$ cells within total CD4$^+$ cells. (B) Frequency of CD9$^+$ cells within each major leukocyte subset of the blood. Data from multiple animals across numerous experiments was amassed and was analysed as per Fig. 2A. The horizontal line represents the mean.

outlining lymphatic vessels (white arrow 1). B cell follicles were observable as spherical regions of DAPI stained cells, which were largely absent of CD3$^+$ cells and dimly expressed SLA-DR in a uniform manner. Distinct CD3$^+$ T cell zones were found on the periphery. Some CD9 positive cells were present within the centre of the B cell follicles (white arrows 2).

3.3. Within porcine T cells, CD9 expression is most frequent on CD4$^+$ and CD4$^+$FoxP3$^+$ T cells

Since CD9 was variably expressed upon T cell subsets, we determined to investigate this further, due to the importance of T cells in the establishment of an immune memory responses. In order to obtain an overview of CD9 expression within different tissue compartments, we characterised lymphocytes taken from peripheral blood, spleen, mediastinal lymph node and lung of five 14-week-old animals. Because CD4$^+$ T cells contained the largest fraction of CD9$^+$ cells, we investigated this population in more detail. CD4$^+$ regulatory T cells (Treg) were distinguished by FoxP3 expression and analysed separately to CD4$^+$FoxP3$^-$ T cells for CD9 expression (Fig. 3A). CD9 expressing cells within total CD8β and γδ T cells were also investigated and identified by the gating illustrated in Fig. 2A.

Among the five animals in this experiment, the mean frequency of CD9$^+$ γδ T cells ranged from approximately 11.0% of total γδ T cells in the blood and lung to 21.5% in the spleen (Fig. 3B). Across the investigated T cell subsets, the frequency of CD9$^+$ cells was lowest within CD8β$^+$ T cells for all organs tested (between 2.23% in blood and 17.3% in the spleen, mean values). CD4 T cells contained the highest frequencies of CD9$^+$ cells in all organs tested. Median values of CD4$^+$FoxP3$^-$ T cells were 21.2% in blood, 33.4% in spleen, 21.6% in lymph node and 39.2% in lung. CD4$^+$FoxP3$^+$ Tregs contained the highest proportion of CD9$^+$ cells with mean values of 35.7% in PBMC, 52.9% in spleen, 33.9% in lymph node, and 67.9% in lung. Due to the consistently higher frequency of CD9$^+$ cells within Tregs, in comparison to FoxP3$^-$ CD4 T cells, the median fluorescence intensity for CD9 was also compared between these two subsets (Fig. 3C). Tregs of the lung had a distinct increase in the median fluorescence intensity (MFI) of CD9 expressing cells, as opposed to all other organs tested.

3.4. CD9 expression is upregulated following in vitro T cell activation with Con A

Having identified differences in the frequencies of CD9$^+$ T cells among CD4, CD8β and γδ T cell subsets, we questioned whether these subsets have the same capacity for *de novo* expression of CD9. Hence, CD9 negative cells of CD4, CD8β, and TCR-γδ T cells, were sorted, respectively, and were analysed for CD9 expression following a 48 h incubation with ConA, in the presence of pIL-2 (Fig. 4). In order to discern T cells that were activated under these conditions staining was also performed for CD25, which is generally increased upon T cell activation. Across all three major T cell subsets, variable levels of CD9-expressing cells were detected, with a lower MFI on CD8β$^+$CD25$^+$ cells, reflecting the *ex vivo* phenotypic differences between these subsets. Nevertheless, there was a marked increase of CD9 expression on CD25$^+$ cells of all three T cell subsets following activation.

3.5. CD9 expression is only marginally more common to CD25^{hi}IL-10$^+$CD4 T cells

Since CD4$^+$FoxP3$^+$ regulatory T cells contained the highest frequency of CD9 expressing cells, across all analysed T cell subsets (Fig. 3B), we postulated that CD9 could serve as a marker of a distinct phenotype of regulatory T cells. As IL-10 production is a major feature of Treg cells, CD9 expression was analysed upon cells with the functional capacity for IL-10 production. CD25^{high} CD4 T cells represent FoxP3$^+$ cells (Suppl. Fig. 5A, left panel). This population has been shown to suppress the proliferation of CD3-stimulated responder cells during co-culture to a greater extent than CD4$^+$CD25^{dim}T cells, which had a greater capacity for IL-10 production (Käser et al., 2011). Intracellular IL-10 was detected within CD25 high, dim, and low CD4 T cells following 24-h stimulation with PMA and Ionomycin, in the presence of Brefeldin A (Fig. 5A), and CD9 expression on these populations was compared. CD25 expression only was chosen to discriminate Tregs without FoxP3 detection, in order to avoid the technical difficulties of combining intracytoplasmic staining for IL-10 and intranuclear staining protocols. However, to demonstrate that the expression levels of FoxP3 and CD25 within CD25^{high}Foxp3$^+$ CD4 T cells were not affected by treatment of cells with PMA and Ionomycin, or cultivation in medium overnight, an intranuclear stain for FoxP3 was done on cells treated this way (Suppl. Fig. 5B + C). In addition, there was no upregulation of CD25

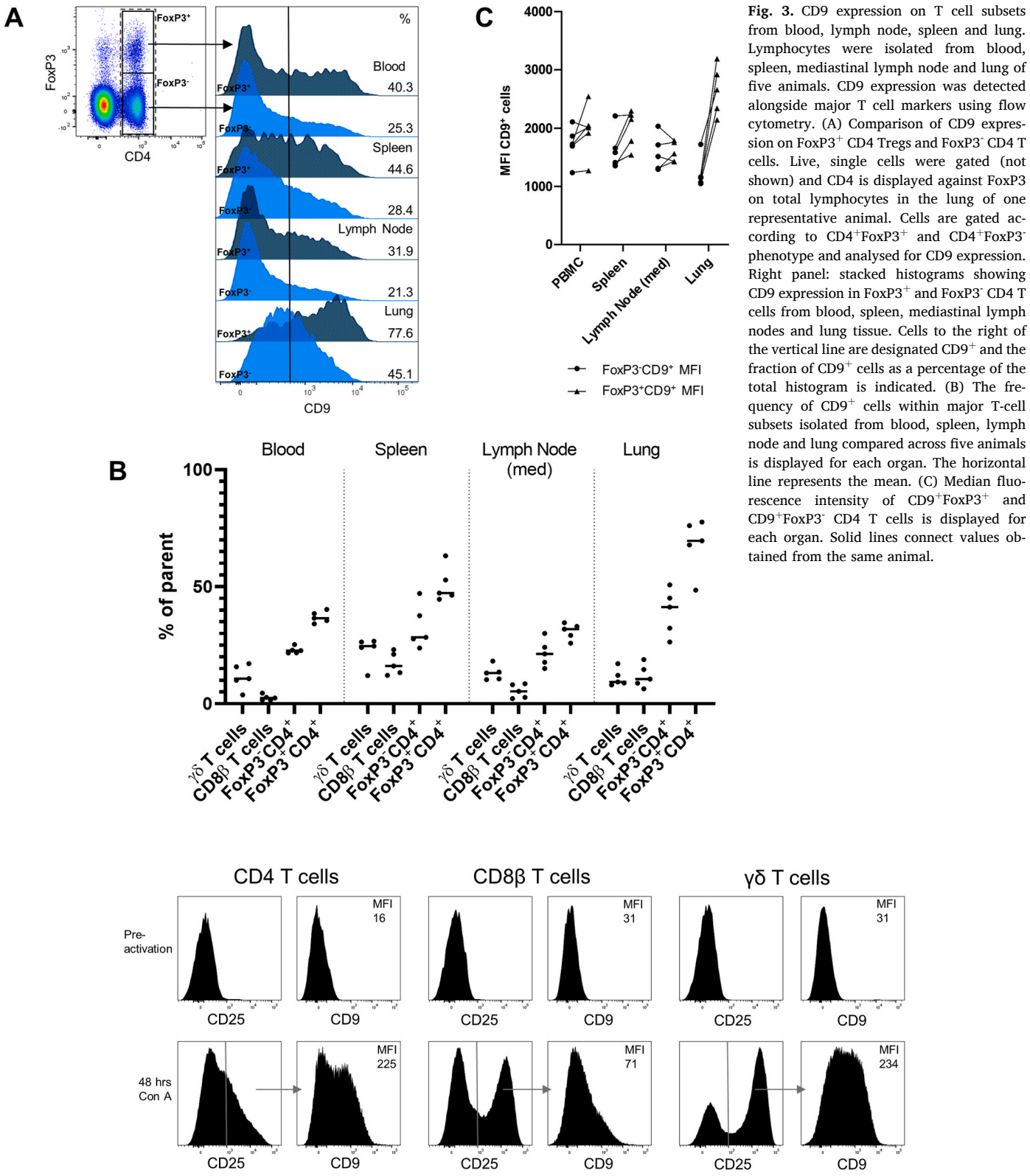

Fig. 3. CD9 expression on T cell subsets from blood, lymph node, spleen and lung. Lymphocytes were isolated from blood, spleen, mediastinal lymph node and lung of five animals. CD9 expression was detected alongside major T cell markers using flow cytometry. (A) Comparison of CD9 expression on FoxP3$^+$ CD4 Tregs and FoxP3$^-$ CD4 T cells. Live, single cells were gated (not shown) and CD4 is displayed against FoxP3 on total lymphocytes in the lung of one representative animal. Cells are gated according to CD4$^+$FoxP3$^+$ and CD4$^+$FoxP3$^-$ phenotype and analysed for CD9 expression. Right panel: stacked histograms showing CD9 expression in FoxP3$^+$ and FoxP3$^-$ CD4 T cells from blood, spleen, mediastinal lymph nodes and lung tissue. Cells to the right of the vertical line are designated CD9$^+$ and the fraction of CD9$^+$ cells as a percentage of the total histogram is indicated. (B) The frequency of CD9$^+$ cells within major T-cell subsets isolated from blood, spleen, lymph node and lung compared across five animals is displayed for each organ. The horizontal line represents the mean. (C) Median fluorescence intensity of CD9$^+$FoxP3$^+$ and CD9$^+$FoxP3$^-$ CD4 T cells is displayed for each organ. Solid lines connect values obtained from the same animal.

Fig. 4. T cells upregulate CD9 on the cell surface following *in vitro* activation. Lymphocytes were isolated from blood and CD9$^-$ fractions of separate CD4, CD8β and TCR-γδ subsets were sorted by FACS. Sorted cells were stimulated with Con A in the presence of IL-2 for 48 h and then stained for flow cytometry. Top row: Histograms display the expression level of CD25 and CD9 in cells immediately following sorting, prior to *in vitro* cultivation. Numbers indicate the median fluorescence intensity (MFI) of CD9 within the total sorted fraction. Bottom row: Histograms display the expression level of CD25 and CD9 within CD25$^+$ cells following 48 h of activation using Con A + IL-2. The MFI of CD9 expression within CD25$^+$ cells is given. Data from one representative animal of three is shown.

on the FoxP3^{-}CD25^{med} T cell population under these experimental conditions (Suppl. Fig. 5D). Data from multiple experiments on fresh lymphocytes isolated from blood and lung on IL-10 production combined with a phenotyping for CD4, CD25, and CD9 is displayed in Fig. 5B. Results showed a general trend in the mean frequency of CD9^{+} cells to be highest within the CD25^{high}IL-10^{+} population and lowest within the CD25^{-}IL-10^{+} population from both organs (Fig. 5B). However, there was no substantial enrichment of CD9-expressing cells within IL-10-producing CD4 T cells (Fig. 5B).

3.6. CD9 expression according to T cell differentiation status

Since CD4^{+} T cells contained the largest fraction of CD9^{+} cells *ex vivo,* this population was investigated in more detail. Lymphocytes

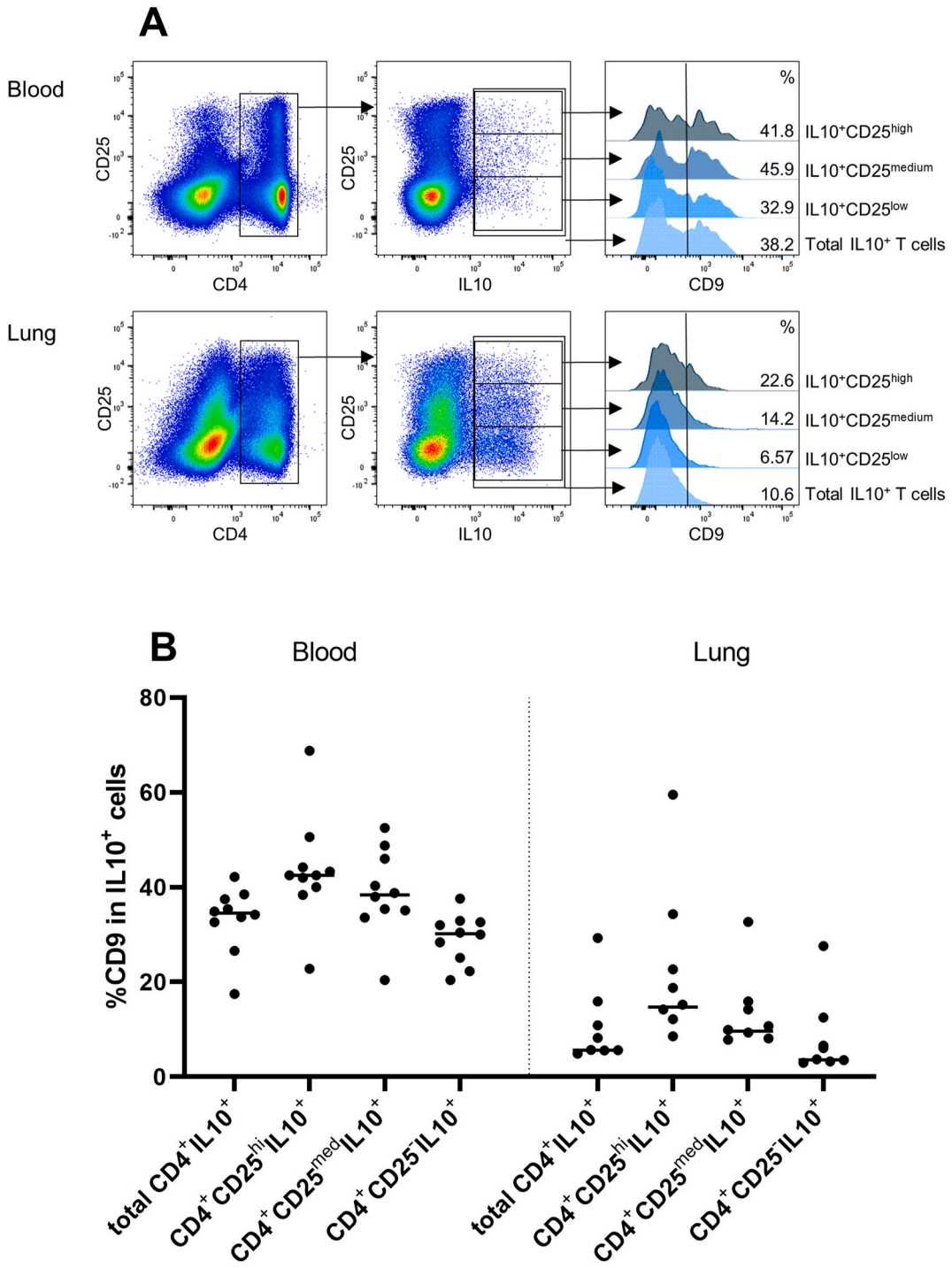

Fig. 5. CD9 expression of IL-10-producing CD4 T cells of the blood and lung. Lymphocytes were isolated from blood or lung and immediately placed into culture. After overnight incubation in medium, cells were treated with PMA, Ionomycin and Brefeldin A for 4 h, then stained for flow cytometry. (A) Representative analysis of lymphocytes from the blood and lung of one animal. Live, single (not shown), CD4^{+} T cells were gated and further divided into IL-10^{+}CD25^{high}, IL-10^{+}CD25^{med}, and IL-10^{+}CD25^{-} cells. CD9 expression of each group is displayed in the stacked histograms. Numbers give percentages of CD9^{+} cells. (B) Combined data from several experiments (n = 10 for blood, n = 8 for lung) showing the frequency of CD9^{+} cells within IL-10-producing CD4^{+} cells subdivided on the basis of CD25 expression level. The horizontal line represents the mean.

isolated from blood, spleen, mediastinal lymph node and lung of five animals were analysed for CD9 expression alongside markers for $CD4^+$ T cell differentiation status (Fig. 6). In the pig $CD4^+$ T cells, which have not previously been primed by exposure to cognate antigen, do not express CD8α and are considered naïve (Saalmüller et al., 2002). $CD4^+CD8\alpha^+$ T cells can be further divided on the basis of their CD27 expression, into effector memory (Tem) cells which are $CD4^+CD8\alpha^+CD27^-$ and into central memory (Tcm) cells with a $CD4^+CD8\alpha^+CD27^+$ phenotype (Reutner et al., 2013). For these analyses $CD4^+FoxP3^+$ Tregs from total $CD4^+$ T cells were excluded (Fig. 6A). Within all organs tested, the frequency of $CD9^+$ CD4 T cells was highest within the $CD8\alpha^+CD27^+$ fraction, corresponding to central memory cells, and lowest within $CD8\alpha^-CD27^+$ cells, corresponding to naive CD4 T cells (Fig. 6B and Suppl. Fig. 6). The MFI of CD9 positive cells was consistently lowest on naive cells and fairly even across both $CD4^+CD8\alpha^+CD27^+$ and $CD4^+CD8\alpha^+CD27^-$ memory subsets (Fig. 6C). Unique to PBMC, the MFI of $CD9^+$ CD4 T cells was highest upon effector memory cells, despite $CD9^+$ cells being less frequent within this functional subset. Since CD9 was most frequently expressed on memory CD4 T cells, the frequency of CD9 positive CD4 T cells was correlated with the frequency of cells of CD4 T cell subsets in individuals (Fig. 7). Data from multiple analyses of peripheral blood T cells was pooled and the frequency of $CD9^+$ cells within total T cells was compared with the frequency of Tcm, Tem and naive cells within total CD4 T cells. A clear positive correlation was found between the frequency of $CD9^+$ T cells and frequency of central memory T cells with significance $P = 0.0001$ (Fig. 7A), but not for Tem and naive CD4 T cells (Fig. 7B and C, respectively).

3.7. Long-lived CD4 T cells that respond to in vitro recall antigen are $CD9^+$

So far, we observed that $CD9^+$ cells are particularly represented within CD4 T cells with a memory phenotype *ex vivo*. Next, we examined whether CD9 expression correlated with functionality of long-term memory subsets through the capacity for cytokine production by influenza A virus specific memory CD4 T cells. To test CD9 expression on memory CD4 T cells, samples were used that had been generated during previous swine influenza A virus infection trials, as described above. Samples used for a first experiment were obtained during a trial in which animals had been infected once at 10 weeks of age and blood sampled up to 42 days later (Talker et al., 2016). The frequency of $CD4^+CD9^+$ T cells with the capacity to coproduce $IFN\gamma^+TNF\alpha^+$ in response to *in vitro* re-exposure to whole influenza A virus was assessed from PBMC sampled at two time points post *in vivo* infection, from two animals (Fig. 8Ai). Intracellular $IFN\gamma^+$ and $TNF\alpha^+$ was detected by flow cytometry and analysed alongside CD9 expression. The frequency of $IFN\gamma^+TNF\alpha^+$ co-producing CD4 T cells stayed the same for swine (SW) 16 and increased in SW17 between day 12 and day 42 post infection (Fig. 8Aii). Nevertheless the frequency of $CD9^+$ cells within $IFN\gamma^+TNF\alpha^+$ CD4 T cells was strongly increased in PBMC taken at six weeks post infection, compared with those taken at two weeks post infection for both animals (Fig. 8A, plots iii and iv). For SW16 there were 29.1% $CD9^+IFN\gamma^+TNF\alpha^+$ cells at day 12, rising to 47.5% at day 42 post challenge, and for SW17 the frequency of $CD9^+IFN\gamma^+TNF\alpha^+$ cells was 23.4% at day 12, rising to 45.8% at day 44. To corroborate this further, CD9 expression upon *in vitro* antigen recall, within samples taken at later time points post *in vivo* infection with swine influenza A virus, was investigated. To this end frozen PBMC stocks from two pigs generated during another investigation (Talker et al., 2015) were used, wherein animals had been infected with swine influenza A virus twice, once at four weeks of age, and again

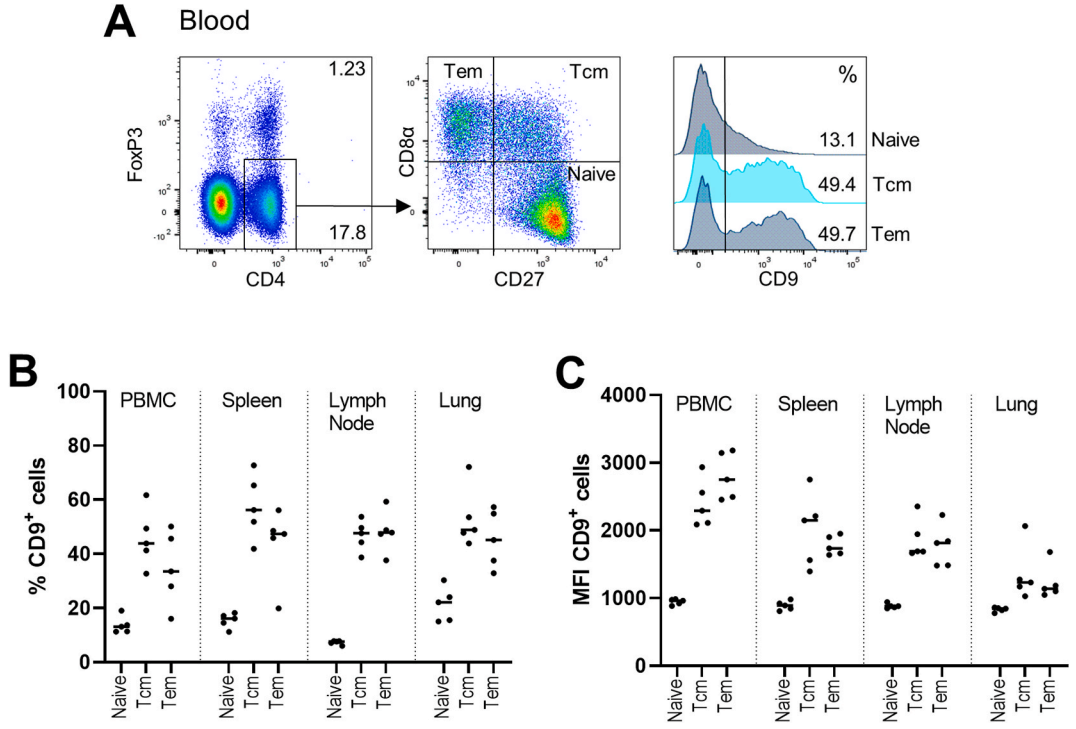

Fig. 6. CD9 is most frequently expressed upon central memory CD4 T cells. Lymphocytes isolated from blood, spleen, mediastinal lymph node and lung of five animals were analysed for CD9 expression upon CD4 T cells and co-stained for markers of differentiation. (A) Representative sample showing PBMC of one animal. $CD4^+FoxP3^-$ cells were selected and further divided into $CD8\alpha^-CD27^+$ (naive), $CD8\alpha^+CD27^+$ central memory (Tcm) and $CD8\alpha^+CD27^-$ effector memory (Tem) subsets. CD9 expression was then analysed for each subset, as indicated in the stacked histograms. The percentage of $CD9^+$ cells within each subset is displayed to the right of the vertical gate. (B) The percentage of $CD9^+$ cells is displayed for each differentiation stage, in all four organs, analysed as per the representative sample above. Each dot represents one individual. (C) As in (B), but the median fluorescence intensity (MFI) of CD9 is shown.

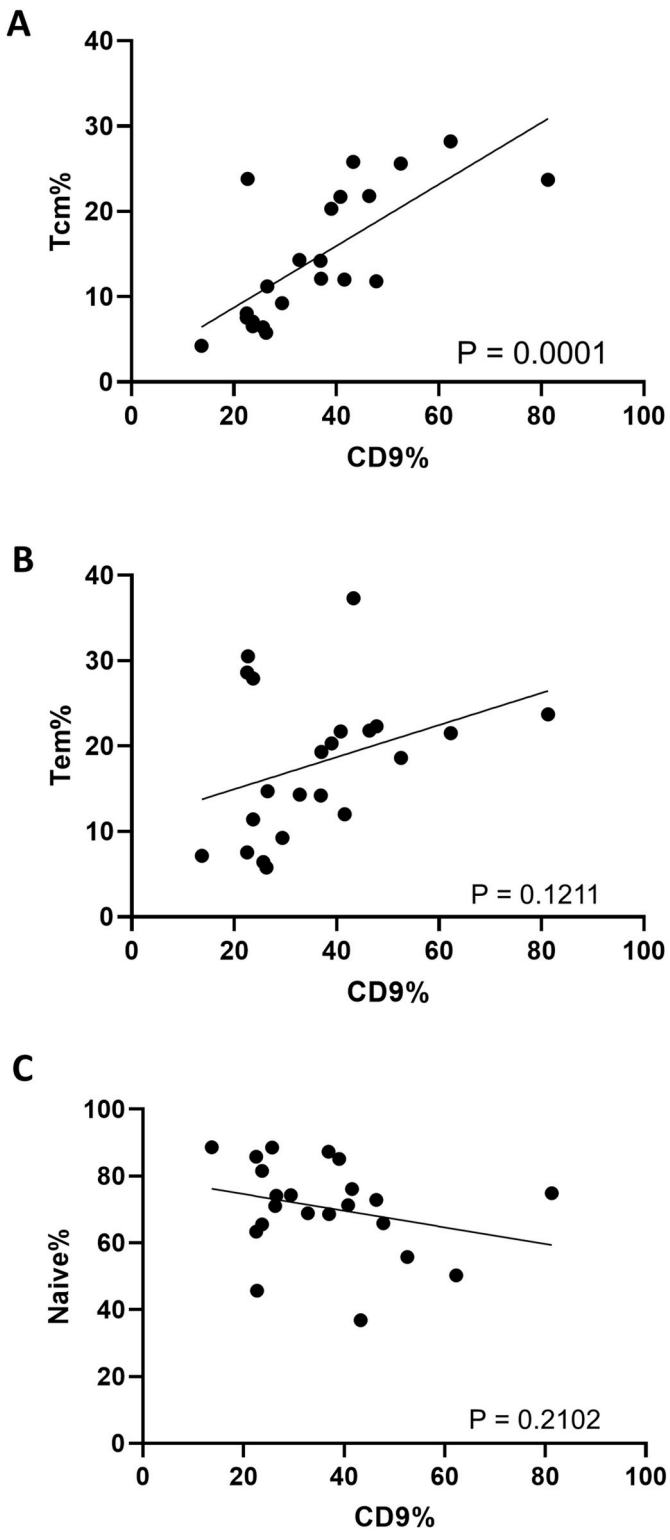

Fig. 7. Correlation analysis of $CD9^+$ CD4 T cells with the frequency of central memory, effector memory and naive CD4 T cells. Correlation was tested using a Spearman's analysis. Data from multiple phenotyping experiments of T cells isolated from blood was pooled and analysed for the frequency of $CD9^+$ cells within total T cells, these values are given on the x-axes. This was correlated with the frequency of (A) $CD8\alpha^+CD27^+$ (central memory, Tcm) T cells (B) $CD8\alpha^+CD27^-$ (effector memory, Tem) T cells and (C) $CD8\alpha^-CD27^+$ (naive) T cells, within CD4 T cells. Each data point represents one individual animal.

28 days later. Here, blood was sampled up to 62 days after primary infection. The capacity for CD4 T cells to coproduce IFNγ and TNFα rose substantially after the second infection (day 35, equivalent to day 7 post-secondary infection) and peaked between days 46 and 56 post re-infection (Fig. 8 Bi). This response was diminished by day 62 (Fig. 8Bii), consistent with previous observations (Talker et al., 2015). A preliminary experiment tested the frequency $CD9^+IFN\gamma^+TNF\alpha^+$ CD4 T cells responding to recall antigen in samples taken following primary infection only. PBL from SW7 contained 63.5% of $CD9^+IFN\gamma^+TNF\alpha^+$ at day 15 and this increased to 77% at day 28, reflecting the trend exemplified in Fig. 8A. However, SW9 showed no increase in $CD9^+IFN\gamma^+TNF\alpha^+$ in samples taken during this short time period. Therefore PBMC were analysed which had been obtained following the second challenge with influenza A virus, administered at day 28 post primary challenge; here a distinct increase in the frequency of $CD9^+$ cells within the $IFN\gamma^+TNF\alpha^+$ responsive population was observed. Specifically there was an increase from 61.8% at day 37 to 100% at day 63 for SW7 and 78.8% $CD9^+IFN\gamma^+TNF\alpha^+$ at day 35 to 91.2% at day 62 for SW9.

4. Discussion

In this work we present two mAb clones, 2E12 and 3B3, which have been raised against porcine immune cells, and their specificity for CD9 has been verified by evidencing reactivity against porcine CD9 transduced mouse Bw5147 cells. To our knowledge, one other anti-porcine CD9 mAb has been generated (Heinz et al., 2002). Clone 1038H-4–6 (IgM) stains 90% of bone marrow cells and not haematopoietic stem cells (Heinz et al., 2002). However, this mAb has not been made commercially available, to our knowledge. An anti-human CD9 mAb (MM2/57) was reported to be cross reactive with porcine CD9 (Saalmüller et al., 2005; Saalmüller and Aasted, 2007) and has been used for characterisation of porcine extra-cellular vesicles (Barranco et al., 2019). Here, it has been demonstrated that the reactivity of MM2/57 against CD9 on porcine PBMC and pCD9 transduced mouse Bw5147 cells was suboptimal for use as a cell surface phenotyping reagent. Of note, in the reporting of the animal homologue section of Human Leucocyte Differentiation Antigens (HLDA) 8 Workshop (Saalmüller et al., 2005; Saalmüller and Aasted, 2007), the authors used a notation scheme of "+", to indicate of a "positive staining pattern" and "++", to indicate a "clear positive result". For swine, clone MM2/57 was noted with "+", i.e. having a "positive staining pattern", which is consistent with the partial staining results obtained in our study. MM2/57 was also highlighted as a reagent with cross reactivity against various other species (cow "+", horse "+", cat "++", mink "++", llama "+", and rabbit "+"), which is reflective of the known inter-species homology of CD9, where the extracellular loop contains both conserved and variable regions (Xing et al., 2010).

The two new pCD9-specific mAbs, 2E12 and 3B3, were used to characterise CD9 expression on various porcine leukocyte subsets in the blood. Tohami et al. (2004), also reported the expression of hCD9 within human peripheral blood monocytes, T cells, and B cells, as both MFI and percentage of parent population. In human, peripheral blood CD9 is expressed upon a quarter of monocytes and stains for it result in the highest MFI on B cells, although these were only a small (1.8%) population within total B cells (Tohami et al., 2004). One other study compared hCD9 expression across leukocyte subsets on *ex vivo* whole blood and found it was detectable on B cells, CD8 T cells and CD4 T cells, and was upregulated following two days of PBMC culture, particularly on monocytes (Pugholm et al., 2016).

MM2/57 and two other anti-human CD9 mAb clones (LT86A and RHIA) are cross reactive with cattle leukocytes (Saalmüller and Aasted, 2007; Sopp et al., 2007). Of note, CD9 expression on cattle $CD2^+$ T lymphocytes ranged from 7.9 to 12.7%, and 15.9–43.8% on $CD21^+$ B cells, depending on the anti-CD9 clone used (Sopp et al., 2007), indicating a cellular distribution of bovine CD9 similar to the pig.

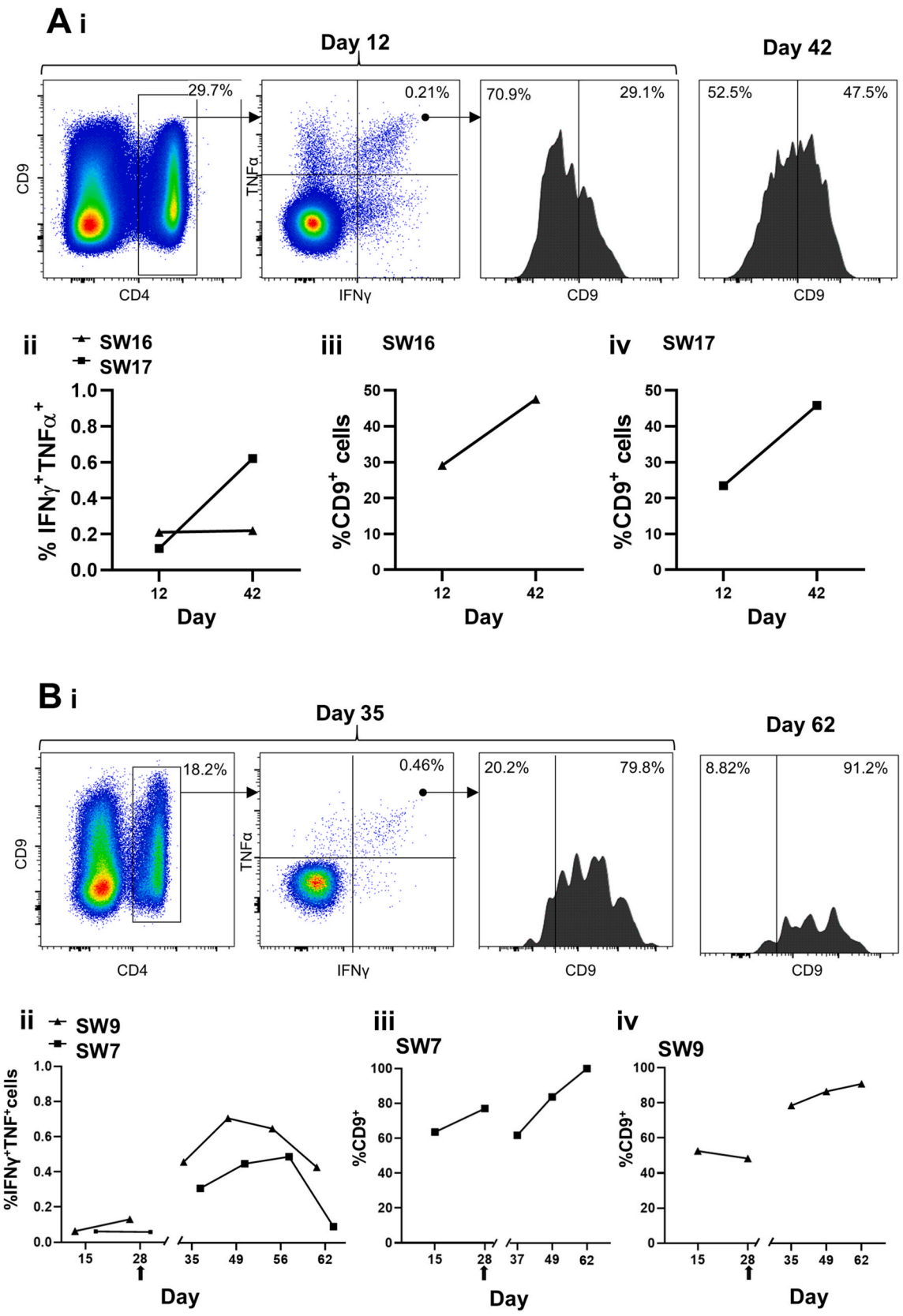

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Fig. 8. Long-lived CD4 T cells that respond to *in vitro* recall antigen are $CD9^+$. (A) Cryopreserved PBMC were used from two animals, which had been bled on day 12 and 42 post infection with influenza A virus. Thawed cells were plated and were stimulated over night with influenza A virus and Brefeldin A was added to the culture for the final 4 h. Cytokine response was assessed using intracellular staining against IFNγ and TNFα and analysed alongside markers CD4 and CD9. (i) representative analysis of flow cytometry data from one animal. CD4 T cells were gated and intracellular staining of IFNγ and TNFα is displayed. Cells that are positive for both cytokines were analysed for CD9 expression. CD9 expression at day 12 is compared with day 42 (histograms) post infection. (ii) Frequency of $IFN\gamma^+TNF\alpha^+$ cells within CD4 T cells at day 12 and 42 for swine (SW) 16 and 17. (iii) Frequency of $CD9^+$ cells within the $IFN\gamma^+TNF\alpha^+$ cells at day 12 and day 42 post infection for SW16 and SW17 (iv). (B) Cryopreserved PBMC from two animals, which were infected twice with influenza A at four and eight weeks of age and bled at the indicated time points, were stimulated with influenza A virus. (i) The same flow cytometry analysis strategy as in (Ai) was applied. (ii) Frequency of $IFN\gamma^+TNF\alpha^+$ cells within CD4 T cells is plotted against the day of sampling for two animals. The day of a second infection with influenza A virus is indicated with an arrow below the x-axis. Percentages of $CD9^+$ cells within $IFN\gamma^+TNF\alpha^+$ cells before and after challenge are shown for SW7 (iii) and animal SW9 (iv).

In mouse, CD9 negatively regulates LPS induced macrophage activation and knockout increases macrophage infiltration to the lung (Suzuki et al., 2009). Murine CD9 (mCD9) is expressed on all peritoneal macrophages but is downregulated upon activation by IFNγ via Stat1 signalling (Wang et al., 2002). Murine CD9 is therefore regarded to be a negative regulator of inflammatory macrophage phenotype. Nevertheless, mCD9 colocalises with MHC-II on dendritic cells and this is proposed to enable cross-linking of the TCR with heterologous MHC-II I-E/I-A multimers (Unternaehrer et al., 2007). CD9 expression may therefore be an indicator of antigen presenting cell (APC) subsets with a higher capacity for T cell activation. Since expression of exogenous CD9 in a mouse B cell line also resulted in the coprecipitation of MHC-II I-E and I-A (Unternaehrer et al., 2007), this suggests that CD9 expressing B cell subsets may also have increased T cell stimulatory function. Here, it was found that CD9 is uniformly expressed on porcine monocytes, which are antigen presenting cell precursors, but also on a distinct population of $CD9^+$ porcine B cells. It may therefore be that a $CD9^+$ phenotype also represents a subset of porcine B cells with enhanced antigen presentation or T cell activating function. Indeed the presented *in situ* analyses with lymph node tissues suggest the presence of $CD9^+$SLA-DR^+ cells within the porcine lymph node germinal centre. This is remarkably similar to immunohistochemistry for CD9 in human tonsil germinal centres, where $CD9^+$ and $CD9^-$ B cells are distinctly localised within the light and dark zones respectively, the former of which are plasma cell precursors (Yoon et al., 2013). Here hCD9 stabilises VLA4 with VCAM-1 expressed on follicular dendritic cells, the downstream signal of which supports longevity of plasma cells (Yoon et al., 2014). The immune fluorescence histology shown here using anti-pCD9 also indicated a high expression of CD9 on porcine lymphatic endothelial cells. This is consistent with human and mouse, in which the tetraspanin plays a key role in lymphangiogenesis (Iwasaki et al., 2013) and facilitates adhesion for migrating leukocytes (Yeung et al., 2018). A polyclonal anti-CD9 antibody (N1), raised against swine CD9 has also detected CD9 expression on endothelia and epithelia of various porcine tissues, and this was compared with the distribution of human CD9; however, detection of CD9 was not achieved in the lymph node whereby a different staining method was used (Yubero et al., 2011).

It was shown that porcine CD4 T cells in blood and tissues also contained a prominent fraction of $CD9^+$ cells. Of note, Tohami et al. (2004) found CD9 expressing cells constituted only 2.2% of total peripheral blood T cells in human. Another anti-human CD9 mAb (5H9) has been previously developed (Kobayashi et al., 2004) and was used to detect hCD9 expressing cells on $CD45RA^+$ and $CD45RO^+$ T cells. The frequency of $CD9^+$ cells within the $CD4^+CD45RO^+$ memory fraction of *ex vivo* blood from healthy human PBMC was low (Kobayashi et al., 2004), consistent with observations that CD9 is only expressed at a low level on *ex vivo* peripheral T cells from human (Pugholm et al., 2016; Tohami et al., 2004). However, approximately half of $CD45RA^+$ PBMC were $CD9^+$. The authors concluded, therefore, that CD9 expression was mainly a feature of naive T cells (Kobayashi et al., 2004). However it must be noted that, although they represent a small fraction of T cells, T_{EMRA} cells were not distinguished.

To the best of our knowledge distribution of $CD9^+$ T cells throughout tissues has not been investigated under homeostatic conditions. However, functional studies into the impact of tetraspanins on migration and integrin modulation, which affects cellular localisation, sheds some light on the relevance of CD9 function on lymphocyte activity in the tissues. The presence of CD9 can both inhibit and promote migration depending on the cell surface expression profile of the cell upon which it is expressed (Jiang et al., 2015). By the use of human cell lines, it was shown that CD9 negatively modulates the function of integrins LFA-1 (αLβ1) and VLA-4 (α4β1) through regulating their clustering on the cell surface as well as being implicated in downstream interactions with cytoskeleton associated proteins (Reyes et al., 2015). CD9 expression on monocytes and B cells is negatively associated with lung inflammation in mouse and human (Brosseau et al., 2018b; Suzuki et al., 2009), suggesting the increased frequency of $CD9^+FoxP3^+$ CD4 T cells isolated from porcine lung may represent an equivalent presence of $CD9^+$ Tregs during steady state.

Early studies investigated CD9 as a costimulatory molecule, which was determined to be independent of CD28 due to its cumulative synergistic effect on T cell proliferation (Toyo-oka et al., 1997) It has since become clear that the mechanism of anti-CD9 induced proliferation is due to the function of tetraspanins in orchestrating colocalisation of T cell activating molecules on the cell surface membrane. It has been shown that CD9 supports β1 integrin signalling at the immune synapse (Rocha-Perugini et al., 2014). Following our observation that CD9 is more frequently expressed upon $CD8\alpha^+$ CD4 T cells of the pig, it can be postulated that pCD9 is upregulated on T cells following activation. Here, a clear upregulation of CD9 upon porcine $CD25^+$ T cells following Concanavalin A treatment was demonstrated, regardless of whether cells belonged to CD4, CD8, or γδ T cell subsets. One other study found that CD9 is increased on lymphocytes following culture of human PBMC with phytohaemagglutinin and IL-2 (Reyes et al., 2015). Moreover, the frequency of $CD9^+$ T cells was correlated with the frequency of $CD8\alpha^+CD27^+$ CD4 T cells – a fraction which contains both central memory cells and early effector cells. As CD9 is elevated shortly after T cell activation this could explain why this fraction has a higher representation of $CD9^+$ CD4 T cells than the $CD4^+CD8\alpha^+CD27^-$ effector memory population does. CD8 T cells also contained higher $CD9^+$ cell frequencies within the equivalent $CD27^+$ effector population (data not shown). Such an upregulation of CD9 following primary T cell activation may function to render the cells more prone to repeated stimulation, or indicate a potential lineage commitment to long lived memory cells. Indeed, *in vitro* treatment of T cells with soluble anti-5H9 inhibits proliferation in human T cells in response to both autoantigen and recall antigen (Kobayashi et al., 2004).

Following our observation that CD9 is most frequently expressed on porcine $FoxP3^+$ T cells, this investigation initially focussed on $IL\text{-}10^+$ CD4 T cells, because this cytokine is a major hallmark of Treg functionality. However, we did not find correlations between the capacity for IL-10 production and CD9 expression in CD4 T cells, regardless of a Treg phenotype. However, it remains to be determined if CD9 expression plays a role in $FoxP3^+$ cells that exert their function through direct cell-cell interaction and not through IL-10 production. This hypothesis is based on our observation that lung tissue contains a population of $FoxP3^+$ T cells that have the highest CD9 MFI, and the consideration that CD9 functions in stabilising cellular interactions. Another possibly relevant mechanism of Treg function lies in the role of CD9 in extracellular vesicle formation and function, since these also transmit

regulatory signals to target cells.

Next, CD9 expression on CD4 T cells responding to a specific recall antigen was analysed. Since the capacity to produce two or several effector cytokines has been shown to correlate positively with the control of infection (Seder et al., 2008), pCD9 expression was analysed upon cells with both intracellular IFNγ and TNFα. It was found that the capacity for CD4 T cells to coproduce IFNγ and TNFα peaks after approximately four weeks post challenge and then diminishes (Talker et al., 2015). At a late time point post challenge cells which exhibit Ag specific cytokine function represent an influenza-specific memory population that does not consist of early effector cells. It was found that by six weeks post primary infection, or five weeks following a secondary challenge, the vast majority of $IFN\gamma^{+}TNF\alpha^{+}$ memory CD4 T cells are $CD9^{+}$; potentially representing a subset of long lived functionally competent cells. Considered in parallel with our observation that CD9 expression correlates highly with frequency of *ex vivo* $CD8\alpha^{+}CD27^{+}$ cells, the increased frequency of $CD9^{+}$ cells following influenza A virus infection may reflect the temporally changing ratios of central memory and effector memory subsets, within total virus-specific CD4 T cells, over the duration of the anamnestic response – in which Tcm numbers peak later. These preliminary observations suggest that CD9 may be a marker that can be used to distinguish between short lived effector cells and long lived memory subsets *in vivo*, with CD9 being an indicator of both longevity and functional capacity. Evidence for CD9 expression being a conclusive correlate of protection would require further *in vivo* trials, by analysing T cells following large scale vaccination trials, or perhaps through the use of an animal model in which $CD9^{+}CD8\alpha^{+}CD27^{+}$ and $CD9^{-}CD8\alpha^{+}CD27^{+}$ CD4 T cells can be adoptively transferred and traced for survival. Indeed, given the functional roles of tetraspanins discussed above, it follows that CD9 may be present upon long lived memory cells in order to be immediately available to stabilise the immune synapse and lower the threshold of TCR signalling, for rapid reactivation of memory cells. Further, CD9 could give a survival advantage to effector memory cells during any TCR engagement required for their survival.

In summary, in this work two novel monoclonal antibodies were used to detect CD9 expression on porcine leukocytes. CD9 was found to be uniformly expressed on monocytes; though the functional implication of this may vary for different antigen presenting cells and their respective stage of differentiation. B cells expressing CD9 may belong to a variety of functional subsets and this requires further investigation. This study aimed to elucidate the variable expression pattern of CD9 on T cells, by correlating this with T cell differentiation status and effector function. CD9 is upregulated following polyclonal stimulation and this is consistent with our observation that it is most frequently expressed upon CD8α-expressing porcine CD4 T cells. There were variable expression levels of CD9 across CD4 T cell subsets, analysed within four organ compartments. Due to the role of tetraspanins in orchestrating integrin function and migration events, further investigation into CD9 as a marker of T cell migratory capacity is warranted. Finally, it was observed that CD4 T cells, that were isolated following two challenges with influenza A virus, and had retained the capacity to respond to *in vitro* recall antigen, are in the majority $CD9^{+}$. The functional implication of the CD9 molecule in this long-lived memory cell phenotype remains to be determined.

Declaration of competing interest

All other authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dci.2021.104080.

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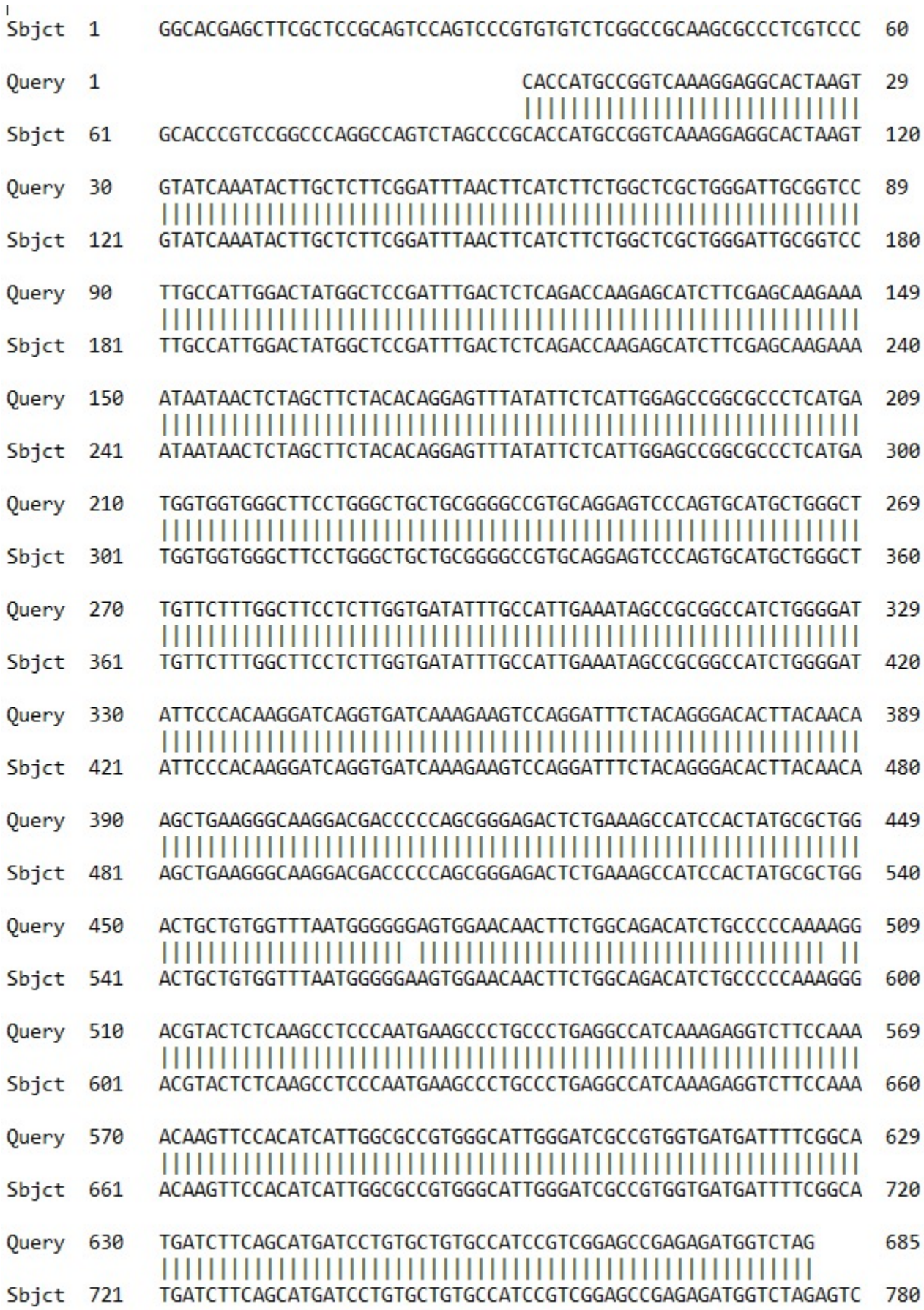
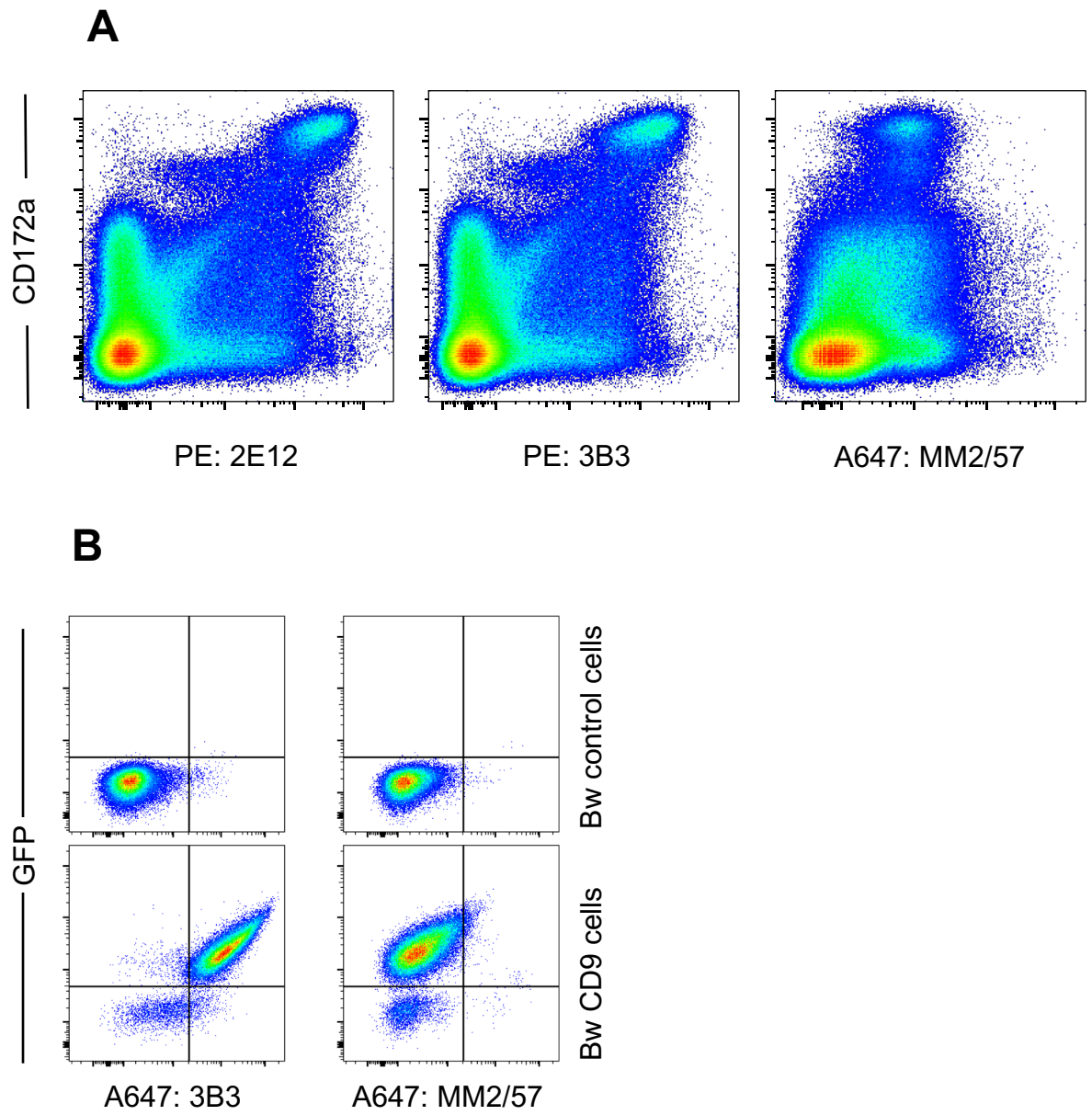
Suppl. Fig. 1. Sequence of porcine CD9 mRNA cloned into expression vector



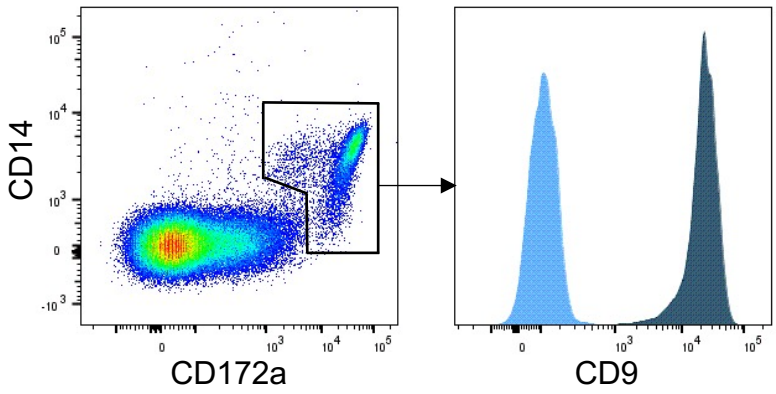
Subject: In house sequence of porcine CD9
Query: S. scrofa CD9 molecule, mRNA NCBI Reference Sequence: NM_214006.1

Suppl. Fig. 1. Sequence of porcine CD9 mRNA cloned into expression vector

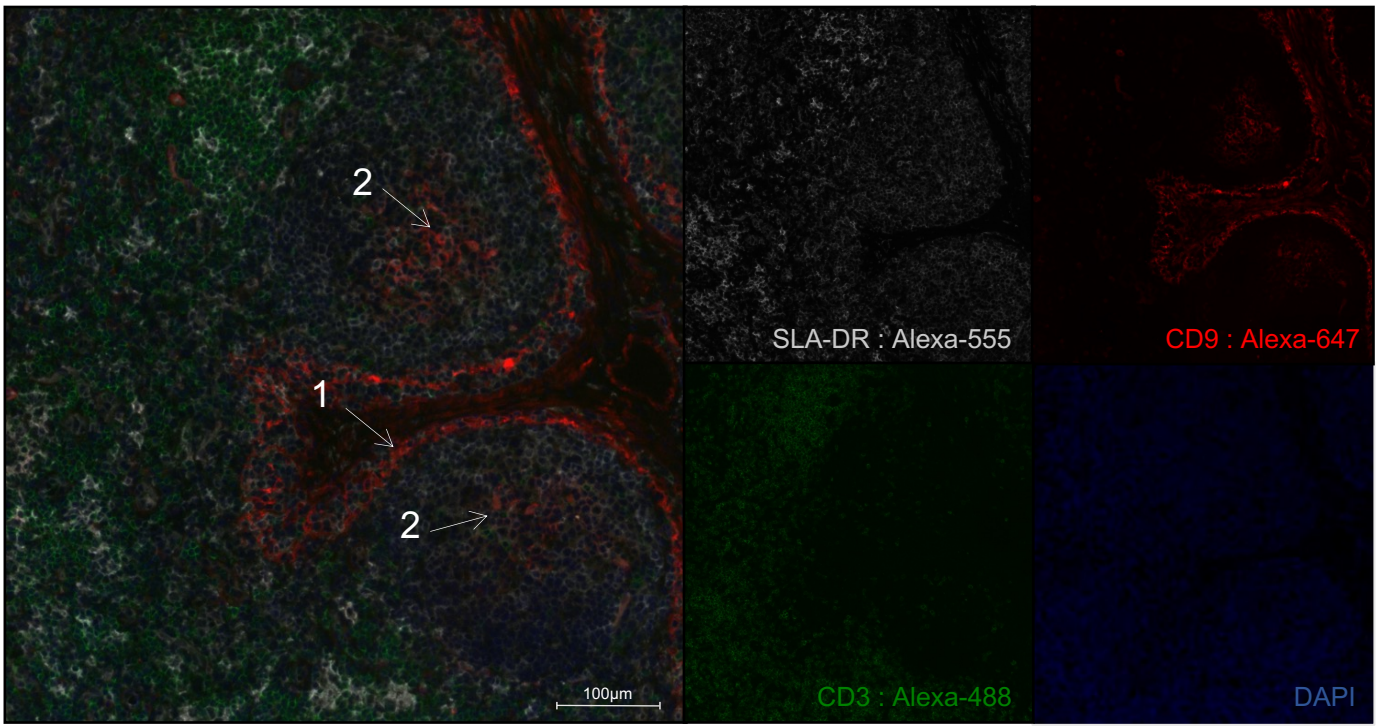
Primers designed against the open reading frame of porcine CD9 were used to amplify and sequence cDNA isolated from PBMC of a pig from a conventional breeding farm. This was aligned with the NCBI Reference sequence NM_214006.1 for *Sus scrofa* CD9 using NCBI BLAST.



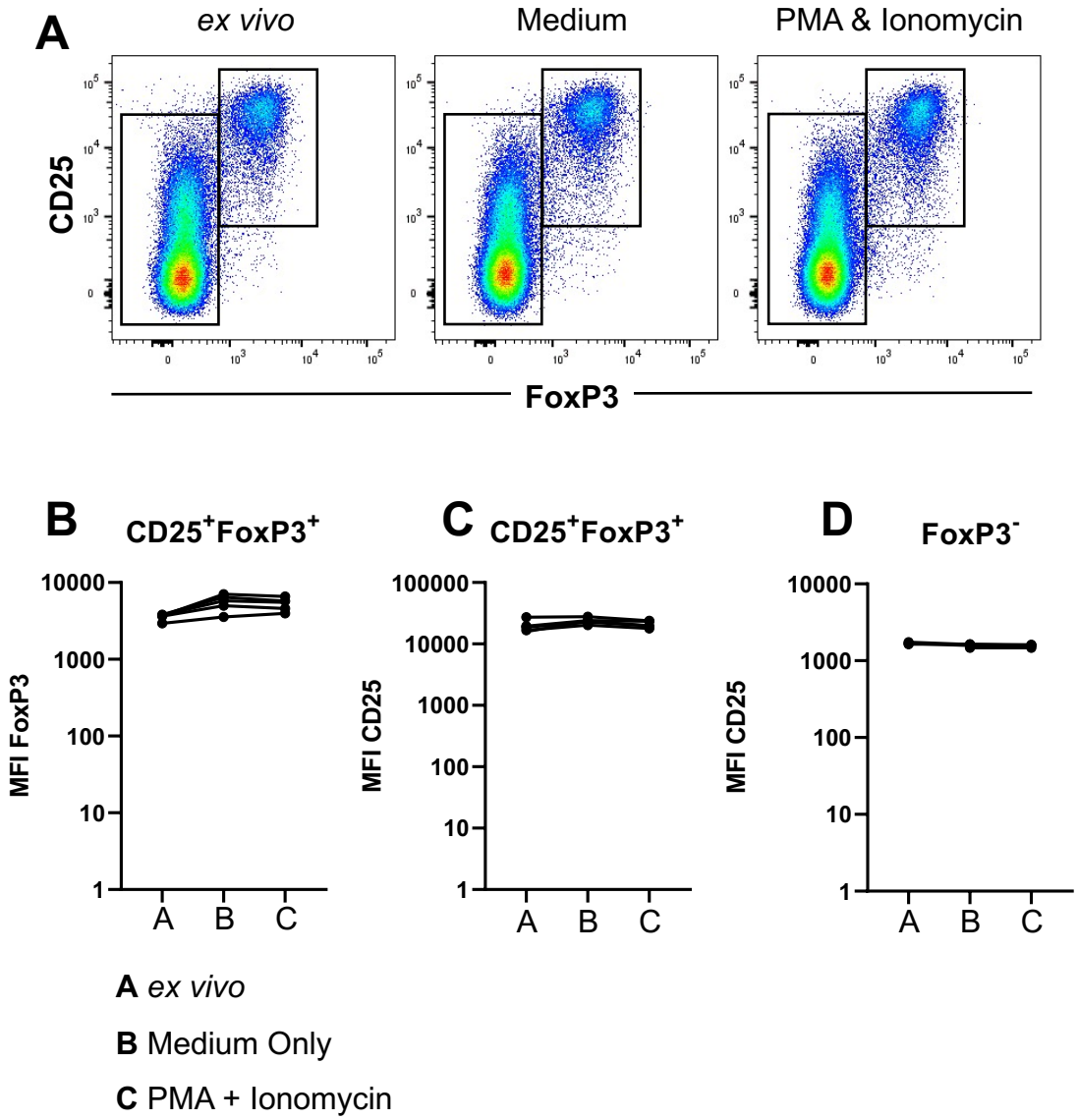
Suppl. Fig. 2. Antibody MM2/57 shows a poor staining pattern compared to monoclonal antibodies 3B3 and 2E12. (A) CD9 was either stained with mAb clone 3B3 or 2E12 and co-stained with CD172a on peripheral blood leukocytes. 3B3 and 2E12 were detected using goat-anti mouse IgG-1-PE (Southern Biotech). This is compared with the commercially available anti-human CD9 mAb MM2/57 (Bio-Rad). MM2/57 was detected using goat anti-mouse anti-IgG1 Alexa 647 (Thermo Fisher). Testing different concentrations of MM2/57 (0.25 – 5 µg) in a staining volume of ~ 40µl) did not change the staining pattern (data not shown). (B) Staining of mouse Bw5147 cells expressing porcine CD9 using 3B3–A647 conjugate compared with MM2/57–A647 conjugate (Bio-Rad). 5µg of mAb MM2/57 was used per stain.



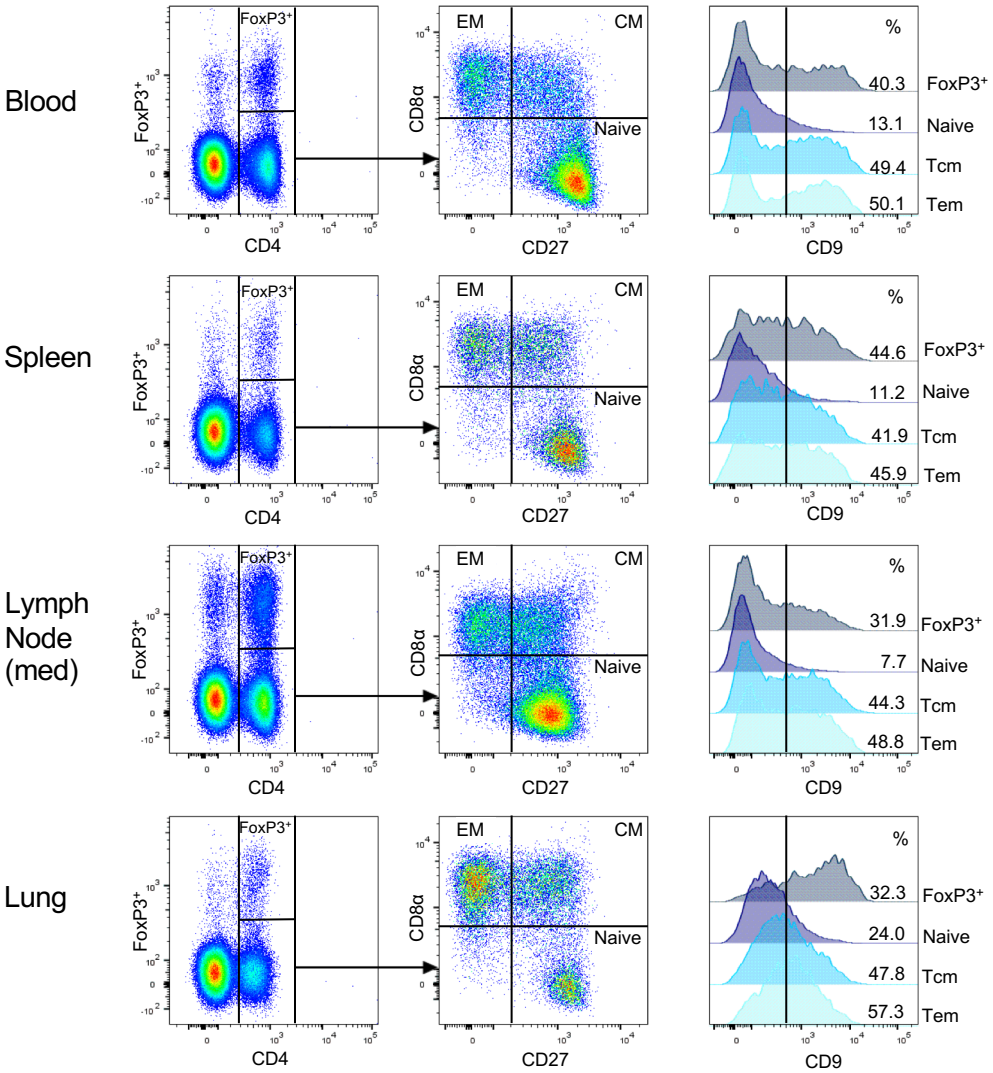
Suppl. Fig. 3. CD14^{+}CD172^{+} leukocytes are CD9^{+}. Freshly isolated PBMC were stained for CD9 alongside CD14 and CD172a and analysed by flow cytometry. A large FSC-A/SSC-A gate was applied to include monocytes and dead cells were excluded (not shown). CD14^{+}CD172a^{high} cells were gated (left panel) and CD9 expression is displayed as a histogram (dark blue) overlaid with a CD9 FMO control (pale blue).



Suppl. Fig. 4. *In situ* CD9 expression within porcine lymph nodes. Immunofluorescence histology of inguinal lymph node tissue using mAb 2E12 in combination with SLA-DR, CD3 and DAPI. Right: Single channel images are displayed with the respective antibodies and fluorophore. Left: Overlay of all channels. White arrows indicate areas of interest within the B cell follicle and endothelium.



Suppl. Fig. 5. PMA + Ionomycin treatment does not affect CD25 and FoxP3 expression. (A) Pseudocolour plots demonstrating the analysis of one representative animal of six. Cells were pre-gated on live, blood-derived CD4 T cells (not shown) and analysed for CD25 and FoxP3 co-expression prior to *in vitro* cultivation, after overnight cultivation in cell culture medium only, and after overnight cultivation including a four hour treatment with PMA + Ionomycin during the final stage of cultivation. (B) Median fluorescence intensity (MFI) of $FoxP3^+$ cell within the $FoxP3^+CD25^+$ population, for each condition. (C) MFI of $CD25^+$ cell within the $FoxP3^+CD25^+$ population. (D) MFI of CD25 upon total $FoxP3^-$ cells. N=6, data of each individual is represented by adjoined marks.



Suppl. Fig. 6. CD9 expression within CD4 T cell subset. As per Fig. 6 (main manuscript) representative data set showing lymphocytes isolated from blood, spleen, lymph node and lung of one animal. Lymphocytes were analysed for CD9 expression upon CD4 T cells and co-stained for markers of differentiation. $CD4^+FoxP3^-$ cells were selected and further divided into $CD8\alpha^-CD27^+$ (naive), $CD8\alpha^+CD27^+$ central memory (Tcm) and $CD8\alpha^+CD27^-$ effector memory (Tem) subsets. CD9 expression was then analysed for each subset, as indicated in the stacked histograms. The percentage of $CD9^+$ cells within each subset is displayed to the right of the vertical gate.

SUPPLEMENTARY TABLE 1 List of secondary reagents and isotype control antibodies

Specificity and conjugation		**Supplier**
anti-IgG1 PE	goat anti-mouse	Southern Biotech
anti-IgG1 BV421	rat anti-mouse	BioLegend
anti-IgG1 Alexa 647	goat anti-mouse	Thermo Fisher
anti-IgG2a BV421	goat anti-mouse	Jackson ImmunoResearch
anti-IgG2a PeCy7	goat anti-mouse	Southern Biotech
anti-IgG2a PE	goat anti-mouse	Southern Biotech
anti-IgG2a PerCP-eFlour710	rat anti-mouse	Thermo Fisher
anti-IgG2b Alexa 488	goat anti-mouse	Thermo Fisher
anti-IgG2b BV421	goat anti-mouse	Jackson ImmunoResearch
anti-IgG2b PE	goat anti-mouse	Southern Biotech
Streptadvidin-BV605		BioLegend
Streptadvidin-Alexa 488		Thermo Fisher
Streptavidin-PE		Thermo Fisher
Isotype controls	**Clone**	
mouse IgG1κ	P36281	Thermo Fisher
mouse IgG2aκ	eBM2a	Thermo Fisher
mouse IgG2bκ	eBMG2b	Thermo Fisher

SUPPLEMENTARY TABLE 2 Antibody Panel for Fig. 2

Purpose	Antigen	Clone	Isotype	Fluorochrome	Labelling strategy[1]	Source of primary antibody
CD4 T cells	CD4	74-12-4	IgG2b	PE	Secondary antibody	In-house
	CD9	2E12	IgG1	BV421	Secondary antibody	In-house
NK cells	CD3	BB23-8E6	IgG2b	PE	Secondary antibody	Southern Biotech
	CD8α	11/295/33	IgG2a	PerCP-eFluor710	Secondary antibody	In-house
	CD9	2E12	IgG1	BV421	Secondary antibody	In-house
	CD16	G7	IgG1	FITC	Direct Conjugate	Bio-Rad
	NKp46	VIV-KM1	IgG1	Alexa 647	Direct Conjugate	In-house
CD8β T cells	CD8β	PG164A	IgG2a	PerCP-eFluor710	Secondary antibody	Kingfisher
and γδ T cells	CD9	2E12	IgG1	BV421	Secondary antibody	In-house
	TCR-γδ	PPT16	IgG2b	PE	Secondary antibody	In-house
B cells and	CD9	2E12	IgG1	BV421	Secondary antibody	In-house
monocytes	CD79α	HM57	IgG1	PE	Direct conjugate	Dako
	CD172a	74-22-15A	IgG2b	Alexa 488	Secondary antibody	In-house
Supplementary	CD172a	74-22-15A	IgG2b	BV421	Secondary antibody	In-house
Figure 2	CD14	MIL2	IgG2a	PE	Secondary antibody	Bio-Rad
	CD9	2E12	IgG1	Alexa 647	Secondary antibody	In-house

[1]Details on secondary reagents are given in Suppl. Table 1

SUPPLEMENTARY TABLE 3 Antibody Panel for Fig. 3

Purpose	Antigen	Clone	Isotype	Fluorochrome	Labeling strategy[1]	Source of primary antibody
CD4 T cells	CD4	74-12-4	IgG2b	FITC	Direct Conjugate	BD Biosciences
	CD8α	11/295/33	IgG2a	PerCP-eFluor710	Secondary antibody	In-house
	CD9	3A1 3B3	IgG1	BV421	Secondary antibody	In-house
	CD27	b30c7	IgG1	Alexa 647	Direct Conjugate	In-house
	FoxP3	FJK-16s		PE	Direct Conjugate	Thermo Fisher

[1]Details on secondary reagents are given in Suppl. Table 1

SUPPLEMENTARY TABLE 4 Antibody Panel for intracellular IL10 detection (Fig. 5 and Suppl. Fig. 5)

Purpose	Antigen	Clone	Isotype	Fluorochrome	Labeling strategy[1]	Source of primary antibody
Staining for IL-10 competent CD4 T cells Fig. 5	CD4	74-12-4	IgG2b	BV421	Secondary antibody	In-house
	CD9	2E12	IgG1	Alexa 647	Direct Conjugate	In-house
	CD25	3B2	IgG1	Alexa 488	Direct Conjugate	In-house
	IL10	945A4C 437B1	IgG1	PE	Biotin-Streptavidin	Thermo Fisher, biotinylated in-house
Staining for Suppl. Fig. 5	CD4	74-12-4	IgG2b	BV421	Secondary antibody	In-house
	CD25	3B2	IgG1	Alexa 647	Secondary antibody	In-house
	FoxP3	FJK-16s	IgG2a	PE	Direct Conjugate	Thermo Fisher

[1]Details on secondary reagents are given in Suppl. Table 1

SUPPLEMENTARY TABLE 5 Antibody Panel for sorting CD9 T cell subsets (Fig. 4)

Antigen	Clone	Isotype	Fluorochrome	Labeling strategy[1]	Source of primary antibody
CD4	74-12-4	IgG2b	FITC	Direct conjugate	BD Biosciences
CD8β	PPT23	IgG2a	BV421	Secondary antibody	In-house
CD9	3B3	IgG1	PE	Secondary antibody	In-house
TCR-γδ	PPT16	IgG2b	Pe-Cy7	Biotin-Streptavidin	In-house
CD25	3B2	IgG1	Alexa 647	Direct conjugate	In-house

[1]Details on secondary reagents are given in Suppl. Table 1

SUPPLEMENTARY TABLE 6 Intracellular cytokine detection following antigenic stimulation (Fig. 8)

Purpose	Antigen	Clone	Isotype	Fluorochrome	Labeling strategy[1]	Source of primary antibody
Analysis of samples following single infection	CD4	74-12-4	IgG2b	Alexa 488	Secondary antibody	In-house
	CD9	3B3	IgG1	Alexa 647	Secondary antibody	In-house
	IFNγ	P2G10	IgG1	PE	Direct conjugate	BD Biosciences
	TNFα	Mab11	IgG1	BV605	Direct conjugate	BioLegend
Analysis of samples following two consecutive infections	CD4	74-12-4	IgG2b	FITC	Direct Conjugate	BD Biosciences
	CD9	2E12	IgG1	BV421	Secondary antibody	In-house
	IFNγ	P2G10	IgG1	PE	Direct conjugate	BD Biosciences
	TNFα	Mab11	IgG1	BV605	Direct conjugate	BioLegend

[1]Details on secondary reagents are given in Suppl. Table 1

SUPPLEMENTARY TABLE 7 Evidence of specificity of in-house antibodies

Antibody target & clone	Reference
CD4, 74-12-4	(Pescovitz et al., 1984)
CD8α, 11/295/33	(Jonjić and Koszinowski, 1984)
CD8β, PPT23	(Yang and Parkhouse, 1997)
CD25, 3B2	(Bailey et al., 1992)
CD27, b30c7	(Reutner et al., 2012)
CD172a, 74-22-15A	*Class switch variant of 74-22-15 (Alvarez et al., 2000)
NKp46, VIV-KM1	(Mair et al., 2012)
TCR-γδ, PPT16	(Yang et al., 2005)

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Identification of IL-10 competent B cells in swine

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ABSTRACT

Progress in the phenotypic characterisation of porcine B cells is ongoing, with recent advances in the identification of B1 cell subsets and plasma cells. However, regulatory B cells, commonly identified by interleukin (IL)-10 production, have not been studied in pigs so far. Here we investigate IL-10 expression in B cell subsets in response to CpG-oligodeoxynucleotides, phorbol 12-myristate 13-acetate and ionomycin stimulation *in vitro*. Our results reflect similar findings in human and mice. We identify a small subset of IL-10 competent B cells, present within both porcine B1 and B2 cell subsets across blood, spleen, mediastinal lymph nodes and lung tissue, with varied differentiation statuses. The capacity for IL-10 production coincided with CD95 expression, suggesting an activated phenotype of IL-10 competent B cells. These findings support the emerging paradigm that B cell IL-10 production is a function of various B cell subsets influenced by activation history and microenvironmental factors.

1. Introduction

Regulatory B cells (Bregs) have been well established as essential for immune modulation in mouse and human and this has been reviewed extensively (Cerqueira et al., 2019; Mauri and Ehrenstein, 2008; Mizoguchi and Bhan, 2006). However, the absence of a universal Breg phenotype or master transcription regulator has made the definition of Bregs, and elucidation of their developmental origins, more challenging than their T cell counterparts (Catalán et al., 2021; Glass et al., 2022). Although subsets of Bregs have various effector mechanisms, IL-10 production is widely considered to be a hallmark of most regulatory B cells and is therefore the main method for identifying putative Bregs in humans (Cossarizza et al., 2019). The capacity for B cells to produce IL-10 is also of interest in the context of immune evasion, in which the inappropriate induction of IL-10 exacerbates disease in the tumour microenvironment and during infection (Chekol Abebe et al., 2021).

IL-10 has been demonstrated to be a key cytokine for B cell regulation of various murine models of inflammatory disease (Kalampokis et al., 2013; Yanaba et al., 2008), after B cell derived IL-10 was shown to be involved in the control of experimental autoimmune encephalomyelitis (EAE) and autoimmune experimental arthritis following autoantigen stimulation (Fillatreau et al., 2002; Mauri et al., 2003; Mizoguchi et al., 2002). Moreover, defective B cell dependent immunoregulation is associated with increased pathology in several human inflammatory diseases in which IL-10 is a key regulatory mechanism, including systemic lupus erythromatosus (SLE) (Blair et al., 2010) and rheumatoid arthritis (Daien et al., 2014), reviewed in (Zhu et al., 2021).

Various surface phenotypes of Bregs have been described, which overlap and are shared with non-regulatory B cells, such Breg diversity reflects the multiple B cell subsets established and has been summarized elsewhere (Boldison and Wong, 2021; Glass et al., 2022; Lin et al., 2014; Zhu et al., 2021). Extensive work has been done on IL-10 producing B cells in mouse that express phenotypic markers typical of innate like B cell subsets: B1 cells (which are $CD1d^{+}CD5^{+/-}$ and upregulate CD11b), and marginal zone B cells. IL-10 competent transitional 2 marginal zone precursors (T2-MZP) and marginal zone B cells are $CD1d^{high}CD9^{+}CD11b^{-}CD21^{high}IgM^{high}$ (Bankoti et al., 2012; Evans et al., 2007; Won and Kearney, 2002). The designation "B10 cells" was coined in reference to a population of splenic $CD1d^{hi}CD5^{+}CD19^{hi}$ cells

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that controlled T cell mediated inflammation in CD19 deficient mice, in an IL-10 dependent manner (Yanaba et al., 2008). These cells were distinguished from Breg with other regulatory mechanisms as their function was solely dependent on IL-10, and later, due to a variable phenotype, were defined by this function alone, though a large proportion express the B1a markers CD5 and CD11b (Tedder, 2015). Further, following CD40 triggering and LPS stimulation up to 20% of mouse peritoneal cavity B1a cells produce IL-10 and have a regulatory function (Margry et al., 2014). The distinction between innate-like B cell subsets is less clear in humans, however an equivalent B10 population has been described, which is instrumental in the control of multiple inflammatory diseases, where B10 and pro-B10 cells constitute ~7% of human blood B cells (Iwata et al., 2011). B10 cells lose their capacity for IL-10 production prior to differentiation into plasma cells (Maseda et al., 2012), however IL-10 producing plasma cells with other origins are described (Cossarizza et al., 2019). For example, during EAE, plasmablasts in the draining lymph nodes limit autoimmune inflammation and are responsible for IL-10 production under the regulation of IRF4 (Matsumoto et al., 2014). IL-10 competent cells have also been described as B2 cells that are $IgM^{+}IgD^{+}$ when mature and undergo isotype class switching (Mizoguchi and Bhan, 2006). A role for B2 cells in immunoregulation is associated with tolerogenic immunoglobulin classes, as seen in the $CD25^{+}CD73^{-}CD71^{+}$ population designated B_R1 cells, which reduce allergic reactions in an antigen specific manner via tolerogenic IgG4 and IL-10 (van de Veen et al., 2013).

Breg appear to gain their potential for immune-regulation following B cell receptor (BCR) dependent selection against self-antigen and differentiate into IL-10 producing cells following further stimulation in an inflammatory microenvironment, resulting in a plastic functional state (Lykken et al., 2015; Rosser and Mauri, 2015; Tedder, 2015). However, both naive and antigen experienced B cells can be induced to express IL-10 following Toll-like receptor (TLR) stimulation (Lampropoulou et al., 2008). The precise activation mechanisms and functional mechanisms that have been described for Bregs appear to vary depending on the subset of regulatory B cells studied and disease context; as such B cell dependent regulation may be related to the mechanism of pathology (Mizoguchi and Bhan, 2006; Rosser and Mauri, 2015). For example, T cell mediated inflammation may be controlled in an antigen specific manner via crosstalk between antigen specific B and T cells (Tedder, 2015; Yoshizaki et al., 2012). Nevertheless, *in vitro* induction of IL-10 expression using the TLR9 agonist CpG, followed by phorbol 12-myristate 13-acetate (PMA) and ionomycin treatment is a well-established method for identifying B cells *ex vivo* which have already been programmed *in vivo* to be IL-10 competent (Lykken et al., 2015). We followed this approach to characterise IL-10 competent B cells in the pig. To the best of our knowledge, IL-10 competent B cells have not been investigated in swine so far. We identified various B1 and B2 cell subsets with the capacity for IL-10 production, reflecting the heterogeneity of IL-10 competent B cells that have been observed in mouse and human.

2. Materials and methods

2.1. Animals

For all experiments (excluding age comparison), organs and heparinised blood were obtained from healthy six-month-old fattening pigs from a local slaughterhouse. At Austrian abattoirs all live pigs are inspected by a veterinarian before slaughter to assure that no obvious clinical signs are present; in addition, all carcasses and inner organs are inspected during meat inspection to assure that no gross lesions are present; no routine monitoring is done for pathogens during slaughter, except for random sampling for notifiable diseases. Animals are exsanguinated following high voltage electric anaesthesia, in accordance with the Austrian Animal Welfare Slaughter Regulation. Since organ and blood collection were done on dead animals, no federal animal ethics approval was required, in accordance with Austrian law. For experiments where an age comparison was performed: Heparinised blood was taken from 12-week-old weaners (Large White x Landrace, cross-bred at the VetFarm) re-housed at the University Clinic for Swine at 10 weeks of age, both University of Veterinary Medicine Vienna.. Federal ethics approval was obtained, GZ 2020–0.073.976. Blood samples were also taken from sows (Large White), likewise housed at VetFarm. Sows were the following ages: Sow 659, 26 months; Sow 730, 15 months; Sow 679, 20 months; Sow 733, 15 months; Sow 646, 28 months. All sows were sampled during mid-gestation (day 30–100). Federal ethics approval was also obtained, GZ 68.205/002-V/3b/2019.

2.2. Lymphocyte isolation

Lymphocytes were isolated from whole blood by density centrifugation on Pancoll® (human; density, 1.077 g/mL; PAN Biotech, Aidenbach, Germany) at 920×*g*, 30 min, and washed twice in phosphate-buffered saline (PBS, PAN Biotech) and once in wash buffer consisting of Roswell Park Memorial Institute (RPMI) 1640 cell culture medium (PAN Biotech), containing 5% (v/v) fetal calf serum (FCS, Gibco™, Thermo Fisher Scientific) prior to culture. The procedure for lymphocyte isolation from organs (spleen, mediastinal lymph nodes and lung) was followed as detailed elsewhere (Mair et al., 2016). For the lung, tissue samples from the cranial right and left lung lobe were collected and used at equal amounts per animal. Lymphocytes were used immediately following isolation, without cryopreservation, for all assays.

2.3. Culture of PBMC and stimulation of cytokine production

All PBMC (peripheral blood mononuclear cells) were cultured in RPMI 1640 (PAN-Biotech) supplemented with 10% (v/v) FCS and 100 IU/mL penicillin and 0.1 mg/mL streptomycin (PAN-Biotech) and incubated at 37 °C with 5% CO_2. Cells were plated at 3×10^5 cells per well in a round-bottom 96-well plate (Greiner Bio-One, Kremsmünster, Austria) and incubated with CpG ODN 2216 (InvivoGen, San Diego, CA, USA). CpG was used at a final concentration of 10 μg/mL for all experiments, excluding the dose response experiment, and earlier experiments in which it was used at 25 μg/mL. Following overnight cultivation in CpG-containing medium, cells were additionally stimulated using 50 ng/mL PMA (Sigma-Aldrich) and 500 ng/mL ionomycin (Sigma-Aldrich) in the presence of 1 μg/mL Brefeldin A (GolgiPlug™, BD Biosciences, San Jose, CA, USA) for 4 h. From here on this stimulation protocol is abbreviated to 'CpG + PIB'.

2.4. Staining of lymphocytes for flow cytometry

Following overnight culture, cells from each condition were pooled and distributed into the wells of a round-bottom 96-well plate (Greiner Bio-One, Kremsmünster, Austria) for flow cytometry staining. The multistep staining procedure for the labelling of extra and intracellular epitopes was followed, as described recently (Milburn et al., 2021). Fixable Viability Dye eFluor780 (Thermo Fisher Waltham, MA, USA) was used for exclusion of dead cells in all staining procedures, diluted 1:800 in PBS and incubated at 4 °C for 20 min. Specificity was controlled for using appropriate isotype and fluorochrome matched antibodies. To confirm specific staining patterns for IL-10, Brefeldin A only treated samples were used to aid the setting of gates (Suppl. Fig. 1).

For staining plasma cells, the following panel was used: **anti-CD49d**, BV605 conjugate, clone L25, mouse IgG2b (BD Biosciences, 1:33). Cells were fixed and permeabilised with the eBioscience transcription factor staining Buffer Set (Thermo Fisher, Cat: 00-5523-00). The following antibodies were applied for intracellular staining: **anti-CD79α,** PerCP-Cy5.5 conjugate, clone HM47, mouse IgG1, (BioLegend, San Diego, CA, USA, 1:200), **anti-IRF4**, PE conjugate, clone 3E4, rat IgG1, (Thermo Fisher, 1:40) and **anti-IL10**-AlexaFluor647, clone 945A 4C4 37B1,

mouse IgG1 (Thermo Fisher, conjugated using the Alexa Fluor™ 647 antibody labelling kit, Thermo Fisher, according to manufacturer's instructions. Used at 0.6 μL in 10 μL.).

For B cell characterisation in combination with IgG and CD21, the following staining panel was applied: **anti-CD21**, PE conjugate, clone BB6–11C9.6, mouse IgG1, Southern Biotech (Birmingham AL, USA, 1:16). Then, following fixation and permeabilisation, using BD Cytofix/Cytoperm and BD Perm/Wash (BD Biosciences): **anti-CD79α,** PerCP-Cy5.5 (as above), **anti-IL10**-AlexaFluor647 (as above), **anti-IgG**, biotinylated, clone MT424, mouse IgG2a, Mabtech, Nacka Strand, Sweden; detected using Streptavidin-BV421 (BioLegend, 1:5).

For B1 cell characterisation, the following panel was used: **anti-CD21**, PE conjugate (as above), **anti-CD95**, clone DX3, mouse IgG2a (OriGene Technologies GmbH, Herford, Germany, 1:20), detected using goat anti-mouse-IgG2a-BV421 (Jackson ImmunoResearch, Ely, United Kingdom), **anti-IgM** biotinylated, clone 5C9, mouse IgG1 (hybridoma purchased from ATCC, Manassas, VA, USA, in-house production and biotinylation, used at 1:2.5), detected using streptavidin-BV605 (BioLegend, 1:50) and **anti-CD11R1,** clone MIL4, IgG2b, (BioRad, Hercules CA, USA, 0.15 μL in 10 μL) conjugated in-house to FITC (Thermo Fisher FITC antibody labelling kit). Following fixation and permeabilisation using BD Cytofix/Cytoperm and BD Perm/Wash (BD Bioscience): **anti-CD79α,** PerCP-Cy5.5 conjugate (as above) and **anti-IL10-**Alexa-Fluor647 (as above). All reagents were titrated according to lot to determine optimal staining and dilutions are provided in brackets as a guide only.

2.5. Flow cytometry sample acquisition

Samples were analysed using a FACSCanto II flow cytometer (equipped with 407, 488 and 633 nm lasers, BD Biosciences) with FACS Diva software version 9.0.1 (BD Biosciences). The data was then analysed using FlowJo version 10.6.2 (BD Biosciences). Compensation was applied using single stain controls, which was calculated using FACS Diva software version 9.0.1 (BD Biosciences) automatic compensation and adjusted in FlowJo. Example gating strategies for identifying porcine CD79α$^+$ B cells isolated from blood, spleen, lymph node and lung are given in Suppl. Fig. 2.

3. Results

3.1. A small fraction of porcine B cells is IL-10 competent and is activated in vitro by CpG in a dose dependent manner preceding PMA and ionomycin stimulation

To determine IL-10 competence in porcine B cells, we applied the established *in vitro* method for polyclonal stimulation of PBMC to produce cytokines by using PMA and ionomycin, in the presence of Brefeldin A, followed by intracellular cytokine detection by flow cytometry (Fig. 1). This resulted in 1–2% of total B cells containing detectable intracellular IL-10. Further activation via TLR9, using CpG oligodeoxynucleotide (ODN) 2216 (hereafter referred to as CpG) for 16 h prior to PMA and ionomycin treatment increased IL-10 expression in a dose dependent manner (Fig. 1B). The amount of IL-10 competent cells reached a plateau of between 2% and 6.7% of total B cells, when cells were treated with CpG at concentrations between 5 and 25 μg/mL (no further increase observed at higher concentrations, up to 30 μg/mL tested); therefore, a concentration of 10 μg/mL was used for further experiments. B cells that expressed IL-10 were both less frequent and had a lower expression level of IL-10 than non-B cells that responded to the *in vitro* CpG PMA and ionomycin stimulation, in all organs (Fig. 1 A, Suppl. Fig. 3). This is consistent with previous data in which porcine regulatory T cells represented a population of lymphocytes with high IL-10 expression levels (Milburn et al., 2021).

3.2. Porcine plasma cells do not express IL-10 in response to in vitro CpG PMA and ionomycin

IL-10 competent plasmablasts have been reported in human and mouse (Cossarizza et al., 2019, chapter 6.2). As terminally differentiated effector cells, we also examined the capacity of porcine plasma cells for IL-10 production, in this system. Our group recently established a phenotyping of porcine plasma cells, based on Blimp-1, IRF4 and CD49d (Villanueva-Hernández et al., 2022). Based on these analyses, blood-derived plasma cells can be identified by an IRF4$^+$CD49d^{hi} phenotype (Fig. 2A), which in this study accounted for 0.2 to 3.4% of total lymphocytes (Fig. 2B). Very few IL-10 producing cells were detected within IRF4$^+$CD49d^{high} cells (0.0–0.5%), in contrast to B cells excluding this population (1.4 to 2.7%) (Fig. 2B).

3.3. CD9 is not a marker of IL-10 competent porcine B cells

Porcine CD9 expressing lymphocytes have recently been characterised using novel monoclonal antibodies by our group, and this tetraspanin is present on a distinct population of porcine B cells (Milburn et al., 2021). In the mouse, adoptive transfer of CD9$^+$ B cells ameliorates allergic airway inflammation (Braza et al., 2015) and CD9 has been proposed to be a marker of mouse B10 cell subsets (Brosseau et al., 2018; Matsushita et al., 2016; Ostrowski et al., 2007; Sun et al., 2015). We therefore investigated to what extent CD9 is expressed on porcine IL-10 competent B cells in the blood. We determined that most IL-10 competent B cells are CD9 negative (Fig. 3A + B). The frequency of CD9

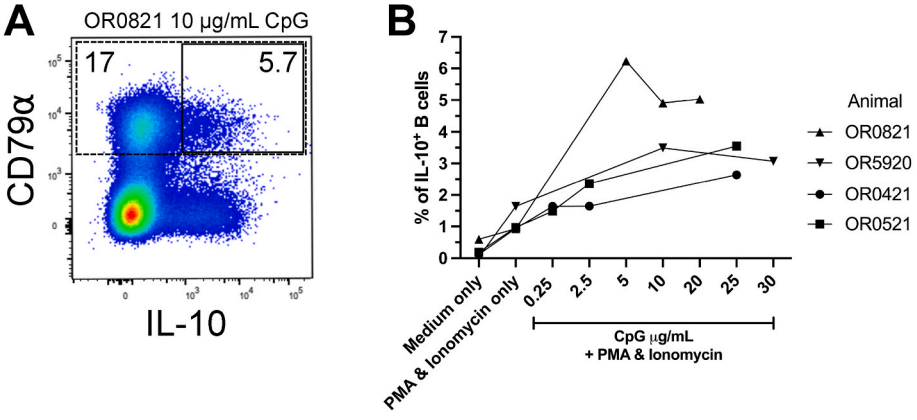

Fig. 1. IL-10 production in B cells in response to PMA/ionomycin and CpG-ODN stimulation. A) PBMC, from 6-month-old pigs from a slaughterhouse, were cultured for 18 h with 10 μg/mL of CpG, and PMA, ionomycin and Brefeldin A for the final 4 h. Cells were then analysed by flow cytometry for intracellular IL-10 alongside CD79α. Representative pseudocolour plot in which the percentage of IL-10$^+$ B cells is indicated (solid gate) within the percentage of total B cells (dashed gate, showing the percentage of CD79α$^+$ cells in total lymphocytes). B) The percentage of CD79α$^+$ cells in which intracellular IL-10 was detected is compared across conditions: cells treated with Brefeldin A only, cells treated with PMA and ionomycin + Brefeldin A, cells treated with CpG for 18 h prior to addition of PMA/ionomycin and Brefeldin A. The concentration of CpG is indicated on the x-axis, a ten-fold increase of CpG concentration was tested on animals OR0421 and OR0521, whereas a twofold increase was tested on animal OR0821.

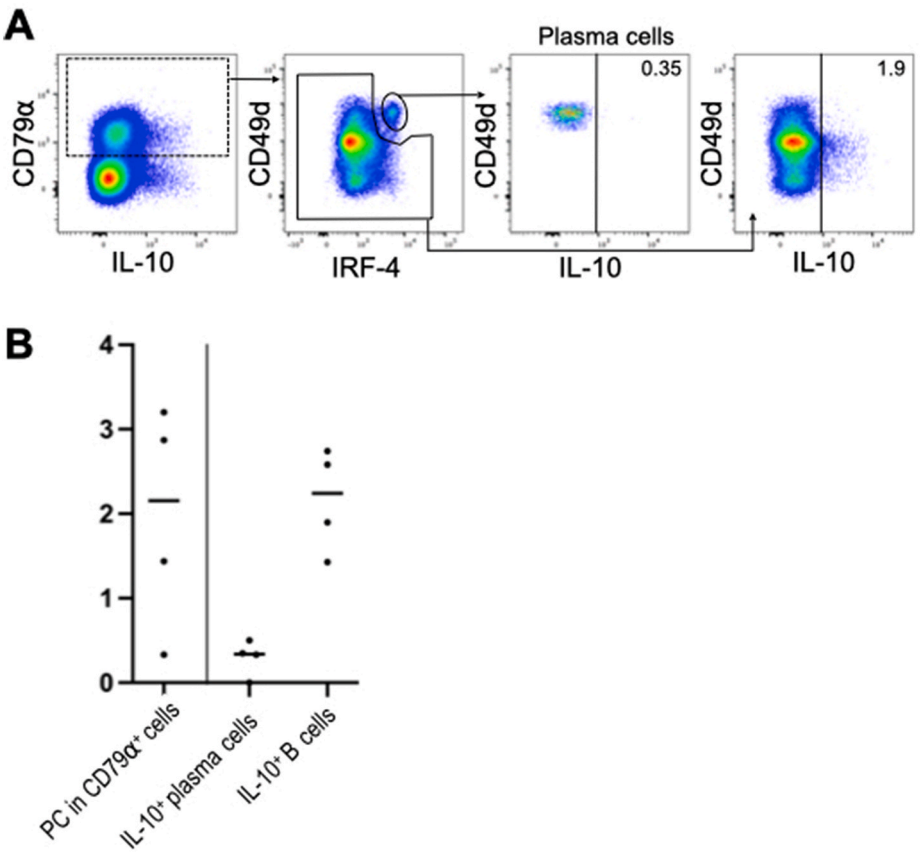

Fig. 2. IL-10 production in plasma cells. A) PBMC were stimulated for 18 h with CpG (10 μg/mL) followed by 4 h PMA and ionomycin, with Brefeldin A. Cells were analysed for intracellular IL-10 expression alongside CD79α, IRF4 and CD49d. Representative pseudocolour plots show the data from one animal and demonstrate the gating strategy used to identify porcine plasma cells. B) Data from four animals is shown. The frequency of plasma cells within total B cells is shown, and the frequency of IL-10$^+$ cells within total plasma cells and IL-10$^+$ cells within B cells excluding plasma cells.

expressing cells within the IL-10 expressing cells ranged from 10 to 35% with a median of 18% (Fig. 3B). Of note, when we first separated CD9$^+$ and CD9$^-$ B cells and investigated capacity for IL-10 production within each of these subsets (Fig. 3C), CD9$^+$ B cells contained a slightly higher frequency of cells with IL-10 competency than CD9$^-$ cells (Fig. 3D). However, given the lack of an obvious association of CD9 and IL-10 competency, we decided not to pursue this further within our *in vitro* approach, and suggest that CD9 is not a marker for IL-10$^+$ B cells in pigs.

3.4. Comparison of IL-10 competent B cells isolated from peripheral blood, spleen, lymph node and lung

In order to compare the IL-10 competence of circulating B cells with B cells obtained from secondary lymphoid tissues or mucosal tissue, we analysed lymphocytes isolated from blood, spleen, mediastinal lymph node and lung (Fig. 4). IL-10 was detected within a greater proportion of B cells isolated from the blood (range 2–8.3%, median 3.7%) and the lung (range 1.4–9.7%, median 3.5%) whereas less IL-10 competent cells were detected in B cells present in the spleen (range 0.7–1.2%, median 0.9%) and lymph node (range 0.6%–1.3%, median 0.7%) with less variation in frequency between individuals (Fig. 4B).

3.5. Age as a factor of IL10$^+$ B cell frequency in the peripheral blood of sows and weaned pigs

To see if age influences the frequency of IL-10 competent cells, we compared blood derived B cells from five weaners (2-month-old), with five sows (between 15- and 28-month-old) (Fig. 5A+B). We co-stained for intracellular IL-10 in CD79α$^+$ B cells alongside surface IgG to discern the predominant phenotype of isotype switched cells (Fig. 5B). We found that the median frequency of IL-10 competent B cells in total B cells was slightly less in the peripheral blood of sows (1.6%) than weaners (2.1%) (Fig. 5A). Weaners had more IL-10 competent cells in both IgG$^+$ (0.9 to 2.0%) and IgG$^-$ (1.8 to 2.8%) B cells, than sows (0.2 to 1.8% and 0.8 to 2.7%, respectively) though there was considerable overlap. In both young and old animals, there were more IL-10 competent cells detected in IgG$^-$ B cells (median: 2.52% and 1.72%, respectively) (Fig. 5B) than in IgG$^+$ B cells (median: 1.4% and 0.55%, respectively). Of note, the observation that most IL-10 competent B cells were IgG negative, coincides with IgG negative cells themselves being the majority (Fig 5Bi).

3.6. B cell differentiation markers and IL-10 competence

To study differentiated B cell subsets, we included CD21 in our analysis alongside IgG expression (Fig. 6). IgG$^+$ isotype switched cells represent an identifiable subset of antigen experienced B2 cells, with a loss in CD21 expression being an indicator of differentiation status. Conversely, IgG$^-$CD21$^-$ cells contain B1 cells, whilst the IgG$^-$CD21$^+$ population is enriched in naive B cells (Braun et al., 2017). All four B cell phenotypes, IgG$^+$CD21$^-$, IgG$^+$CD21$^+$, IgG$^-$CD21$^+$, IgG$^-$CD21$^-$, contained cells with the capacity for IL-10 production (Fig. 6A + B). The distribution of IL-10 competent cells, across the four possible phenotypes for IgG and CD21, is shown in Suppl. Fig. 4 A + B. There was no major difference in the frequency of IL-10 expressing cells within CD21$^+$ or CD21$^-$ cells within IgG$^+$ cells in the blood, but slightly more IL-10 expression in IgG$^+$CD21$^-$ cells in the spleen (Fig. 6B). As described for weaners and sows, blood, lymph node and spleen B cells from six-month-old slaughterhouse animals contained the most IL-10$^+$ cells within IgG$^-$ cells. There was also no major difference in IL-10 expression in CD21$^+$ or CD21$^-$ cells within IgG$^-$ cells of the blood. The IgG$^-$CD21$^-$ B1 cells were most prominent in the blood and lung (14% and 11%, respectively, representative data, Fig. 6A left column) but did not contain substantially more IL-10 competent cells than the IgG$^-$CD21$^+$ predominantly naive B2 cell population.

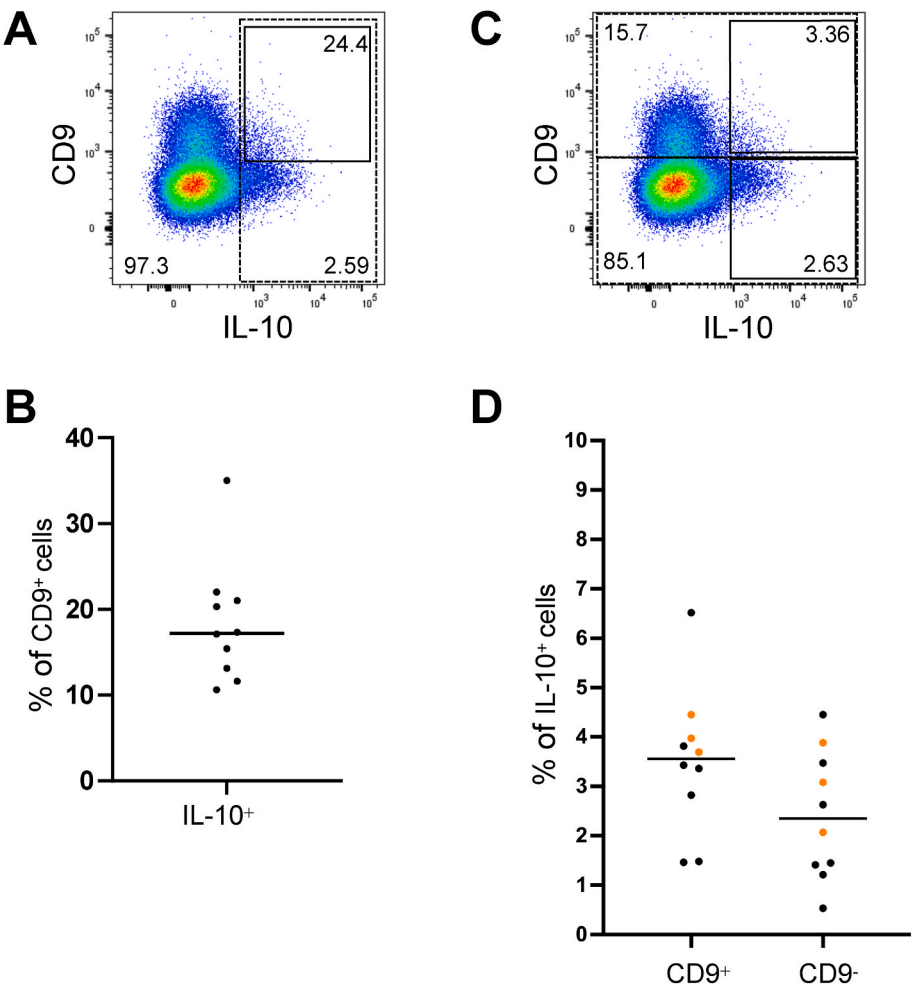

Fig. 3. Association of IL-10 production and CD9 expression. PBMC were cultured for 18 h with CpG and PMA and ionomycin, with Brefeldin A, for the final 4 h. B cells were identified using anti-CD79α labelling (not shown) and analysed for CD9 expression alongside IL-10. A) Total IL-10$^+$ producing cells were gated (gate with dashed lines, number shows percentage within CD79α$^+$ B cells), thereafter CD9$^+$ within IL-10 producing cells were gated (gate with solid lines, number shows percentage of CD9$^+$ cells within parental IL-10$^+$ population. B) Data from experiments with PBMC of 10 different pigs is shown, based on the gating illustrated in Fig. 3A, percentages of CD9$^+$ cells within IL-10$^+$ B cells are shown. C) CD9$^+$ and CD9$^-$ B cells were gated (dashed lines, numbers show % within CD79α$^+$ B cells). Within these two populations IL-10$^+$ cells were identified (gates with solid lines, numbers show percentages of IL-10$^+$ cells within parental CD9$^+$ or CD9$^-$ populations). D) Data from experiments with PBMC of 10 different animals is shown, using gating illustrated in Fig. 3C. Orange data points indicate results for cells stimulated with 25 μg/mL CpG, black dots show results for 10 μg/mL.

3.7. IL-10 expression in porcine B1 cells and recently activated B cells

B cells with an IgG$^-$CD21$^-$ phenotype are predominantly B1 cells, but this population could also contain rare, activated B2 cells of other isotypes. Therefore, we designed a parallel analysis for B1 cells as defined by IgM expression alongside a negative CD21 co-stain (Fig. 7A). We decided to apply this staining panel to B cells in the blood, where IL-10 producing B cells were most frequent (Fig. 4B). CD79α$^+$IgM$^-$ B cells have undergone isotype switching following BCR dependent activation and are therefore a differentiated subset of B2 cells. IgM$^+$CD21$^-$ B cells were identified as B1 cells, and further differentiated based on CD11R1 expression (Braun et al., 2017). IgM$^+$CD21$^+$ cells are predominantly naive B2 cells. Further, we analysed CD95 expression as an indication of activation. In this dataset, the median frequency of IL10$^+$ cells was slightly higher in IgM$^+$CD21$^+$ cells (median 4.26%) than in the IgM$^+$CD21$^-$ cells (median 3.3%) with the fewest IL-10 producers in IgM$^-$ cells (median 0.77%) (Fig. 7B). The latter finding is consistent with the lower frequency of IL-10 producing cells within IgG$^+$ B cells described above (Fig. 6B). To determine the activation status of IgM$^+$ subsets we analysed CD95 expression. IgM$^+$CD21$^-$ cells were in their vast majority CD95$^+$, whereas IgM$^+$CD21$^+$ cells contained a prominent CD95$^-$ population that expressed less IgM than IgM$^+$CD21$^+$CD95$^+$ cells (Fig. 7A). The vast majority of IL-10$^+$ cells were CD95$^+$ in both IgM$^+$CD21$^+$ (range 72.2 to 98.5%, median 86.4%) and IgM$^+$CD21$^-$ cells (range 80.3 to 98.4%, median 94.3%, Fig. 7C). We also further discriminated B1 cell subsets into CD11R1$^+$ and CD11R1$^-$ cells, as proposed by Braun et al. (2017). We found an even distribution of both CD11R1$^+$ and CD11R1$^-$ IL-10 competent B1 cells (range 18.55 to 78.6%, median 50.6%, Fig. 7C, bottom).

4. Discussion

We demonstrate that porcine B cells are capable of IL-10 expression, in response to TLR9 activation followed by polyclonal stimulation using PMA and ionomycin. We further show that the capacity for IL-10 production is a property of B cells belonging to various phenotypic subsets. Induction of IL-10 expression in mouse and human B cells via TLR9 ligation using CpG followed by PMA and ionomycin has been well established (Cossarizza et al., 2019; Iwata et al., 2011; Lampropoulou et al., 2008). Engagement of CD40 using CD40L or activating anti-CD40 antibodies also increases the frequency of IL-10 competent B cells *in vitro*, however this induces the epigenetic accessibility of the IL-10 locus in pro-B10 cells, causing their differentiation into activated B10 cells (Iwata et al., 2011). We avoided this method because we sought to determine the frequency of IL-10 competent B cells in pigs, under homeostatic conditions, without including pro-B10 cells in our analysis. Spontaneous expression of IL-10 in B cells cultured with Brefeldin alone was minimal, indicating a rarity of IL-10$^+$ effector B cells *ex vivo* (Suppl. Fig. 1). An increase to a frequency of up to slightly less than 2% IL-10$^+$ cells within total B cells, upon treatment with PIB (Fig. 1B) was found; this is consistent with similar experiments on human blood derived B cells (Iwata et al., 2011). Our results show that IL-10 competent B cells are present within all porcine B cell subsets studied. This is in accordance with the notion that IL-10 producing B cells represent a functional state of B cells belonging to multiple subsets. We sought to detect porcine IL-10 competent B cells within B1 and B2 cell subsets, according

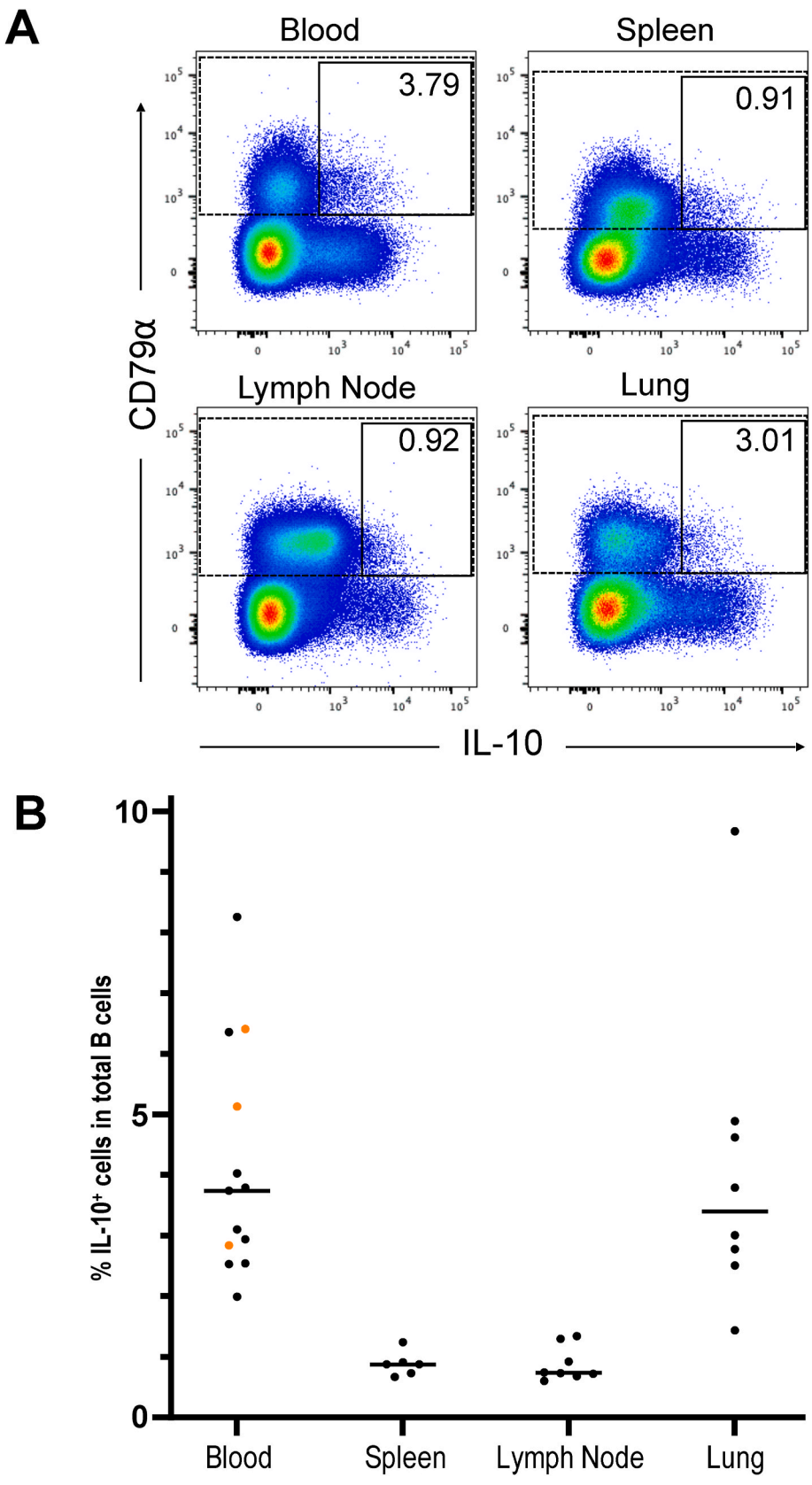

Fig. 4. IL-10 producing B cells in blood, spleen, mediastinal lymph nodes and lung tissue. A) Representative data showing IL-10 expression in lymphocytes isolated from blood, spleen, lymph nodes and lung, and stimulated for 18 h with CpG and 4h with PMA and ionomycin. Total B cells are identified by CD79α expression and indicated by the dashed gate and the percentage of IL-10^{+} cells within CD79α^{+} B cells is indicated within the solid gate. Gates were set according to Brefeldin A only control stains (Suppl. Fig. 1). B) Frequency of IL-10^{+} cells within total B cells, isolated from the blood (13 pigs), spleen (6 pigs), lymph nodes (8 pigs), and lung (8 pigs), of individual six-month-old animals, analysed across multiple experiments. Orange data points indicate results for cells stimulated with 25 μg/mL CpG, black dots show results for 10 μg/mL.

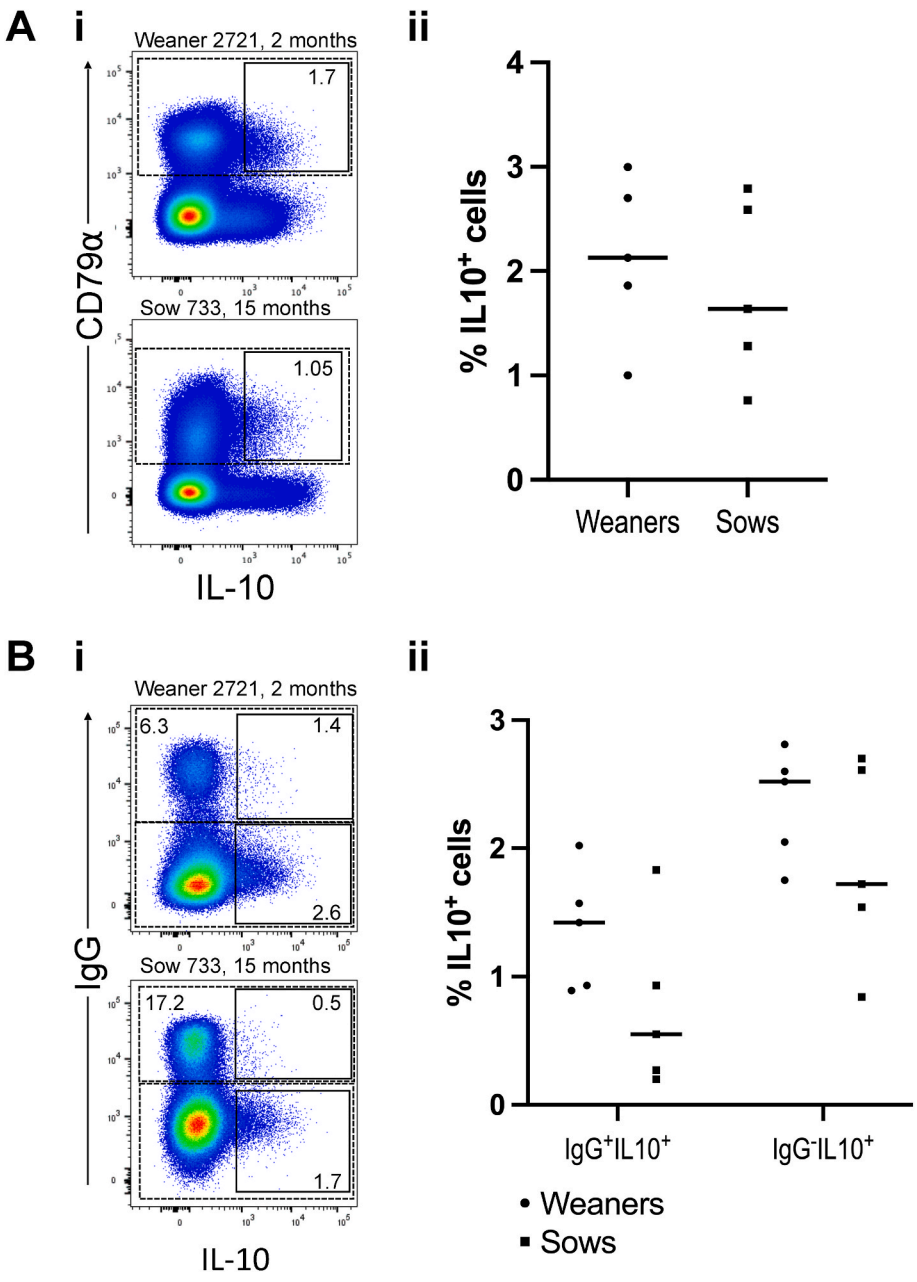

Fig. 5. IL-10 production in blood-derived B cells from weaned pigs and sows. Blood was taken from five 12-week-old weaners and five sows aged between 15 and 28 months. PBMC were cultured for 18 h with 10 μg/mL of CpG, and PMA and ionomycin for the final 4 h. Ai) Representative data from one weaner (top) and one sow (bottom). Total B cells were gated based on CD79α expression. Aii) IL-10 expression in B cells from five weaners (circles) and five sows (squares). Bi) Intracellular IL-10 is displayed against extracellular IgG, and the cells are divided into IgG^+ and IgG^- populations (dashed gates). The frequency of IL-10 positive cells within IgG^+ and IgG^- B cells is indicated within solid gates. Percentages of IgG^+ cells within total B cells are indicated in the top left corner of dashed gates. Bii) Data from five weaners (circles) and five sows (squares). The frequency of $IL\text{-}10^+$ cells within total B cells (Aii), IgG^+ and IgG^- cells (Bii) is displayed.

to B cell phenotyping strategies recently established for pigs. We therefore interrogated whether IL-10 competent cells were present within differentiated B2 cells, by analyzing plasma cells and isotype switched B cells. Little to no IL-10 production was detected in porcine plasma cells, using CpG + PIB. Although mouse $CD5^+CD1d^{high}$ B10 cells retain their capacity to differentiate into plasmablasts, they lose IL-10 competency prior to differentiation into plasma cells (Maseda et al., 2012). This contrasts with IL-10 competent plasmablasts and plasma cell subset that have been found in mouse and human (Cossarizza et al., 2019; Matsumoto et al., 2014). However, BCR mediated stimulation was not tested in our system, which may be necessary for IL-10 expression by some Breg subsets (Baba et al., 2015). The full plasticity of porcine $IL\text{-}10^+$ B cells remains to be determined but we aimed to gain an indication of IL-10 capacity in differentiated B cell subsets so we co-stained for CD21 and IgG alongside intracellular IL-10. IgG is only expressed on B2 cells following isotype switching. CD21 is absent on porcine B1 cells (Braun et al., 2017) but is expressed on the majority of mature naive and antigen experienced B2 cells (Sinkora et al., 2013). However expression patterns of CD21 on activated B2 cells in pigs are yet to be elucidated in detail. Interestingly, mouse Breg express high to intermediate CD21, and $IL10^+$ marginal zone B cells and transitional 2-marginal zone precursor B cells express high levels of CD21 and IgM (Cossarizza et al., 2019, chap. 6.2). We found the greatest frequency of IL-10 competent cells in IgG^- B cells in the blood, so we investigated non-class switched B cells by staining for IgM alongside B1 cell markers. Mouse B1 cells are $CD5^+CD11b^+$ and upregulate CD1d upon activation, this is a phenotype typically shared by murine B10 cells, which are most frequent in the peritoneal cavity and spleen (Yanaba et al., 2008). CD5 is not a marker for B1 cells in pigs (Wilson and Wilkie, 2007). Here we define porcine B1 cells as $CD79\alpha^+IgM^+CD21^-CD11R1^{+/-}$ according to the phenotyping described by Braun et al. (2017). Our results indicate that the frequency of IL-10 competent B cells is fairly evenly distributed between IgM^+CD21^+cells and IgM^+CD21^-B cells (Fig. 7B). However, isotype switched cells contained consistently less IL-10 in both sets of experiments that analysed either IgG^+ cells or IgM^- cells. Although other rarer B cell subsets would not be discriminated using these markers, we

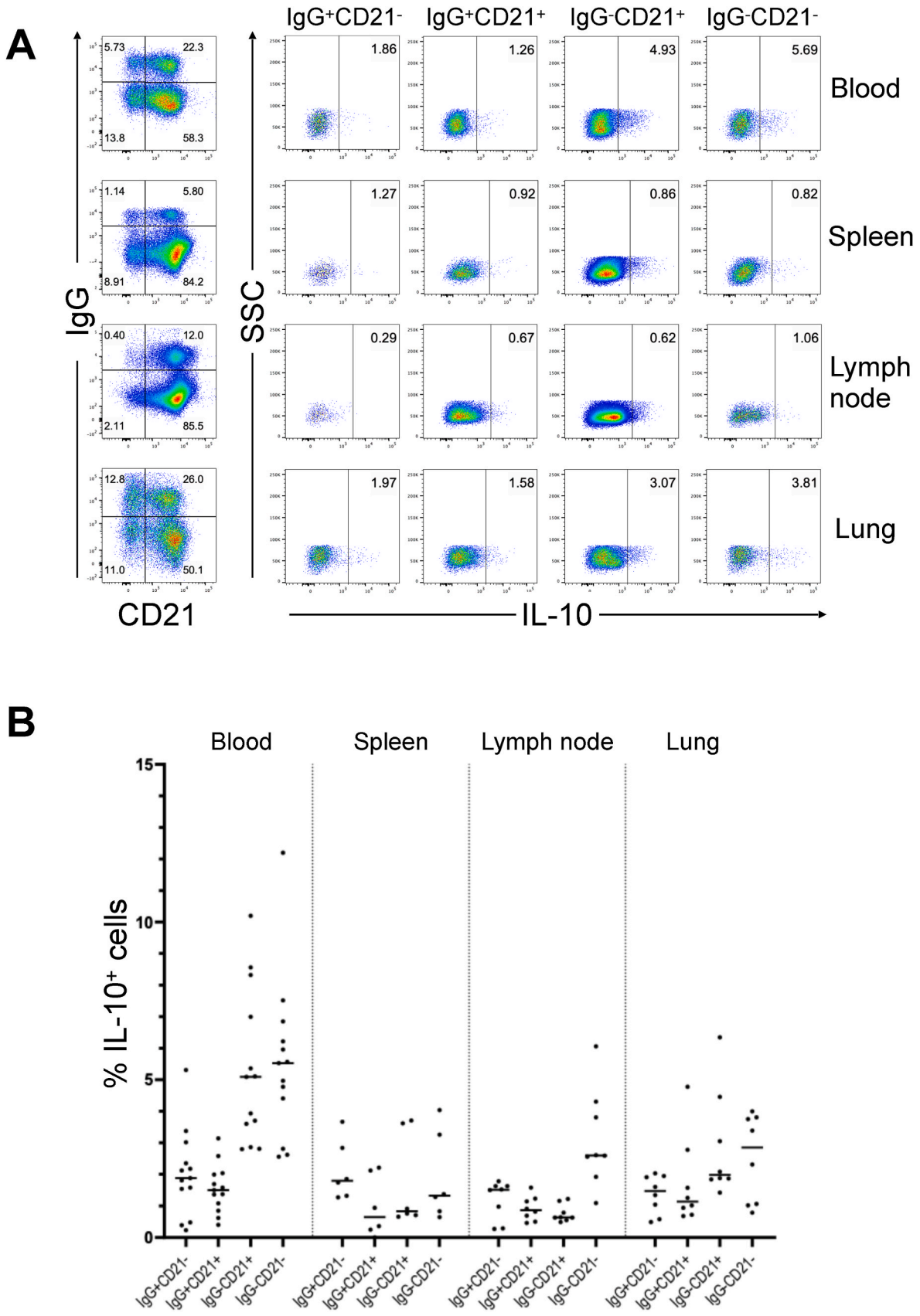

Fig. 6. IgG and CD21 phenotyping of IL-10 producing B cells in blood, spleen, mediastinal lymph nodes and lung tissue. A) Representative data from one animal, CD79α$^+$ (pre-gated, not shown) B cells are divided into four populations on the basis of IgG and CD21 expression, and the frequency of IL-10$^+$ cells within each population is illustrated against side scatter. B) Frequency of IL-10$^+$ B cells within CD21/IgG defined phenotypes. Data from multiple experiments using cells obtained from the blood (13 pigs), spleen (6 pigs), mediastinal lymph nodes (8 pigs) and lung (8 pigs) of 6-month-old animals.

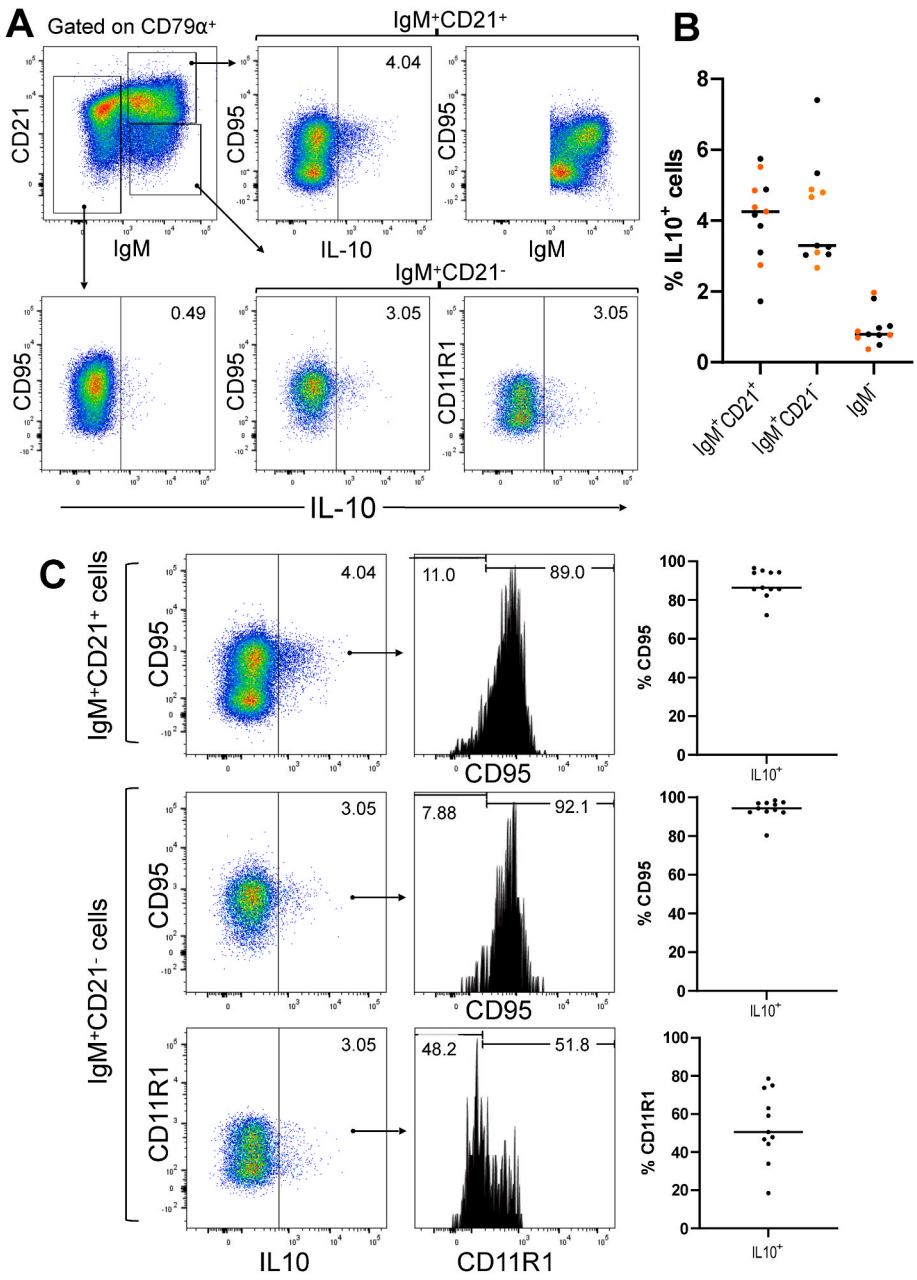

Fig. 7. IL-10 producing B cell subsets associated with B1 and B2 cell phenotypes. PBMC were stimulated for 18 h with CpG and with PMA and ionomycin for the final 4 h, then analysed for IL-10 production along markers for B cell subsets. A) Representative data from one animal, pre-gated on CD79α$^+$ cells (not shown). B cells expressing IgM and CD21 were gated into three populations: IgM$^+$CD21$^+$ cells – enriched in naive B cells, IgM$^+$CD21$^-$ cells – enriched in B1 cells, and IgM$^-$CD21$^{+/-}$ cells – isotype switched B2 cells. Intracellular IL-10 was detected and is displayed for each subset against CD95. IgM$^+$CD21$^-$ cells were further divided into CD11R1$^+$ and CD11R1$^-$ subsets. B) Data from experiments with PBMC from eleven pigs, displaying the frequency of IL-10$^+$ cells within IgM$^+$CD21$^+$, IgM$^+$CD21$^-$ and IgM$^-$ populations. Orange data points indicate results for cells stimulated with 25 μg/mL CpG, black dots show results for 10 μg/mL. C) Representative data from one animal, displaying the frequency of CD95 or CD11R1 expressing IL-10$^+$ cells as histograms alongside pseudocolour plots. Right: Data from eleven animals showing the percentage of CD95 and CD11R1 cells within IL-10$^+$ cells.

gained an indication of whether IL-10$^+$ cells are enriched within these phenotypes. Interestingly, IgG$^+$CD21$^-$ cells had a tendency towards slightly higher frequencies of IL-10 competent B cells than IgG$^+$CD21$^+$ B cells in all organs (Fig. 6B). However, most notably IgG$^-$CD21$^-$ B cells, representing mainly B1 cells, contained the most IL-10 competent cells in all organs (except the spleen) and this was marked in the lymph node. Nevertheless, we detected a slightly higher median frequency of IL-10 competent cells within IgM$^+$CD21$^+$, mainly naive B2 cells, than IgM$^+$CD21$^-$ B1 cells (Fig. 7B). Naive B2 cells have not encountered antigen via the BCR, however intial activation via TLR is sufficient to induce Breg function (Cerqueira et al., 2019; Lampropoulou et al., 2008). As B1 cells do not typically undergo class switch recombination, following BCR-mediated activation, CD95 expression was used as a marker of activation status. The vast majority of IL-10$^+$ cells were CD95$^+$, indicating that IL-10 competence is a feature of only activated B cells, indeed IL-10 was almost absent in IgM$^+$CD21$^+$CD95$^-$ naive B2 cells, under these conditions. This is consistent with the model that IL-10 competency is acquired in an antigen dependent manner (Baumgarth, 2016; Tedder, 2015). We would speculate that IgM$^+$CD21$^+$CD95$^-$ are bona fide naive B cells, whereas IgM$^+$CD21$^+$CD95$^+$ could be either naive cells that were stimulated *in vivo* in a non-BCR dependent manner, or are antigen experienced IgM$^+$ cells that have not yet undergone isotype switching. Within IgM$^+$CD21$^-$ B1 cells, we found IL-10 competent cells to be evenly distributed between CD11R1$^+$ and CD11R1$^-$ despite CD11R1$^+$ B1 cells being more differentiated and more proliferative in response to CpG stimulation (Braun et al., 2017).

Porcine IL-10 competent B cells were substantially more frequent in the blood and the lung, than in the spleen and lymph node, where naive B cells prevailed. This is in agreement with the concept that antigen exposure plays a role in programming IL-10 competence (Tedder, 2015). Also, there was a higher degree of inter-animal variation in the frequency of IL-10$^+$ cells isolated from the blood and lung, compared with IL-10$^+$ cells of the lymphoid organs which were more consistently between 0.5 and 1.5%. This suggests that in pigs B cell derived IL-10 might be most relevant in the periphery, or that the rarer IL-10$^+$ cells in the lymphoid organs represent populations which migrate to the peripheral

sites of inflammation following encounter of self-antigen. Yanaba et al. (2008) found mouse B10 cells were predominantly localized in the spleen and peritoneal cavity, with a frequency of 1 to 2%, with few detected in mediastinal lymph nodes — consistent with the frequency of porcine splenic IL-10$^+$ B cells found in our study. Furthermore, during mouse allergic airway inflammation cells generated in the spleen migrate to the lung and are essential to the control of inflammation there (Braza et al., 2015). Similarly, B cells originating from the mediastinal lymph nodes control chronic intestinal inflammation in mouse (Mizoguchi et al., 2002). In these studies, the presence of Bregs is dependent upon inflammatory status, whereas we studied healthy animals from a slaughterhouse. However, the housing conditions of conventional fattening farms lead frequently to subclinical infections and inflammatory conditions, which may explain the elevated IL-10$^+$ B-cell frequencies in blood and lung tissue.

We addressed the question of whether B cell IL-10 competence changes with age. We noted a marginally higher frequency of IL-10$^+$ B-cells in the blood of weaners compared with sows, and this is reflected in both IgG$^+$ and IgG$^-$ B cells. Overall, we detected less IL-10 competent cells in blood from both weaners and sows compared with six-month-old slaughterhouse animals. We speculate that this could be due to differing housing conditions. Animals kept on the VetFarm of the University of Veterinary Medicine Vienna benefit from more space compared to conventional fattening farms in Austria, which may result in less stress and an improved underlying health status. This aspect is probably worth studying in the future in more controlled experimental conditions. Of note, the frequency of B10 cells expands transiently in children, compared with newborn and adult humans (Kalampokis et al., 2017). Related to this, the sows in our study were in mid-gestation, and this might have impacted peripheral lymphocyte subsets in their polarization towards inflammatory or tolerogenic statuses. The function of Bregs in this context would be very interesting to study in the future, now that we have identified swine IL-10 competent B cells, alongside the recently developed technique for studying both foetal placenta and maternal endometrium (Stas et al., 2020). Another interesting future perspective for studying porcine Bregs would be to examine their distribution and function in the gut, where immune tolerance is necessary for avoiding inappropriate inflammation in response to the intestinal microflora. Booth et al. (2009) identified that sorted CD21$^+$ B cells derived from sheep jejunal Peyer's patches secrete IL-10 *ex vivo*. In this setting *in vitro* treatment with CpG was not necessary, indicating a pre-conditioned capacity for IL-10 production at this site, likely caused by activation of TLR9 in the intestine. Similar experiments in pigs to determine the distribution of constitutive IL-10 producing B cells in sites of immune tolerance would therefore be interesting. Whether porcine B cells acquire the capacity for IL-10 expression also in response to inflammatory signals *in vivo*, and in turn moderate the immune response that has been driven by those same signals, seems likely, but remains to be addressed in specific experimental scenarios.

CD9 expression has been found to be a feature of IL-10 producing B cells in various contexts in mice (Braza et al., 2015; Sun et al., 2015). However, although there have been some reports of CD9 expressing Bregs in humans (Brosseau et al., 2018, 2019), any proposal of CD9 being a stable marker for human Bregs is now well contended (Mohd Jaya et al., 2021). We accordingly questioned whether CD9 expression on porcine B cells was associated with their capacity for IL-10 production. We found there to be an almost dichotomous expression pattern between CD9 and intracellular IL-10, with only a small minority (%) of IL-10 competent B cells expressing CD9. Our initial observations at least indicate that the majority of *in vitro* induced IL-10 expressing B cells do not express CD9. Whether CD9 is a feature of IL-10 producing B cells in swine in any disease scenario, or during the resolution of an immune response post infection, in an antigen specific manner, or during regulatory mechanisms requiring direct cell-cell contact, warrants further investigation.

In summary, here we phenotyped porcine B cells that respond to CpG + PIB by expressing IL-10, which was detected intracellularly using flow cytometry. The established phenotyping for porcine B cells used confirmed that IL-10 competent cells may be found within both isotype-switched and naive B2 cells as well as B1 cells. We have discussed IL-10 competence in the context of immunoregulation, drawing parallels with mouse and human IL-10$^+$ regulatory B cells. However, further studies are warranted to functionally characterise the IL-10 competent porcine B cells described here and establish their hypothesised regulatory function both *in vitro* and *in vivo*.

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Declaration of competing interest

All authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.dci.2022.104488.

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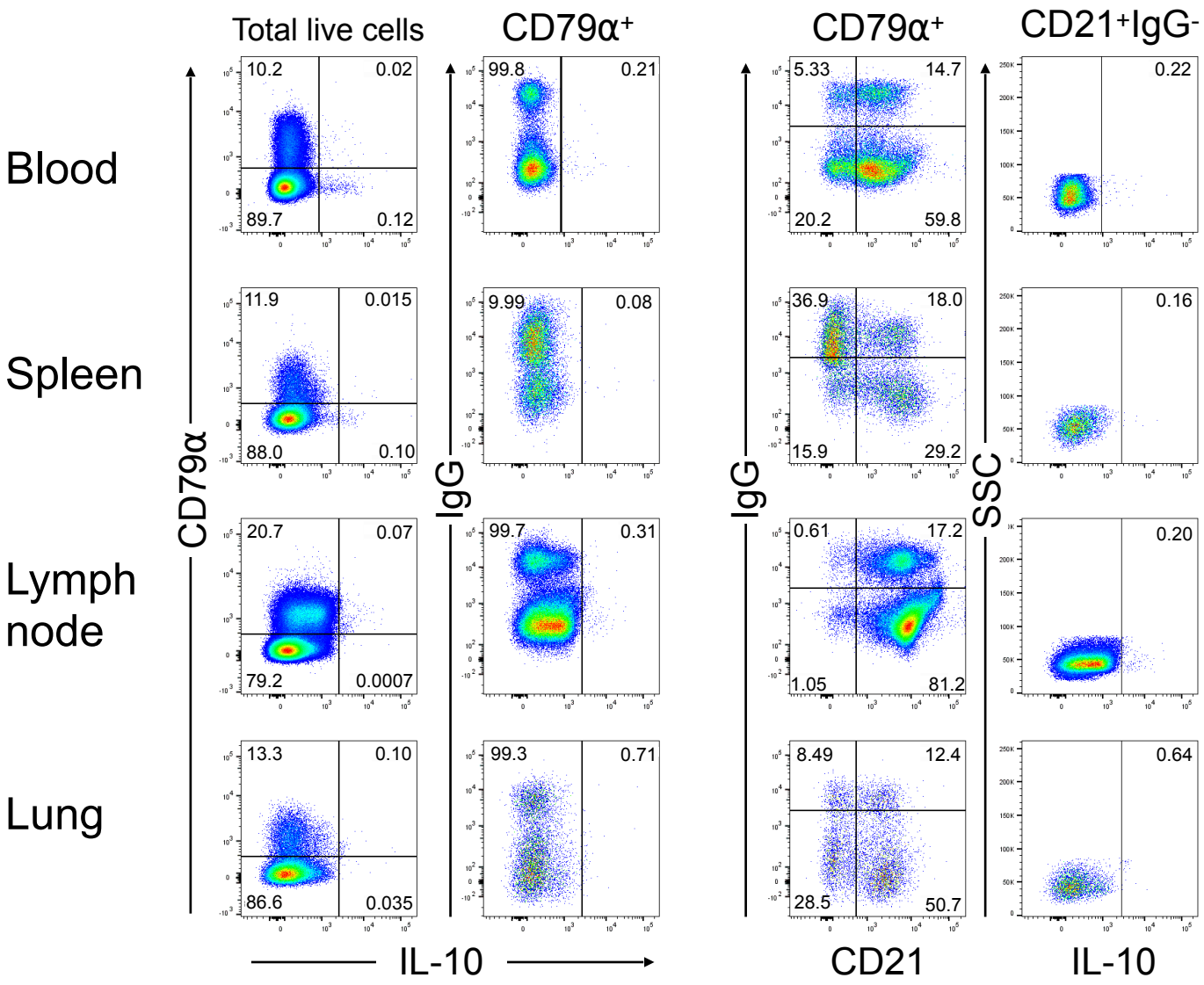
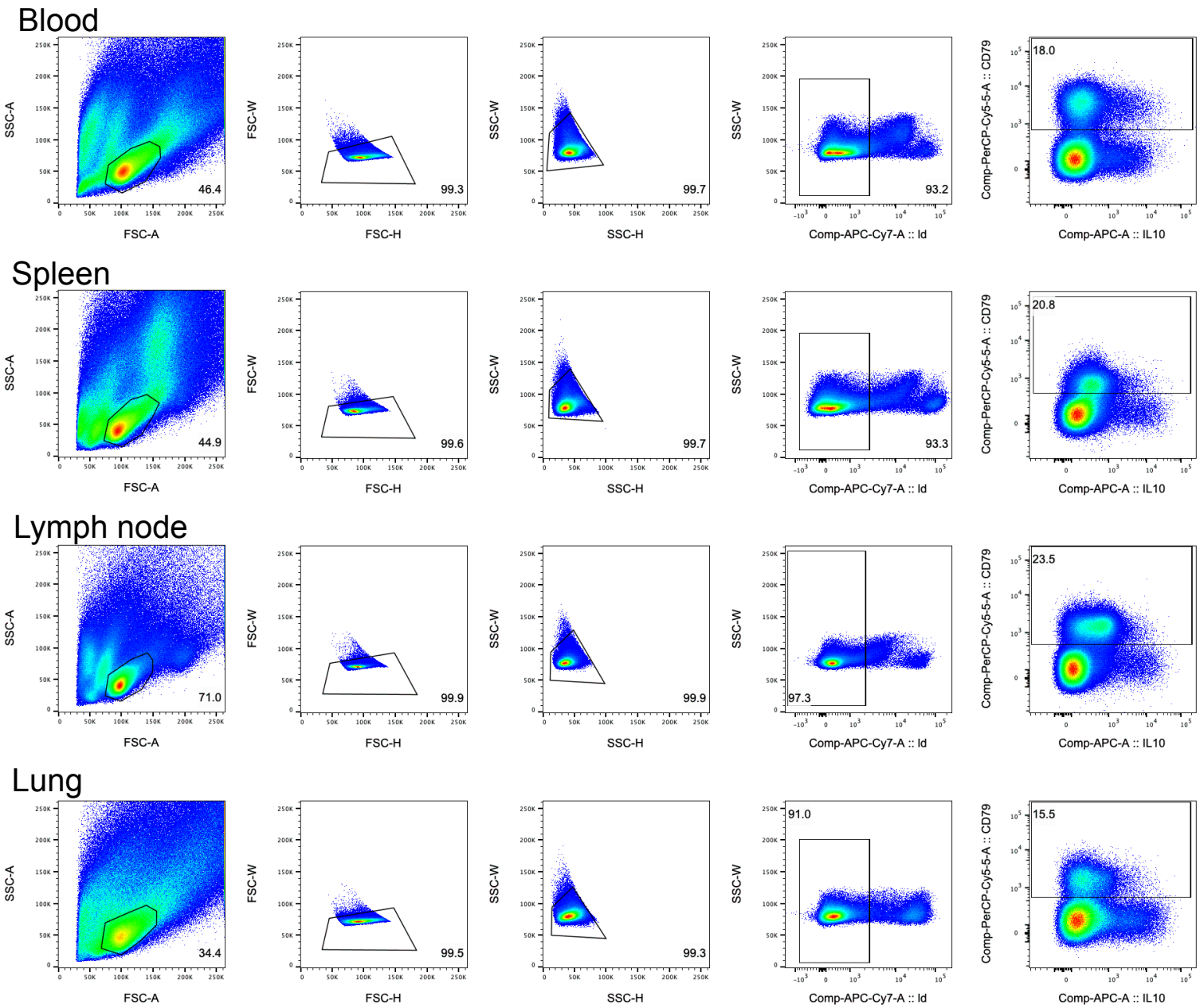
Suppl Fig. 1



Suppl. Fig. 1

Gating controls for flow cytometry analysis of in vitro stimulated cells. Lymphocytes isolated from blood, spleen, lymph node and lung were cultured overnight then only treated with Brefeldin A for 4 hrs prior to intracellular cytokine staining for IL-10 (no PMA + ionomycin or CpG stimulation). Gates were set according to the fluorescence detected, shown in the pseudocolour plots, to enable analysis of IL-10 expression in CpG + PIB stimulated cells (all other figures throughout the manuscript). The same gating strategy was applied for IL-10$^+$ cells within total CD79α$^+$ cells and IgG & CD21 defined subsets. Numbers show percentages of cells in the respective gates or quadrants.

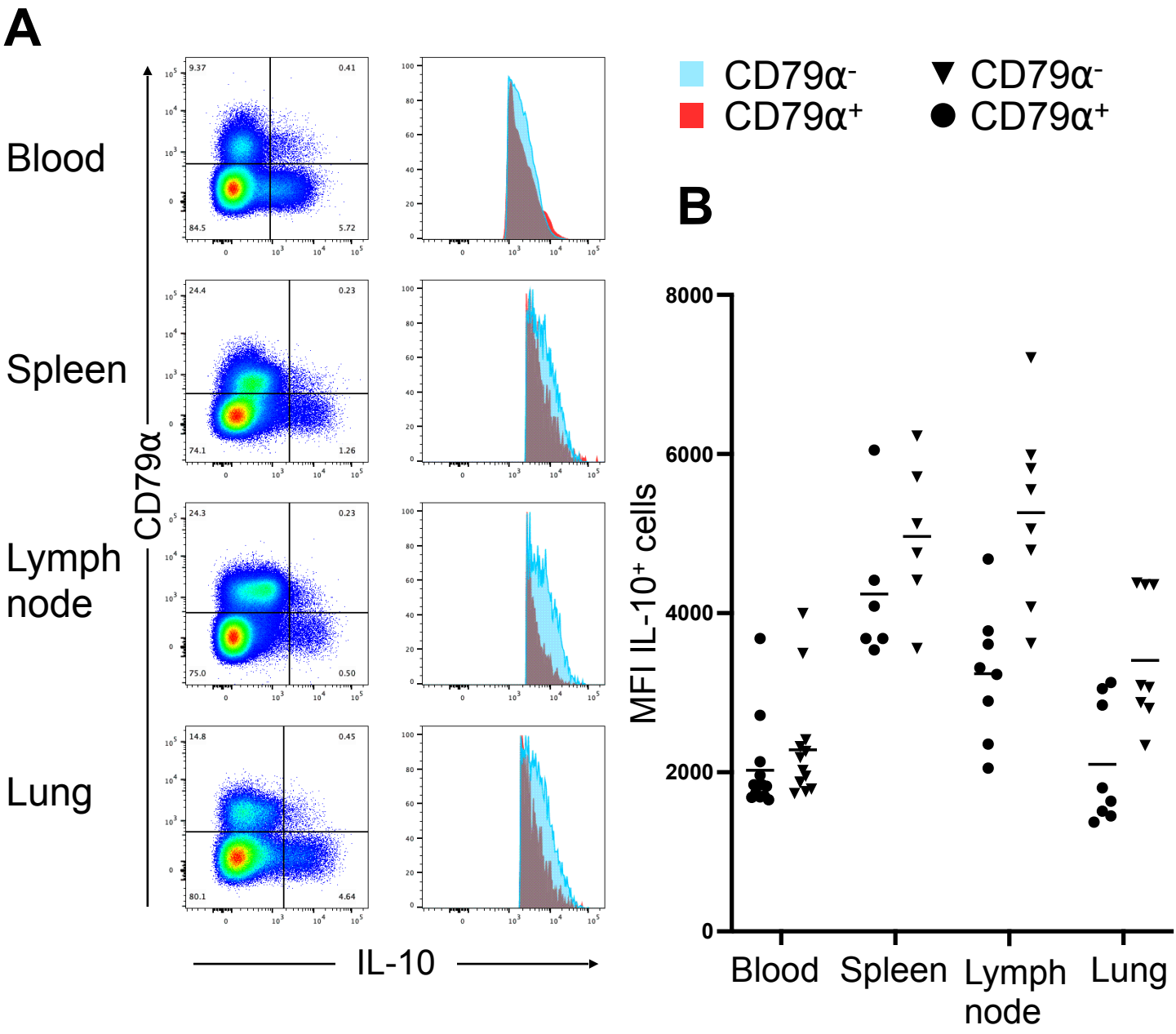
Suppl Fig. 2



Suppl. Fig. 2

Example gating strategy for discriminating porcine B cells within lymphocytes isolated from Blood (top), Spleen (upper middle), Lymph Node (lower middle) and Lung (Bottom). Cells were cultured for 18 hrs with 10 µg/mL CpG and 4 hrs with PMA and ionomycin, with Brefeldin A. Gating hierarchy is from left to right. Lymphocyte + lymphocyte blast cells are identified by a gate on total cells, then doublet exclusion, then exclusion of dead cells by gating on Fixable Viability Dye eFluor780 negative cells, then gating on CD79α^{+} B cells.

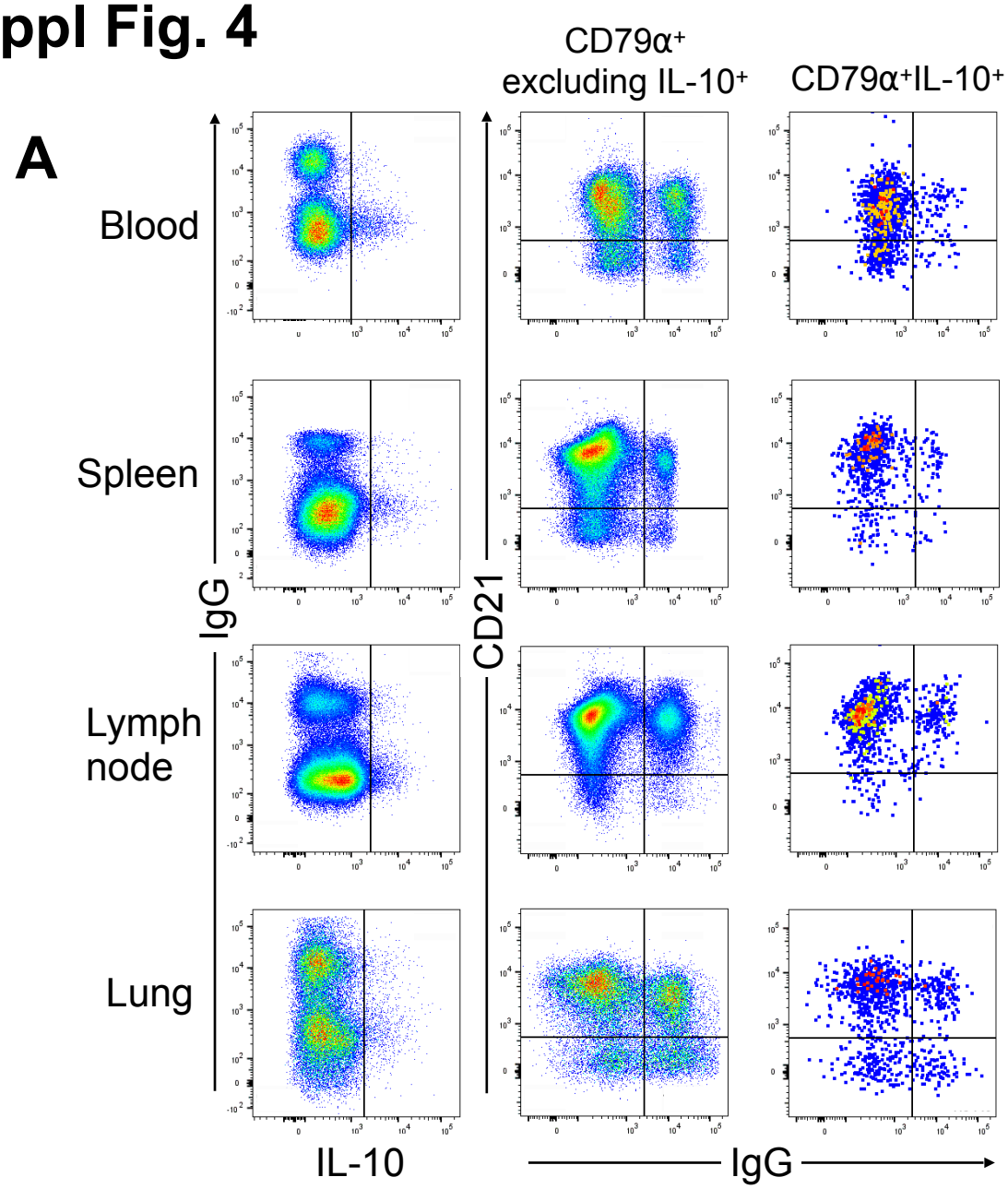
Suppl Fig. 3

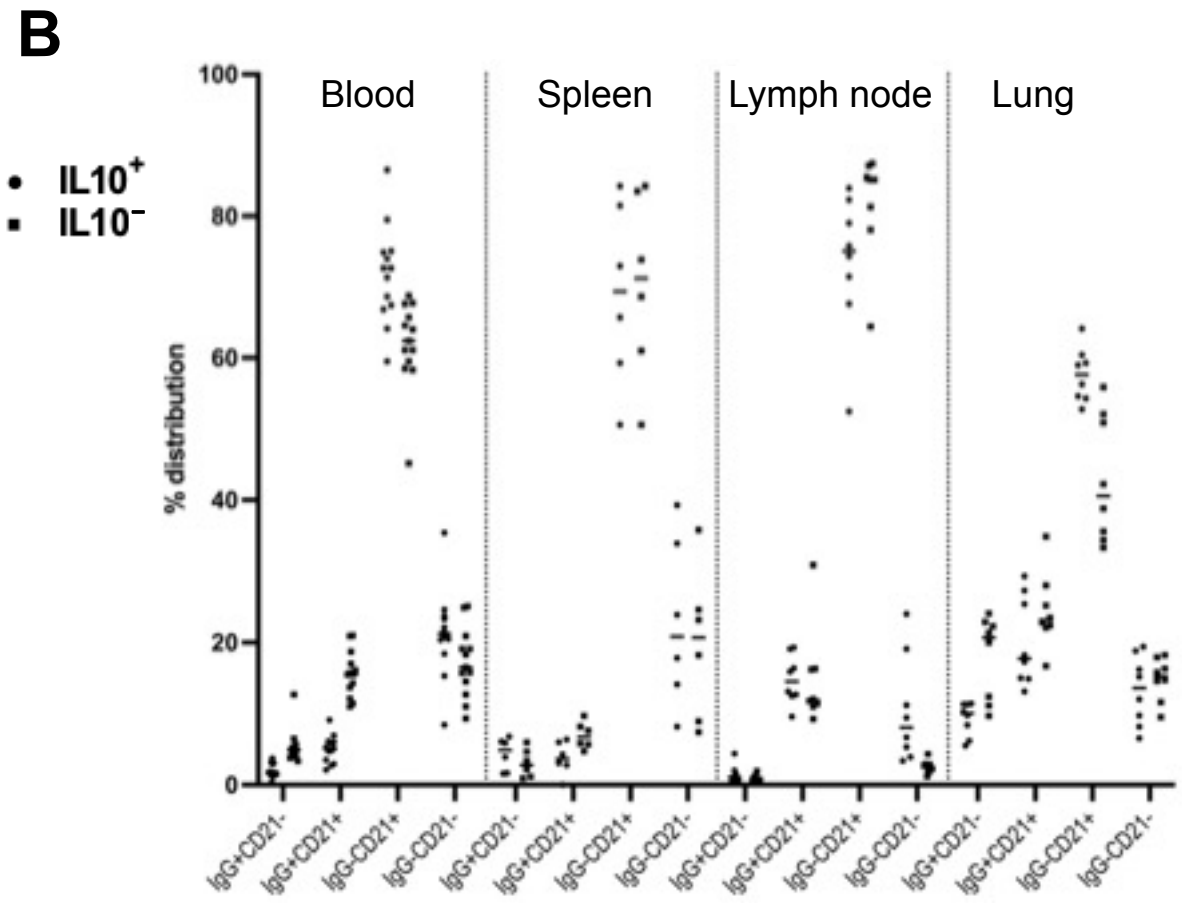


Suppl. Fig. 3

Analysis of IL-10 expression in B cells compared with non-B lymphocytes following 18 hrs of culture with 10 μg/mL CpG and 4 hrs with PMA and ionomycin, with Brefeldin A. A) Representative pseudocolour plots and histograms from blood, spleen, lymph node, and lung. Overlays in histograms show the fluorescence intensity of IL-10$^+$ lymphocytes, red histograms show CD79α$^+$ IL-10$^+$ cells, blue histograms show CD79α$^-$IL-10$^+$ cells. B) Data was collected across multiple experiments and analysed for the MFI of IL-10$^+$ B cells (circles) and IL-10$^+$ non-B lymphocytes (triangles).

Suppl Fig. 4


A
CD79α+ excluding IL-10+
CD79α+IL-10+
Blood
Spleen
Lymph node
Lung
IgG
CD21
IL-10
IgG


B
IL10+
IL10−
Blood
Spleen
Lymph node
Lung
% distribution
100
80
60
40
20
0
IgG+CD21-
IgG+CD21+
IgG-CD21+
IgG-CD21-

Suppl. Fig. 4

Complementary analyses to Fig. 6, showing the distribution of IL-10^{+} and IL-10^{-} B cells across IgG & CD21 defined subsets. A) Representative data from one animal, CD79α^{+} cells (pre-gated, not shown). Left column: IL-10 expression displayed against IgG. IL-10^{+} and IL-10^{-} cells were gated. Middle and right column: the distribution of IgG/CD21defined IL10^{-} B cells (middle) is compared with the distribution of IgG/CD21defined IL-10^{+} B cells (right). B) Data from multiple experiments using cells obtained from the blood (13 pigs), spleen (6 pigs), mediastinal lymph nodes (8 pigs) and lung (8 pigs) of 6-month-old animals. The frequency of IL10^{+} B cells (circles) is compared with the frequency of IL10^{-} B cells (squares), within each CD21/IgG-defined subset.

6 Discussion

6.1 CD9 expression on porcine T cells

6.1.1 The cellular distribution of porcine CD9

In this work we used CD9 monoclonal antibodies to investigate the distribution of tetraspanin 29 on immune cell subsets in swine and analysed whether CD9 could be used as a marker of T cell activation and memory. In conventional pigs, CD9 was variably expressed across lymphocyte subsets, almost universally expressed on monocytes and highly expressed on endothelial cells. Therefore, CD9 expression alone does not define a cell subset.

The high expression of CD9 on porcine endothelial cells, as determined using fluorescence microscopy (publication 1 suppl. fig. 4) is consistent with reports in other species, where endothelial adhesive platforms containing CD9 alongside ICAM-1 and VCAM-1 allow for leukocyte transmigration through the endothelium.[99] CD9 also promotes lymphangiogenesis in mouse and human cells and is required for vascular endothelial growth factor receptor 3 signalling.[137] The ubiquitous expression of CD9 on porcine monocytes is interesting. Human CD9 was detected on a quarter of monocytes isolated from peripheral blood by Tohami et al.,[107] and Pugholm et al.[106] did not detect CD9 on human *ex vivo* monocytes, but they observed significant up-regulation of its surface expression following culture.

The cellular distribution of porcine CD9 also reflected variation between species which was reported in the context of haematopoiesis.[103] In mice, antibodies that target CD9 can be used to enrich for haematopoietic stem cells alongside other markers, following lineage depletion, and these $CD9^+$ haematopoietic stem cells (HSC) fully reconstitute the haematopoietic system of recipient mice.[138] In apparent contrast to this, another monoclonal antibody (which is not

available) reactive against porcine CD9 was used to negatively select for porcine HSC.[139] Nevertheless, these two studies employed different strategies for identifying HSC, and in the case of murine HSC, these express lower levels of CD9 than the lineage depleted cells which were excluded prior to selection of $CD9^{med}$ expressing cells.[138] Therefore, while we may acknowledge species differences in the distribution of CD9 across the haematopoietic system, consistent methods would need to be employed to allow for a genuine comparison.

Due to the complex tissue distribution and multiple functions of CD9, it has not been widely considered as a cell surface marker outside of cancer biology. Moreover, inference of the distribution and function of CD9 expressing cells from one species to another should be approached with caution, due to the variability in the tissue distribution of tetraspanins, and their multiple and overlapping molecular functions.[103] However some studies suggesting functional redundancy may be a misinterpretation of Tspan-Tspan interactions within TEMs.[128]

Within the porcine T cell population, CD9 expression was most frequent on memory T cells, and the frequency of $CD9^+$ cells within total CD4 T cells correlated with the frequency of central memory CD4 T cells, this is the first study to associate CD9 with memory T cells. We also showed that CD9 was upregulated on T cells following activation using ConA (publication 1, fig. 4). Moreover, we detected CD9 on the vast majority of cytokine expressing cells, following antigen specific restimulation, in PBMC isolated from two animals at day 62 following two swine influenza A challenges (publication 1, fig. 8). Such an enrichment of $CD9^+$ cells was particularly interesting under these conditions, because blood taken from animals with only one influenza challenge, or at earlier time points, had lower frequencies of $CD9^+$ Ag specific CD4 T cells within the $IFN\gamma^+TNF\alpha^+$ population. This work evidences that CD9 is upregulated on activated T cells, and further implies that it is expressed on longer lived memory cells. The potential to distinguish cells with a long-lived memory fate from the transient pool

of activated effector cells could aid in predicting the effectiveness of vaccination, in which instance long lived memory cells are desired. Whether CD9 expression by T cells could serve as a correlate of protection should be interrogated by further challenge and vaccination studies.

6.1.2 Functional implications of the expression of CD9 by long-lived memory T cells

The presence of CD9 on longer lived memory cells raises the question of whether this subset is selected for due to any survival advantages offered by CD9 expression, or whether it is rapidly expressed upon re-exposure to cognate Ag. We found a small proportion of naive cells express CD9, however our results indicate that CD9 expression can be induced *de novo* following activation by TCR crosslinking, in the absence of selection (publication 1, fig 6). This does not negate the possibility that CD9 offers a selection advantage, but it does imply signalling downstream of the TCR results in upregulation of this molecule on a subset of activated T cells. Interference of CD9 expression using RNAi or CRISPR knock-down in a porcine T cell line might present an opportunity for interrogating the role of CD9 in porcine T cell activation, migration, Ag specific responsiveness, and *in vitro* survival. However limitations with regards to the immortalised nature of cell lines, which may confound cell signalling results, should be considered. It would nevertheless be interesting to consider the molecular functions that CD9 might be fulfilling at the T cell surface (introduced in chapter 3.7).

The molecular interactions of porcine CD9 are not known. Therefore discussion about the molecular function of CD9 on porcine memory T cells is highly speculative, by virtue of the multitude of processes that its expression may be regulating and the variety of potential partner proteins with which CD9 interacts, as emphasised in the introduction (chapter 3.7). Nevertheless, we may consider information about mouse and human CD9, which has been

reported.[131] Firstly, a consideration of the level of interaction between a tetraspanin and a putative binding partner is necessary for understanding claims alluding to tetraspanin functions based on their associations. Some reports of CD9 interacting with immunoreceptors were based on interactions that are conserved under only weakly dissociative conditions and are likely to be indirect.[97,128] Molecules which directly interact with CD9 are introduced in chapter 3.7. One avenue for further investigation would be to interrogate the capacity of porcine CD9 to modulate integrins expressed by T cells. The implications of these CD9-integrin interactions to the biology of long-lived memory cells could be multiple because the role of CD9 in integrin modulation at the T cell surface differs depending on the context. For example, the role of CD9 in enhancing the recruitment of activated VLA-4 (α4β1 integrin) at the immune synapse, differs from its function in decreasing the overall valency of LFA-1 (αLβ2 integrin) during leukocyte migration (introduced in chapters 3.7.3 and 3.7.4). Both LFA-1 and VLA-4 are expressed across lymphocyte subsets and accumulate in the peripheral ring of the immune synapse (p-SMAC) where they stabilise T cell-APC interactions and co-stimulate T cells.[124] CD9 expression in this context might be required during the re-encounter of cognate antigen and subsequent initiation of an anamnestic response involving rapid re-activation of effector functions, including rapid cytokine release. It might also be involved in modulating the TCR threshold, or the switch to TCR-independent cell survival. Alternatively, repeated interactions with MHC is important for maintaining memory CD4 T cell function [140] in this context, CD9 may play a role in regulating signalling at the TCR complex during maintenance of long lived memory cells.

Interestingly, our data showed that although CD9 was slightly more frequently expressed on T_{CM} cells, a distinct proportion of T_{EM} cells also expressed CD9 and the density of CD9 at the cell surface was highest on T_{EM} cells isolated from the blood (publication 1 fig. 6C). Therefore,

more information is required about the effect of CD9 expression on porcine T cell migration. Given that CD9 downmodulates LFA-1 valency,[121] migration to tissue sites of inflammation and away from re-circulating through lymphoid tissues might be promoted, potentially indicating a subset of central memory cells which are transitioning to effector cells. To investigate this, *in vitro* functional assays, could be complimented by high dimensional phenotyping, discussed below (chapter 6.4). Antibodies reactive for porcine CD49d, CD29, CD11a and CD18 are available (integrin chains α4, β1, αL and β2 respectively).[22,141] This means that experiments to determine CD9-integrin interactions in pigs may be possible using these reagents. Furthermore, spatiotemporal information including the localisation and density of CD9 molecules along with these integrins, may be determined using super-resolution fluorescence microscopy, such work is underway by others and would also be interesting when applied to T cells.[92] We have already shown that one of the CD9 mAb clones described herein can be used for fluorescence microscopy (publication 1 suppl. fig. 4). Finally, these reagents are already in use for phenotyping lymphocyte subsets by flow cytometry and more extensive phenotyping of CD9 expressing memory cells that includes an analysis of integrin expression patterns would be interesting. For example, CD49d (integrin α4) is upregulated on memory CD4 T cells.[141] We could investigate if CD9 expression is associated with variable expression levels of integrins, or even potentially conformational epitopes on activated/inactivated states.

The diversity of CD9 functions at the cell membrane across immune cells is exemplified in the introduction (chapter 3.7.6). Whether or not porcine CD9 could also be a cytokine receptor is yet another potential avenue for investigation. Also, CD9 may have a role in regulating lipid based cell membrane processes. Ligation of CD9 using anti-human mAb clone ALB6 (but not clone ML13) results in rapid loss of membrane phosphatidylserine exposure, on Jurkat T cells, following calcium ionophore treatment, but not following treatment with delayed inducers of

apoptosis.[142] This is interesting because it may have implications for T cell survival. Whether anti-porcine mAbs 3B3 and 2E12 could be used for similar experiments could be tested.

6.1.3 CD9 as a potential marker of long-term self-renewal

It has been recently demonstrated that CD9 expression is induced by STAT5B in haematopoietic stem cells and it was proposed that CD9 can be used as a STAT5B activation dependant marker.[143] STAT5B (but not STAT5A) drives self-renewal [143] and this transcription factor is prominent in lymphoid cells.[143] Since a feature of memory cells is the necessity for long-term self-renewal, it would be interesting to determine if the detection of CD9 on T cells is also indicative of a STAT5B driven axis of survival, quiescence and self-renewal. However, STAT5B promotes antigen restimulation-induced cell death in Treg and T_{EM} in mice and humans,[144] so attention must be given to whether a signalling threshold for cell survival vs cell death could be determined in this context, or different effects of STAT5 expression in memory T cell populations. We found a correlation between CD9 expression and the frequency of memory T cells in the peripheral blood of swine which was significant for $T_{CM,}$ but not T_{EM}. Interestingly, HIV induced expression of virus-host fusion mRNA encoding STAT5B results in the survival of virally infected T_{CM} and Treg, but not other T lymphocyte compartments, further indicating that this pathway may function specifically in T_{CM} rather than T_{EM} (i.e. the virus hijacked a functional pathway).[145] Additionally, CD9 stabilises the cytokine receptor gp130 on glioma stem cells, promoting downstream STAT signalling[146] and interestingly, gp130 is key to long lasting anti-viral T cell immunity.[147]

6.1.4 Future direction for investigating CD9 expression as a potential correlate of protection

Although investigations into the molecular function of CD9 on porcine T cells are an interesting avenue for reductive basic science, future work will likely focus on the utility of this molecule as a cell surface marker for immune memory, due to the more limited nature of cell biology research in swine, and more immediate applications that such knowledge would have for animal healthcare. Very little attention has been given to the function of CD9 expressing lymphocytes in mice and humans outside of B cells. Next steps to expand on our findings in swine would include the analysis of CD9 expression on T cells responding to antigen specific challenge in future challenge or vaccination studies. A limitation of the work presented here is the small sample size in our experiments on CD9 expression alongside cytokine expression on T cells responding to swine influenza A virus Ag specific restimulation. Alongside increasing the number of animals studied, this data may also be strengthened in a number of ways, including by analysing memory cells isolated from the site of challenge (e.g. lung in the case of respiratory infection) and by increasing the time frame post challenge at which samples are collected. We used CD8α and CD27 expression to distinguish differentiation of CD4 T cells [16] (introduced in chapter 3.4). However $CD27^-$ and $CD27^+$ CD4 T cells can further be subdivided according to CCR7 expression, and other memory markers such SLA-DR and loss of CD45RC [18,148] (and chapter 3.4). Since the acceptance of publication 1, another group has characterised CD9 expressing porcine CD4 T cell subsets.[149] In support of our work, this group also associates CD9 expression with a central memory population defined by a $CCR7^+2E3^-$ phenotype. This study also shows that upon *in vitro* antigen specific restimulation, the vast majority of $IFN\gamma^+$ cells are $CD9^+$ in PBMC isolated from animals at day 44 following pseudorabies virus vaccination.[149]

Future studies that aim to strengthen the data showing that CD9 is upregulated on the majority of longer-lived memory cells, could additionally investigate whether this phenomenon is observed upon other T cell subsets, particularly CD8β$^+$ T cells which respond to intracellular virus by the targeted release of cytotoxic molecules such as perforin. It may well be that the frequency of CD9 expressing cells within defined T cell subsets is critical for the outcome — for example the ratio of CD9$^+$ Treg vs CD9$^+$ T_{CM} could be considered. This may especially be important in the lung, as it is a mucosal site of immune challenge, where an effective immune response must be tightly regulated to limit pathology, and where we found the highest proportion of CD9$^+$ T cells co-expressing FoxP3 (publication 1 fig. 3B). Finally, to fulfil the title 'correlate of protection' CD9 expression on lymphocyte subsets must be correlated with protection from disease following re-exposure to the pathogen.

More broadly, it would be ideal to analyse CD9 expression on T cell subsets as a potential corelate of protection during challenge and vaccination studies with viruses other than swine influenza A and pseudorabies viruses, such as PRRSV, CSFV, ASFV, FMDV. Whether CD9 is a feature of Th1 polarised cells or also Th2 and Th17 cells also remains in question. To address this, CD9 expression may be analysed alongside intracellular cytokine staining for IL-17 in T cells isolated from the gut of animals responding to *Salmonella* Typhimurium [7] or blood or lung of animals infected with *Actinobacillus pleuropneumoniae.*[150] Studying porcine Th2 polarisation still relies on analysis of mRNA, however T cells responding to *Ascaris suum* may be analysed for CD9 expression.[37]

6.1.5 Conclusion of discussion of CD9 expression by porcine T cells

CD9 expression is fairly broad and variable, and it is not restricted to a hitherto established functional subset of T cells. However, our analysis of CD9 expression on CD4 T cell memory

subsets and on antigen specific T cells indicates that when used in combination with markers that are already used to define memory T cells, or when detected on populations of T cells responding to specific antigen restimulation, identification of CD9 expression on these cells might be an indication of memory cell longevity and / or function. Further work is needed to confirm this. This work could utilise memory cells obtained following larger and more various challenge and vaccination studies. Deducing the molecular activity of CD9 on porcine T cells was outside the scope of this work. However in light of increasingly detailed models of CD9 function attained from the wider field, future research may interrogate the molecular interactions of CD9 at the cell surface membrane of porcine memory T cells. These two research questions would complement each other in parallel investigations, without being dependant on the hypotheses of each other. The novel reagents presented in this work will be of use to the wider field of porcine immunology for phenotyping the various lymphocyte populations that CD9 is expressed on and will also be of use to the broader field of porcine biology including virology and developmental biology.

6.2 IL-10 competent B cells

6.2.1 What are IL-10$^+$ B cells?

The question of whether IL-10 competence in B cells is a functional state common across B cell subsets, or a particular feature of regulatory B cells (Breg), and whether or not the latter can be assigned to distinct subsets is complex, however we may draw some understanding of what IL-10$^+$ B cell populations in pigs may represent from ongoing research in other species. A fully comprehensive introduction to regulatory B cells, B10 and B1a cells, including their ontogeny, diversity and seminal reports lies outside the scope of this thesis, however regulatory B cells have been widely reviewed[151–155] and appear to be subject to an evolving paradigm. Therefore here, I briefly introduce some of the prominent IL-10 producing B cell subsets that have been studied, for context, and go on to discuss how our data fits in with an emerging paradigm of IL-10$^+$ B cell heterogeneity.

6.2.2 B cells with regulatory functions

Multiple populations of B cells have been shown to regulate immune responses in mice,[65,74,76,111,156–161] are present under conditions of controlled inflammation in humans,[77,113,162–165] and possess various mechanisms for regulating myeloid cells and T cell polarization[158] and survival *in vitro,* including by production of IL-10.[55,158,161] Early studies on Bregs by C. Mauri and colleagues identified the populations of interest to them as a subset within conventional B cells,[160] however B cells from the B1 lineage also possess immunomodulatory functions[72] as do marginal zone (MZ) B cells.[73,166] Breg have since been described as having multiple phenotypes and belong to various B cell subsets.[70] Mauri et al. suggest that a common precursor of the Bregs which they study in mice are T-2 marginal-zone

precursors.[74,166] Nevertheless there is no universal Breg phenotype, no known master transcription factor, and they are not a firmly established lineage comparable to natural Treg. Many reports describe 'subsets' of Breg, which have been summarised according to phenotype,[70] however the only defining feature of Breg as a 'subset' themselves is functional.[55,70] Therefore IL-10 became a surrogate indicator of Breg cells, with emphasis on the notion that IL-10 producing cells must be empirically proven to have regulatory functions,[70] and populations of Breg have various regulatory mechanisms that are independent of IL-10 production.[153,167] It would therefore be premature to suggest that the IL-10$^+$ cells identified here all represent regulatory B cells.

6.2.3 B10 cells

B10 cells have been described as a functionally defined subset of regulatory B cells that control the polarization of an immune response exclusively via IL-10 production, setting them apart from other IL-10 competent B cells including other Breg.[54,55,65] Murine B10 cells can be CD1d$^+$CD5$^+$ and therefore have overlapping phenotypes with B1a and marginal zone B cells.[65,168] These overlapping markers preclude sorting of B10 cells from the B1a or the MZ cell pool; additionally, since different B cell subsets may express similar molecules by virtue of their tissue localisation, distinguishing B10 cells from B1a cells and MZ B cells is difficult.[164] This is exemplified by one limitation in the methodology of a study that suggested CD9 is a marker of B10 cells, on the basis of increased CD9 mRNA on sorted CD1d^{hi}CD5$^+$CD19$^+$ cells, which upon reanalysis, post-sorting, were only enriched in IL-10$^+$ B cells.[112] What is more, the definition of a 'B10 negative' subset used for comparison of differential gene expression is questionable as it was based on simply a CD1d$^-$CD5$^-$CD19$^+$ phenotype[112]. The observed enrichment in CD9$^+$ cells between these subsets might be

explained by the enrichment of CD9 within MZ and B1 cell populations in mice.[68] We also found that $CD9^+$ B cells are somewhat enriched within porcine B1 cells (data not shown). Such discrepancies emphasise that B10 cells remain functionally, rather than phenotypically defined.

Interestingly, the B10 cell population contains cells with both hypermutated and germline BCR, as well as both isotype switched and polyreactive IgM, therefore B10 cells in mice appear to be a heterogenous population consisting of both B1 and B2 cells.[164] An equivalent subset of B10 cells in humans does not belong to a distinct B cell subset but is enriched within memory B cell populations.[162,165] Further studies on porcine B1 cells and IL-10 competent B cells could also consider re-employing methods that were used by Wilson and Wilkie to interrogate $CD5^+$ vs $CD5^-$ B cells in pigs,[58] this time using the new model for B1-like porcine B cell discrimination,[51] it would be interesting if differences in the restricted use of variable gene segments and hypermutations are experimentally discernible in mature cells, since the combinatorial use of variable Ig gene segments in pigs is generally more restricted but highly hypermutated.[42]

6.2.4 IL-10 expression in B1 cells

The existence of IL-10 producing cells within the B1 population has been regarded as a feature of this subset.[58,61] A role for CD5 in promoting autocrine IL-10 signalling is particularly relevant to the B1a subset,[80] which its self has been attributed regulatory properties [64,72] though this population would contain indistinguishable B10 cells.[72,164] Nevertheless IL-10 is a survival factor for activated human B cells[81] and promotes B1 cell activation and proliferation.[61] Therefore the overall role of IL-10 in local immune control by B1 cells is complex, where earlier studies identifying regulatory B1 populations may indeed include B10 cells, and IL-10 may be fulfilling roles beyond counter regulation of antigen specific responses. Murine B1

cells are selected neonatally against self-antigen,[169] so a role in self-antigen mediated peripheral tolerance may not be surprising. Porcine B1 cells have not been divided into B1a and B1b subsets on the basis of CD5 expression, however one reason for this was that there is no difference in IL-10 mRNA transcripts between $CD5^+$ and $CD5^-$ B cells.[58] Further studies into porcine B1 cells may interrogate BCR specificity alongside cytokine profiles, this might in turn shed light on the role of this subset to immune regulation.

6.2.5 IL-10 competent B cells in pigs

Our analysis of IL-10 competent B cell in the peripheral blood of swine so far reflects the situation in humans. There are very few *ex vivo* IL-10 competent B cells in the peripheral blood isolated from healthy humans[162,165] and swine (publication 2, fig. 1), whereas overnight stimulation using CpG-ODN prior to PMA and ionomycin clearly increases the frequency in pigs (publication 1 fig. 4B) and humans.[162,165] *In vitro* activation of human and mouse B cells using TLR agonists was documented to induce IL-10 in various instances: CpG-ODN and CD40L induce IL-10 expression in innate-like B cells and B10 cells, and LPS can induce IL-10 expression in B2 plasmablasts and B1a cells.[72,76,165] Engagement of CD40 *via* CD40 mAb or CD40L increases the number of IL-10 competent B10 cells *in vitro* by inducing differentiation of B10pro cells.[55,164] For our work, we chose to use CpG-ODN followed by 4 h of PMA and ionomycin stimulation, to induce IL-10 expression in porcine B cells, with brefeldin A to inhibit intracellular protein transport. This method omitted quantification of B10pro cells. Therefore although the relevance of *in vitro* induced cytokine production to any *in vivo* scenario is questionable, we have at least identified a pathway for induction of IL-10 expression which matches that of mouse and human reports. Other B cell subsets, such as plasma cells, which we found had very low IL-10 expression under these conditions, may

require different activation to induce IL-10 competency, such as LPS and additional cytokines.[76]

Our data shows that IL-10 production is a feature that is shared across various populations of porcine B cells. These observations are in harmony with recent reports that human *in vitro* induced IL-10^{+} B cells are diverse and do not belong to a single subset.[78,79] Detection of IL-10 alone is not sufficient to determine Breg status, since many IL-10 competent B cells are multifunctional as they co-express inflammatory cytokine, and IL-10 production can be a transient state.[75,78,79,170]

Taken together, our results and the reviewed literature, indicate that the diversity of IL-10 competent B cells, reflects the multiple populations of B cells. Therefore IL-10 producing B cells may originate from heterogenous subsets and rather represent a cell state, [78] that may also include populations which arise from pre-conditioning cells within the total B cell pool to become B10 cells. Meanwhile it has not been ruled out that Breg cells could originate from a common precursor and are induced to acquire regulatory B cell functions and adopt multiple phenotypes further down a differentiation pathway.[166]

6.2.6 B10 conditioning and activation

How B cells are preconditioned to acquire regulatory functions is not fully established. In the case of B10 cells, T cell derived IL-21 and cognate interactions between B cell CD40 and T cell CD40L drives differentiation of B10pro cells into IL-10 producers and expansion of B10 cells, and this subset is able to control antigen specific inflammation.[171] Interestingly, engagement of CD40 also induces upregulation of CD95 on B lymphocytes, including naive cells.[53,172] This might explain the co-expression of CD95 and IL-10 on porcine B cells that we observed (publication 2 fig. 7.). Although T cells are instrumental in the induction of B10 cell

function, they do not appear to be required for the development of B10 cells, which are present in T cell deficient mice.[54] BCR complex signalling contributes to the generation of IL-10 competent B cells along with other costimulatory signals,[164,173] as diverse BCR receptors are a requirement for development of a complete B10 population in mice,[54] and Breg populations are enriched in cells with autoreactive BCR.[75,164] This would suggest a key role for BCR in the conditioning of B10 cells. As such B10pro induction may occur in an antigen specific manner *in vivo*, whilst initiation of B10 effector function can be achieved by TLR ligation and T cell dependant signals — potentially explaining the targeted control of Ag specific T cells.[54,55,71,164] The ability of TLR engagement to induce IL-10 expression in B cells *in vitro* is well established in mice and humans.[54,71,162,174] However, CpG-ODN is sufficient to induce differentiation of human B10pro cells, whereas CD40 engagement of murine B cells is necessary and the contribution of BCR signalling at this stage remains unclear.[164] This highlights questions which remain open also in the context of our work, as we have not yet tested the contribution of CD40 engagement or BCR signals to the promotion of IL-10 competent B cells in pigs. Sophisticated co-culture experiments would also be required for interrogating B cell mediated regulation of Ag specific T cells.

To conclude, also within the porcine immune system, it remains to be fully elucidated which B cell populations transiently express IL-10 (perhaps as an autocrine growth factor), which express IL-10 as part of a polyfunctional cytokine response following stimulation, which have an inherently immunomodulatory nature by virtue of IL-10 release — as is suggested for murine B1a cells, and which have acquired durable — or at least targeted — regulatory function that is tailored to the immune microenvironment from which they are isolated and are potentially dedicated Breg; and finally whether or not any of these scenarios are mutually exclusive. Even without answers to these broader question within the field of Breg

immunology, our capacity to detect intracellular IL-10 within porcine B cells will be useful for interrogating the changing immune response to infection and vaccination, which fits the broader aims of our research.

6.2.7 The possible role of IL-10 producing B cells in vaccination

Ideally, a vaccine should activate multiple arms of the immune system and trigger sufficient cross talk between innate and adaptive immune responses. Whether IL-10 production by subsets of B cells is an indication of appropriate activation following vaccination or challenge is an avenue for future investigation. Transient IL-10 production by B cells can act in an autocrine manner to promote survival of activated B cells, and differentiation of human, but not mouse, B cells into plasmablasts.[80,81] Nevertheless activated B10 cells rapidly differentiate into Ab producing cells, with an Ig diversity indicative of both innate like B cell and B2 origins,[75] therefore intracellular IL-10 detection will indicate sufficient activation of B10 cells prior to their differentiation into antibody secreting cells. Which, in the case of polyreactive Ab production by innate-like B cells could result in early broader protection, and in the case of B2 cells will be highly specific. Conversely, IL-10 is well known to limit acute immune responses, while the predominantly germline encoded IgM of B10 cells [75] and IgG_4 from IL-10 producing B2 cells,[44] might also play a role in immune regulation at later stages of the response.[75] Although Breg appear to function in limiting T cell mediated autoantigen responses, more research may elucidate their contribution to the natural limitation of autoimmunity [175] during responses to infection and vaccination.

Detection of B cell derived IL-10 may also be necessary when evaluating the route of vaccination and the use of different adjuvants. Considering the complexity of the relationship between IL-10 and B cells, the utility of intracellular cytokine staining as a tool for

characterising B cell subsets is clear. The presence of IL-10 during both vaccination and the anamnestic response upon re-challenge has been studied at the tissue level, measuring total cytokine or IL-10 mRNA, and in the context of myeloid cell and Treg derived IL-10.[176] However the importance of B cell derived IL-10 in the context of establishing immune memory is somewhat less studied. In order to demonstrate the generalised considerations introduced here, below I offer details from two vaccination studies, conducted in mice, against pathogens which could be of relevance to swine. These studies employed very different methods of immunization against two different facultative Gram-negative intracellular bacteria. One study used a CpG-containing-DNA vector vaccine, encoding the heat shock protein 65 (Hsp65) of *Mycobacterium tuberculosis*, which can invade and cause persistent infection in macrophages.[177] The other used whole inactivated *Salmonella* Typhimurium [178] which invades the intestinal epithelium in pigs, but causes systemic typhoid-like infection in mice.[7]

In the study by Fontoura et al. 2015,[177] wild type (WT) mice and B cell-deficient mice ($\mu^{-/-}$, BKO) were immunized 3 times with DNA-Hsp65. As a DNA vaccine containing CpG motifs, this system also utilises the auxiliary effect of TLR9 activation. When splenocytes were isolated and restimulated using recombinant Hsp65 protein *in vitro* there were increased frequencies of memory CD4 and CD8 T cells from WT mice than BKO mice, as a result of the DNA vector. Cytokine mRNA was also isolated from total splenocytes following antigen specific restimulation. Vaccination using DNA-Hsp65 induced significantly more IFNγ and IL-12 mRNA expression than with empty vector, in both WT and BKO mice but this effect was drastically enhanced in BKO mice. Crucially wild type mice had more splenic IL-10 mRNA 30 days after DNA-Hsp65 vaccination than BKO mice and when B cells were enriched from WT spleens, they contained more IL-10 mRNA in the vaccinated group than the unvaccinated group. It appears that B cells were responsible for producing IL-10 during

vaccination, which somehow increased the frequency of memory CD8 T cells. The authors suggest that B cell derived IL-10 controls the amount of inflammatory cytokine in the spleen, preventing T cell apoptosis due to over-stimulation, and increasing the duration of available Ag and therefore the opportunity for memory T cell selection.[177] One may therefore consider how IL-10 mediated antigen persistence and T cell activation regulation could be advantageous in the case of a subunit or DNA vaccine, as opposed to during a primary infection, meaning the frequency of IL-10^{+} B cells in primary infection vs vaccination may be considered differently advantageous or detrimental to protection.

A study by Neves et al*.,* 2010[178] identified a pathway for induction of IL-10 expression in murine B cells via the adaptor protein MyD88. *S.* Typhimurium activated CD138^{hi} B cells produced IL-10 *in vitro* via activation of TLR2 and 4 in mice, but not TLR9. IL-10.eGFP reporter mice infected with *S.* Typhimurium develop a prominent splenic population of eGFP^{+} B cells within the activated CD138^{+} population, as early as 1 day post infection. In this study they also tested the *Salmonella* vaccination using mice that were deficient in B cell MyD88 (B-*MyD88* $^{-/-}$) and B-WT chimera control mice. Mice were vaccinated intravenously with live attenuated *Salmonella* and challenged with virulent *Salmonella* 90 days post vaccination. Wild type mice succumbed to infection, with a 50 % survival rate at day 60 following challenge. However, remarkably B-*MyD88* $^{-/-}$ mice had a 100 % survival rate at day 60, evidencing that impaired responses to *Salmonella* vaccination in mice is a result of MyD88 dependant B cell responses. To elucidate the mechanism of protection, vaccinated mice were challenged with attenuated *Salmonella*, and cellular and cytokine responses were comprehensively evaluated 5 days later. *Salmonella* reactive serum antibody levels and T cell cytokine expression were similar in vaccinated mice from both groups before and after challenge, however *B-MyD88*$^{-/-}$ mice had more TNFα^{+} neutrophil and natural killer (NK) cell infiltration to the spleen and

liver. This indicated that memory B cells have a MyD88 dependant mechanism, which includes IL-10 production, that down-modulates innate cell activation early during antigen re-challenge.[178] This could in-turn cause delayed activation of T cells following challenge and impaired vaccine efficacy.[155]

Whether or not these two examples given for laboratory mice can be translated to pigs is not known, especially considering the live attenuated Salmoporc™ vaccine for pigs is effective.[7] Nevertheless, they instantiate how the presence of IL-10$^+$ Breg can have different impacts for vaccine success depending on the unique vaccination context. Ideally, for a full picture of the temporal dynamics of B cell derived IL-10 and its effects in swine vaccines, IL-10$^+$ B cells would be detected at multiple time points following vaccination and upon each subsequent vaccine booster or challenge. However since these dynamics will also vary depending on the vaccine system, particular attention must be paid to the method of vaccination or adjuvantation. For example, the DNA-vector vaccine described above was shown not to activate TLR9, however, the use of such a vector in pigs might induce B cells to express IL-10. Interestingly, an adjuvant consisting of CpG oligodeoxynucleotides significantly induced IL-10 and TNFα mRNA upregulation in total skin tissue in pigs, following intradermal injection,[179] indicating the relevance of interrogating CpG induced IL-10 expression on a cellular level following vaccination with CpG containing vaccines in pigs.

Therefore it appears that IL-10 can cause too much or too little homeostatic down modulation of primary immune responses, having outcomes for T cell memory. Given the multiple possibilities, it seems likely that a dynamic ideal minima and maxima for IL-10$^+$ B cell subsets will be established for each individual scenario, which will vary depending on the location and polarisation of the immune response and whether it is a primary vaccination, booster or challenge response.

6.2.8 Conclusion of discussion of porcine IL-10 competent B cells

In summary, we have successfully used intracellular cytokine staining for IL-10 to identify porcine B cells which express IL-10 in response to TLR9 activation during *in vitro* culture with CpG-ODN followed by PMA and ionomycin. This is consistent with previous methods that have been used for *in vitro* characterisation of IL-10 competent B cells in mice and humans. The IL-10 competent cells share phenotypes which overlap with multiple populations of porcine B cells, including isotype switched memory B2 cells and B1 cells. Further work would be necessary to ascertain whether these cells are capable of immune regulation. *In vivo* regulatory B cells may undergo a distinct program of activation leading to acquisition of Breg functions. Detection of IL-10 competent B cells in the context of a primary response to vaccination or infection could aid in understanding vaccine outcomes.

6.3 CD9 expression on B cells

An avenue for further investigation would be to characterise CD9 expressing B cells in pigs. In this work we focussed on CD9 expression on T cells, and somewhat limited our investigation of CD9 expression to whether or not it is expressed more frequently on naive, B1, or plasma cells in the blood, and whether its expression correlates with intracellular IL-10 expression. We found minor populations of CD9 expressing cells in all porcine B cells subsets analysed from blood, except for plasma cells, and $CD9^+$ cells were slightly enriched within the B1 subset (unpublished data). We also observed four distinct populations of B cells isolated from the blood, according to CD9 and CD49d (**α4** integrin) co-expression (unpublished data). This is quite intriguing as the single positive subsets show that each of these molecules are expressed independently on subsets of B cells. These subsets may have distinct migration or functional differences. Therefore, analysis of CD29 (β1 integrin) expression on these subsets might help to determine whether CD9 is co-expressed with either VLA-4 or other integrins, such as integrin β7, too. Moreover, our ability to phenotype B cells of swine is currently expanding, so an interrogation of CD9 expression on emerging phenotypic subsets may be justified. Likewise, whether CD9 expression is associated with a long lived memory capacity of B cells, as suggested here for T cells, remains to be investigated. Recent evidence suggests CD9 is not a marker of all $IL\text{-}10^+$ B cells,[180] as initially suggested.[112,131] We also did not detect a prominent population of $CD9^+IL\text{-}10^+$ B cells isolated from the peripheral blood of conventional pigs that was cultured with CPG-ODN and PMA and ionomycin (publication 2 fig. 3). This discrepancy might be somewhat explained by the diversity of *in vitro* generated $IL\text{-}10^+$ B cells compared with B cell populations that respond to inflammation *in vivo*.[79]

A further hypothesis which remains to be tested is that CD9 might be expressed on subsets of regulatory lymphocytes which exert their mechanisms via direct cell-cell contacts.[167] Given the role of CD9 at the APC side of the immune synapse[132] a role for CD9 in B cell antigen presentation or B cell — T cell interactions also might be investigated, which would have relevance to both inflammation and immune regulation.

6.4 Extended phenotyping of porcine B and T cells

So far we and others have shown that CD9 expressing T cells are enriched within central and effector memory populations, and represent a minor population of naive cells, and we used different models to phenotypically define these populations. However, a limitation to dividing CD4 T cells into three major phenotypic populations, is that these subsets actually contain a mixture of cells with potentially different fates, and transient states of plastic cells. Minor populations of T cells expressing combinations of cell surface markers exist,[16,23] which may represent transitional populations. High dimensional analyses of datasets generated using larger combinations of cell surface markers may reveal interesting distributions of CD9 on as yet undefined T cell subsets. This should ultimately be related to function. Subsets may be further divided with increasing complexity, but this limitation will remain. Therefore in future immunology may be less fixated on subsets and more concerned with populations of cells that carry out functions in distinct clinical settings. Acknowledging this, porcine immunologists are still faced with a dirge of mAbs specific for suitable molecules that can be used as markers of major cell subsets, particularly in the case of B cells, so efforts in this field will continue to address this, as the field is still in a stage where defining major populations is necessary. The possibility to do full spectrum cytometry will enable us to develop larger staining panels, and high dimensional analysis algorithms offer an unbiased approach for identifying phenotypic

populations. More extensive phenotyping of porcine B cell subsets may include antibodies specific for other molecules which have also been reported to be expressed on fractions of porcine B cells. Such phenotyping might include analysis of B cells expressing: CD1, CD5, β7 integrin, CD52,[11] SWC7 (on cells isolated from lymphoid organs), and CD200R1,[181] alongside the markers introduced above. Additionally, a more thorough study of immunoglobulin isotypes expressed by porcine B cell subsets would be interesting. B1 cells in mice have been shown to isotype switch to IgA and also a low frequency of IgG2b cells [57] so methods which limit B1 identification to IgM^+ cells could exclude these minor populations.

For this extended phenotyping to have utility, identified populations should then be further analysed for their physiological distribution and functional roles, and monitored over the duration of an immune response during infection and vaccination studies. It is also unlikely that the IL-10 competency of B cells will be considered alone, as increasingly a case for assessing the polyfunctionality of lymphocytes emerges, which involves detection of more than one intracellular cytokine.[78] Therefore future perspectives for evaluating vaccinations in pigs could include both analysis of memory B cell populations and B cell intracellular cytokine analysis. Particular focus should be given to the temporal dynamics of IL-10 production by B cell subsets over the duration of an immune response. Whilst the overall presence of other IL-10 producing leukocytes should also be considered as these will also contribute to the cytokine milieu, the potential for Ag specific regulation of T-cell responses by Breg is interesting.

7 Thesis conclusion and outlook

This thesis addressed the need for more reagents to study the immune memory response in swine. Two novel monoclonal antibodies specific for porcine CD9 were identified and used to characterise CD9 expressing T cells. CD9 is variably expressed across T cell subsets but is upregulated following activation and is present on the majority of memory cells that respond to virus antigen by cytokine production. The molecular functions of CD9 on porcine immune cells remain to be investigated and are likely to be multiple. Future prospects would evaluate whether CD9 expression on memory T cells can be used as a marker that correlates with protection following vaccination. We also identified IL-10 competent B cells in swine for the first time. We did not find a clear association with CD9 expression and IL-10 competence in B cells treated with CpG-ODN followed by PMA & ionomycin. CD9 expressing B cells in pigs remain to be fully characterised. IL-10 competent B cells had a variety of phenotypes but were more frequently IgM^+. Future prospects would explore the contribution of IL-10 competent B cells to the establishment and control of immune memory responses. This would likely be in a context of expanded phenotyping panels and multifunctional analysis of lymphocytes.

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9 Glossary of abbreviations used in this thesis

Ab antibody

ADAM a disintegrin and metalloproteinase

Ag antigen

APC antigen presenting cell

ASC antibody secreting cell

BCR B cell receptor

BKO B cell knock out (in this case $\mu^{-/-}$)

BLAST Basic Local Alignment Search Tool

Breg regulatory B cells

CCR c-c motif chemokine receptor

CD cluster of differentiation

CpG-ODN CpG containing oligodeoxynucleotides

cDNA copy DNA (from reverse transcription of RNA)

ConA concanavalin A

CXCR c-x-c motif chemokine receptor

EWI E: glutamine W: tryptophan I: isoleucine containing immunoglobulin-like family of proteins

HLA human leukocyte antigen

HsP-65 heat shock protein 65

ICAM intracellular adhesion molecule

IFN- interferon

Ig immunoglobulin

IL- interleukin

LEL large extracellular loop (also called EC2 - extracellular domain 2)

LFA leukocyte function antigen

MZ B cell marginal zone B cell

mAb monoclonal antibody

MHC major histocompatibility complex

MyD88 myeloid differentiation primary response 88 protein

NCBI National Center for Biotechnology Information

PBMC peripheral blood mononuclear cells

PCR polymerase chain reaction

PI4K phosphatidylinositol 4-kinase

PKC protein kinase C

PMA phorbol 12-myristate 13-acetate

rhIL- recombinant human interleukin

SEB Staphylococcal enterotoxin B

SEL small extracellular loop (also called EC1 extracellular domain 1)

SLA swine leukocyte antigen

SMAC supramolecular activation cluster

T_{CM} central memory T cells

TCR T cell receptor

T_{EM} effector memory T cells

TEM tetraspanin enriched microdomain

TGF - transforming growth factor

Th1 T helper 1 cells

TLR toll-like receptor

TNF- tumour necrosis factor

Treg regulatory T cells

VCAM vascular cell adhesion molecule

VDJ variable diversity and joining (gene segments)

VLA very late antigen

WT wild type

Members of the CD Lab for an Optimised Prediction of Vaccine Success in Pigs

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"That'll do pig, that'll do!"

— Farmer Hoggett, Film: Babe 1995