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Blocking antigen-presenting cell/T cell interaction by monoclonal antibodies

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Table of contents

1	Introduction	1
1.1	Importance of the pig as immunological model.....	1
1.2	The immune system with emphasis on the pig	1
1.3	T Cells.....	5
1.3.1	Naive and memory Th cells	5
1.3.2	Cytolytic T cells	6
1.3.3	TCR- $\gamma\delta$ T cells	6
1.4	The immunological synapse	7
1.4.1	CD2.....	8
1.4.2	CD4.....	9
1.4.3	LFA-1 (CD11a/CD18)	9
1.4.4	CD50.....	10
1.3.5	MHC-II	10
1.5	Aim of this study.....	11
2	Materials and methods.....	12
2.1	Material	12
2.1.1	Reagents	12
2.1.2	Antibodies used for integrin phenotyping by FCM	13
2.1.3	Antibodies used for proliferation assay and subsequent FCM staining	14
2.1.4	Scientific equipment	15
2.1.5	Samples	16
2.1.5.1	OVA samples	16
2.1.5.2	PCV2 samples.....	16
2.1.5.3	Further samples.....	16
2.2	Methods	17
2.2.1	Thawing of samples	17

2.2.2	FCM staining	17
2.2.3	Violet staining and proliferation assay	18
2.2.4	Dialysis	19
2.2.5	Data analysis	20
3	Results.....	21
3.1	Integrin phenotyping.....	21
3.2	Proliferation assay.....	23
3.3	Effects of antibody blocking on different T-cell subsets.....	26
3.4	Effects of antibody blocking on Th memory cells	29
4	Discussion	31
4.1	Summary of results	31
4.2	Problem resolution	32
4.2.1	Sodium azide (NaN ₃).....	32
4.2.2	Cell-dependent factors	33
4.2.3	Epitope binding	33
5	Summary	34
6	Zusammenfassung	36
7	References	38
8	Figures and tables	48

Abbreviations

APC	Antigen Presenting Cell
BCR	B Cell Receptor
CCR	C-C motif chemokine receptor
CD	Cluster of Differentiation
cSMAC	Central Supra-Molecular Activation Cluster
CTL	Cytolytic T Lymphocyte
DC	Dendritic Cell
DC-SIGN	Dendritic Cell-Specific Intercellular Adhesion Molecule-3-Grabbing Non-integrin
DMSO	Dimethyl Sulfoxide
dSMAC	Distal Supra-Molecular Activation Clusters
FCM	Flow Cytometry
FBS	Fetal Bovine Serum
FSC	Forward Scatter
ICAM	Intercellular Adhesion Molecule
IK	Immunological Kinapse
IS	Immunological Synapse
LFA	Lymphocyte Function-Associated Antigen
mAb	Monoclonal Antibody
MAC	Membrane Attack Complex
MHC	Major Histocompatibility Complex
NaN ₃	Sodium Azide
NK cells	Natural Killer Cells
OVA	Ovalbumin
PAMP	Pathogen Associated Molecular Pattern
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PCV2	Porcine Circovirus Type 2
pMHC	Peptide-MHC
PRR	Pattern Recognition Receptor
pSMAC	Peripheral Supra-Molecular Activation Cluster

SMAC	Supra-Molecular Activation Cluster
SSC	Sidewards Scatter
T _{CM}	Central Memory T cells
TCR	T Cell Receptor
T _{EM}	Effector Memory T cells
T _{FH}	Follicular T-Helper Cell
Th cells	T-Helper Cells
T _{reg}	Regulatory T Cell
VLP	Virus-Like Particle

1 Introduction

1.1 Importance of the pig as immunological model

Many advances in human immunology are based on research using mice as laboratory animals (Radaelli et al. 2018). Dawson compared orthologs of genes with an immunological function between mice, humans, and pigs. During this investigation, Dawson showed a significant preservation of orthology of about 80% between human and pig, whereas there was only a similarity of 6% between human and mouse. Although gene orthology studies have to be reviewed critically, as the presence of similar sequences does not necessarily correlate with a similar function, Dawson demonstrated that more research is needed in porcine immunology to possibly establish a more human-fitting research model (Dawson 2011). Furthermore, the scientific community is trying to introduce pigs as possible organ donors for humans and demonstrated huge success in 2022 by transplanting a pig heart to a human (Sykes and Sachs 2022). This is possible due to the quite similar anatomy/physiology between pigs and humans, for example considering lung or kidney tissue and heart anatomy. Nevertheless, xenotransplantation between these two species still leads to complications in terms of graft rejection, thus requiring genetic modifications performed in pigs to better meet humanized situations (Meurens et al. 2012, Sykes and Sachs 2022). Researching the immunological processes in pigs more profoundly can help to possibly resolve these problems and can further aid in porcine vaccine development and disease prevention, leading to less stress in farm animals and therefore increasing meat quality (Martínez-Miró et al. 2016, Opriessnig et al. 2021).

1.2 The immune system with emphasis on the pig

The immune system consists of multiple defence barriers. The different parts of the immune system are generally divided into two groups: The innate immune system and the adaptive immune system. Each of these systems can again be subdivided in a humoral and a cellular response part (Murphy and Weaver 2018). The innate immune system reacts quickly to pathogens in a rather unspecific manner, whereas the adaptive immune system needs prior activation, leading to a delayed response. This response however is very specific and ideally leads to eradication of the pathogen and development of long-lasting memory (Alberts et al. 2017, Chi et al. 2024).

In the case of pathogen contact, the first barrier consists of the humoral innate immune system. A classic example would be the complement system, but there are also other very important

components like the contact system, acute phase proteins or other antimicrobial peptides that are also well described in the pig (Mair et al. 2014). These systems respond to bacterial and viral threats, leading to opsonization, neutralization or lysis of targets via the membrane attack complex (MAC). Besides of that, the complement system also targets apoptotic cells and is able to interact with B cells, inducing the formation of antibodies in mice but not in humans (Kovács et al. 2021, Shishido et al. 2012).

The cellular innate immune system resembles the second layer of the defence and the bridge to the adaptive immune system. It consists of macrophages, dendritic cells (DC), granulocytes, mast cells, natural killer (NK) cells and $\gamma\delta$ -T cells, where each cell type serves different purposes (Mair et al. 2014). Monocytes and macrophages are situated all over the body and the blood and provide protection by phagocytosis of antigens and by activating the adaptive immune system through the major histocompatibility complex (MHC) on the extracellular surface. Macrophages detect pathogens by pattern recognition receptors (PRR) that detect pathogen-associated molecular patterns (PAMP) (Guerriero 2019). PAMPs are considered to be conserved molecular characteristics of a pathogen. Nevertheless, microbes also adapt to this immune recognition and try to avoid detection by the innate immune system by slightly modifying or downregulating their PAMPs (Vance et al. 2009).

Dendritic cells can also be found in many locations in the body like the skin, blood or lymphatic organs. These cells again recognise antigens by PRRs and start their uptake. After antigen degradation this leads to extensive and long-lasting antigen presentation on the cell surface via MHC (Toka and Golde 2013, Zanna et al. 2021). In the pig, the conventional dendritic cell, plasmacytoid dendritic cell, and monocyte-derived dendritic cell subsets are described and their phenotypes and functions were recently compared to major DC-subsets of human and mice (Summerfield et al. 2015).

Another big group of innate cells are granulocytes comprised of neutrophils, eosinophils, and basophils. These cells play an important role in defence against bacteria, parasites, and fungi. Pathogens are detected by granulocytes through PRR and destroyed through phagocytosis or by releasing effector peptides and oxygen radicals into the extracellular space, therefore affecting potential pathogens (Geering et al. 2013, Sharma et al. 2022). These effector peptides also affect surrounding host tissue, potentially leading to extensive tissue destruction (Chollet-Martin et al. 1996, Kobayashi et al. 2003). All three granulocyte types have been described in pigs, but still detailed information is limited (Blanc et al. 2020, Uston et al. 2007).

Recently, a new way of differentiating between circulating neutrophils and eosinophils was described in the pig using flow cytometry (FCM), possibly enabling more research in this topic (Blanc et al. 2020).

Mast cells home many effector compounds and are involved in different physiological mechanisms like angiogenesis or bone remodelling. Upon activation through other immune cells or pathogens, mast cells release their granules. Mast cells are commonly known for their role in allergic reactions (Krystal-Whittemore et al. 2015, Mair et al. 2014).

NK cells are mostly known for destroying cells in distress like cancer cells and cells infected by viruses. NK cells recognize healthy cells via the MHC-I complex which represents an inhibitory signal for the NK cell, therefore the cell is not destroyed. Infected or oncogenic cells try to escape immune recognition by downregulating their MHC-I expression, but the activity of for example cancer suppressor genes lead to an increased expression of NK-cell activating proteins on the cell surface. Therefore NK cells can recognize and destroy cells, even if no MHC-I is being expressed, which makes these cells very unique (Paul and Lal 2017). Recently, porcine NK cells were investigated in great detail, demonstrating that these cells can also induce T-cell proliferation (Pelsmaeker et al. 2018). Furthermore, different functional subsets have been described, according to their cluster of differentiation (CD) 335 expression (Mair et al. 2012).

$\gamma\delta$ -T cells have different functions that are associated with the innate but also with the adaptive immune system. $\gamma\delta$ -T cells can for example produce large amounts of cytokines and express molecules that are typically associated with antigen-presentation (Takamatsu et al. 2006). During viral or bacterial infections, some $\gamma\delta$ -T cells also demonstrate cytolytic activity (Kim et al. 2021, Olin et al. 2005). More details on porcine $\gamma\delta$ -T cells are given below.

The adaptive immune system gets activated by antigen-presenting cells (APCs). Professional APCs are considered to be DCs, macrophages and B cells (Eiz-Vesper and Schmetzer 2020). Even though B cells are part of the adaptive immune system, in recent years, it has been shown, that B cells partake in pathogen uptake and antigen presentation to T cells (Hong et al. 2018). All APCs present a peptide-MHC (pMHC) complex on the cell surface after pathogen uptake and processing, leading to recognition by T cells via the corresponding T-cell receptor (TCR) or by B cells via the B-cell receptor (BCR) (Capitani and Baldari 2022, Yuseff et al. 2013). APCs present antigens to cytolytic T lymphocytes (CTL) via the MHC-I molecule, whereas T-helper cells (Th) get activated via peptide presentation by the MHC-II molecule.

Therefore, depending on the antigen, the MHC class can have a significant impact on the type of immune response (Rock et al. 2016).

CTLs target cells that have been infected by intracellular pathogens like viruses. All cells provide information about their internal protein synthesis via the MHC-I molecule on their surface. If foreign pathogens start synthesizing their own proteins in the host cell, some of these non-autologous proteins get represented on the MHC-I, leading to destruction of the infected cell by an activated effector CTL (Alberts et al. 2017, Rock et al. 2016).

Th cells have many different subsets including T_H1 , T_H2 , T_H9 , T_H17 , follicular Th cells (T_{FH}), and regulatory T cells (T_{reg}). These cells have varying functions but generally recognise peptide-MHC-II complexes. After the activation of Th cells, these cells influence many other cells from the innate and adaptive immune system. A well-known function is the thymus-dependent activation of B cells, leading to production of antibodies. Whereas most Th cells lead to increased immune system activity, T_{reg} cells suppress immune system activity, to prevent excessive immune reactions (Alberts et al. 2017).

B cells represent the other significant group of the cellular adaptive immune system. They express the BCR on their surface, which is unique for each B cell, binding a different antigen (Alberts et al. 2017). Depending on the antigen, B cells can be activated without the help of T_{FH} cells or can be activated thymus-dependently, where the B cell presents the detected antigen on its MHC-II molecule, leading to recognition by T_{FH} cells (Allman et al. 2019). Through bi-directional signalling, the T cell activates the B cell, which starts the differentiation process of B cells to effector plasma cells. Plasma cells produce antibodies, which resemble the humoral part of the adaptive immune system. Antibodies lead to the highly specific neutralization of the pathogen (Alberts et al. 2017, Yuseff et al. 2013).

Besides of the effector subsets, T and B cells also differentiate into memory cells providing long-lasting protection against an antigen. Furthermore, during reinfection with the same antigen, these memory cells generally have a lower threshold of activation, leading to a quick and strong secondary response (Ratajczak et al. 2018).

In the following chapters, we discuss porcine T-cell subsets relevant for this thesis and immune cell communication in more detail.

1.3 T Cells

T cells are bone-marrow derived lymphocytes belonging to the adaptive immune system. The early development of T cells occurs when lymphoid progenitor cells originating from the bone marrow enter the thymus (Sinkora and Butler 2009). In the thymus, T cells undergo negative and positive selection, to prevent excessive immune response against self-antigens and to prevent T cells having a non-reactive TCR (Kurd and Robey 2016).

In the pig, two subsets of T cells can be observed: T cells carrying a TCR comprised of an alpha and beta chain (TCR- $\alpha\beta$) and T cells carrying a TCR consisting of a gamma and delta chain (TCR- $\gamma\delta$) (Gerner et al. 2015). Unlike humans or mice, in which predominantly TCR- $\alpha\beta$ cells are found in peripheral blood samples, pig peripheral blood samples contain high amounts of TCR- $\gamma\delta$ T cells (Bertho and Meurens 2021, Gerner et al. 2022).

All T cells can be distinguished from other immune cells by their CD3 expression. The TCR is dependent on the signalling molecule CD3 to transfer an activation signal from the TCR and start an intracellular signalling cascade. This cascade can for example lead to the activation of the NF- κ B pathway, causing T-cell activation and expression of genes involved in immune response (Deng et al. 2022, Liu et al. 2017).

Furthermore, T cells can be differentiated by their expression of coreceptors like CD4 and CD8. CD4 and CD8 are molecules comparable in their function, stabilizing the TCR-pMHC bonding where CD4 binds specifically to MHC-II molecules, whereas CD8 binds to the MHC-I (Alberts et al. 2017). In the pig, CD8 can be found on different T-cell subsets, either as a CD8 $\alpha\beta$ heterodimer or as a CD8 $\alpha\alpha$ homodimer (Gerner et al. 2022).

1.3.1 Naive and memory Th cells

When looking at TCR- $\alpha\beta$ Th cells, we can observe different subtypes depending on the CD4 and CD8 expression. In the pig, CD4⁻CD8 α ⁻, CD4⁻CD8 α ⁺, CD4⁺CD8 α ⁻ and CD4⁺CD8 α ⁺ cells have been described (Gerner et al. 2015). After antigen contact, CD4⁺CD8 α ⁻ cells increase the expression of the homodimer CD8 $\alpha\alpha$ and during rechallenging with the same antigen, these CD4⁺CD8 α ⁺ cells are reactivated and proliferate. These observations led to the conclusion that CD4⁺CD8 α ⁺ cells represent memory Th cells in swine and CD4⁺CD8 α ⁻ cells represent naïve Th cells. Besides this characteristic being important for memory T cell detection, the purpose of the CD8 $\alpha\alpha$ molecule on this cell type remains a subject for further study (Gerner et al. 2022,

Summerfield et al. 1996). It is possible to distinguish Th memory cells further between central memory T cells (T_{CM}) and effector memory T cells (T_{EM}) depending on their C-C motif chemokine receptor 7 (CCR7) or CD27 expression (Reutner et al. 2013).

1.3.2 Cytolytic T cells

CTLs have a TCR- $\alpha\beta$ and express CD8 $\alpha\beta$ on their surface (Lagumdzic et al. 2022). Therefore, CTLs are dependent on MHC-I binding for T-cell activation, detecting their distinct pMHC. After activation through an APC, CTLs scan MHC-I molecules on cells, to identify intracellular pathogens or cancer cells. During scanning and CTL killing, the effector CTL has to form cell contact with its target cell, to achieve TCR-pMHC binding and cell killing (Halle et al. 2017). If a CTL detects an altered/infected cell, its effector granules containing perforin and granzyme are polarized to the site of cell-cell contact. Granules can then be released at the site of cell-cell contact, leading to effector proteins destroying the target cell. Besides of that, CTLs produce cytokines, affecting further immune response (Alberts et al. 2017, Halle et al. 2017).

In the pig, naïve CTLs have been described as perforin⁻ and CD11a^{dim}. Effector CTLs on the other hand may be represented by a CD11a^{high} and perforin⁺ phenotype (Martini et al. 2022). A more precise classification for effector CTLs was recently demonstrated, dividing this group in intermediate differentiated CTLs and terminally differentiated CTLs, depending on their level of CD11a and CD27 expression (Lagumdzic et al. 2022). Two porcine memory CD8 T-cell subtypes have also been described: T_{CM} and T_{EM} , important for long-term protection against viral threats (Gerner et al. 2022, Martini et al. 2022).

1.3.3 TCR- $\gamma\delta$ T cells

As previously mentioned, TCR- $\gamma\delta$ T cells have many different functions, some resembling properties of the innate immune system, some having properties of the adaptive immune system (Mair et al. 2014). Generally, TCR- $\gamma\delta$ T cells can detect foreign and self-antigens via the TCR, or receptors that are typically present on NK cells, and through PRRs. The main difference to TCR- $\alpha\beta$ T cells lies in the fact that TCR- $\gamma\delta$ T cells can detect antigens in an MHC-independent manner and can therefore react not only to peptide antigens but also to non-peptide antigens. Put simply, the TCR on this cell type behaves more like an antibody, detecting many different molecule types (Vantourout and Hayday 2013, Willcox et al. 2020).

Porcine TCR- $\gamma\delta$ T cells can be divided in different subsets according to their CD2 and CD8 α expression. TCR- $\gamma\delta$ T cells that express neither of these molecules are described to resemble more naïve cells, whereas CD2⁺CD8 α ⁻ cells follow behaviour of memory-like cells. CD2⁺CD8 α ⁺ cells seem to resemble a more mature form of TCR- $\gamma\delta$ T cells (Sedlak et al. 2014a, Stepanova and Sinkora 2013). After activation, TCR- $\gamma\delta$ T cells produce large amounts of cytokines, interacting with different immune cells (Sedlak et al. 2014b, Willcox et al. 2020). Furthermore, they have cytolytic abilities, which are important in cancer cell destruction and viral diseases (Kabelitz et al. 2020).

1.4 The immunological synapse

During immune system activation, APCs get in contact with T and B cells. This contact between APCs and the adaptive immune system is called the immunological synapse (IS) or the immunological kinapse (IK), depending on the duration of the cell-cell contact (Basu and Huse 2017, Moreau et al. 2015). If the TCR only shows low affinity to the antigen presented on an APC, the T cell does not come to a complete stop and therefore only stays in contact with the APC for several minutes. This short interaction is an IK (Moreau et al. 2015). It has been shown that dendritic cells interact with up to 5000 different T cells per hour. During all these contacts, a T cell can have multiple brief interactions (IK) until there is a high affinity bond between a TCR and a pMHC: an IS is formed (Moreau et al. 2015, Obst 2015).

Nevertheless, it has to be mentioned, that adhesion molecules are essential to achieve this high affinity bond by pulling cells closer together for prolonged contact between the TCR and the pMHC (Dustin 2014). It has been shown, that T cells lacking the important adhesion molecule lymphocyte function-associated antigen 1 (LFA-1) have to be exposed to antigen amounts 100 times as high as normal to achieve activation (Bachmann et al. 1997).

In the IS, three types of receptors play an important role: antigen recognising receptors (TCR), costimulatory receptors and adhesion molecules (Dustin 2014). The IS can also be categorized in different supra-molecular activation clusters (SMAC). The central SMAC (cSMAC), the peripheral SMAC (pSMAC) and the distal SMAC (dSMAC). Each of these areas have their own protein composition (Basu and Huse 2017, Monks et al. 1998). The cSMAC consists primarily of the TCR and other costimulatory molecules like CD28 or CD2 (Dustin 2014, Siokis et al. 2018). The pSMAC surrounds the cSMAC and is comprised of costimulatory molecules like CD2 but mainly of adhesion molecules like LFA-1 (Comrie et al. 2015, Dustin 2014). LFA-

1 is formed by CD11 and CD18, which will both be discussed later in more detail. The dSMAC contains inhibiting molecules like CD45 but also additional microclusters of TCRs, which move towards the centre, forming the cSMAC (Capitani and Baldari 2022, Johnson et al. 2000, Ritter et al. 2013). A graphical overview of the immunological synapse is provided in Figure 1 and molecules relevant for this study are described in more detail in chapters 1.4.1-1.4.5.

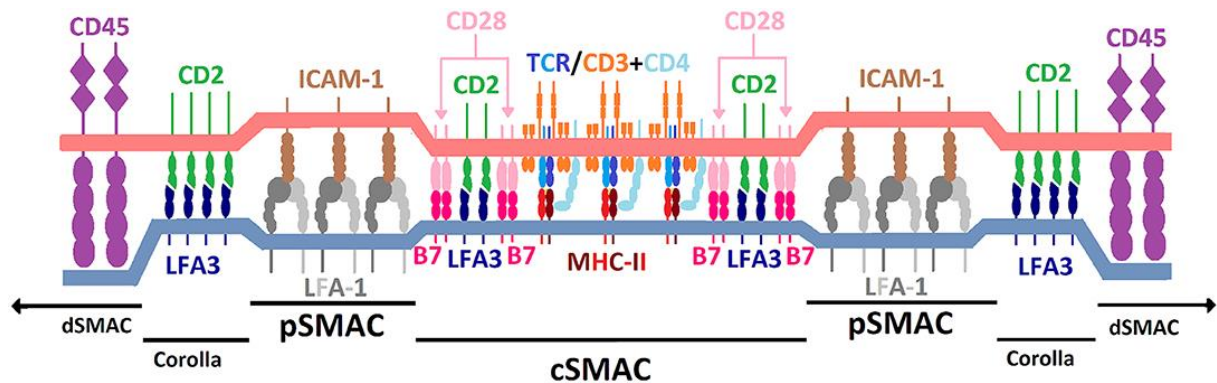


Figure 1: Graphic representation of the immunological synapse

This figure provides a simplified overview over the molecules and structures involved in the immunological synapse, demonstrating the three areas of the IS, the cSMAC, the pSMAC and the dSMAC. Molecules are shown with their corresponding binding partners. The cell membrane of an APC is demonstrated in blue, whereas the T-cell membrane is depicted in red. From: Binder et al. (2020)

1.4.1 CD2

CD2, also called LFA-2, represents an adhesion and costimulatory molecule, present in the pSMAC and cSMAC (Binder et al. 2020). In humans, CD2 can be found primarily on T cells, NK cells and DCs. The CD2 molecule is a glycoprotein of the immunoglobulin family binding to its main ligand LFA-3 (or CD58). LFA-3 is situated on cells of the innate and adaptive immune system: Granulocytes, macrophages, dendritic cells, B cells and T cells (Krensky et al. 1983, Springer et al. 1987). CD2 is assumed to participate in scanning APCs, reducing the distance between APC and T cell, facilitating potential TCR pMHC binding (van der Merwe et al. 1995). When CD2/CD58 binding occurs, intracellular signalling pathways get activated in the T cell, partially matching with TCR and CD28 signalling pathways (Leitner et al. 2015). Furthermore, the intracellular domain of the CD2 molecule can bind to actin filaments, restructuring the cytoskeleton and affecting further IS assembly (Binder et al. 2020). Interestingly, in humans, memory CTL reactivation is primarily based upon CD28/CD80 costimulation. If this pathway is

missing, CD2/CD58 costimulation leads to comparable levels of reactivation, whereas other pathways do not seem effective in memory cell reactivation. This evidence suggests importance of CD2 in memory cell reactivation (Binder et al. 2020, Leitner et al. 2015).

The presence of CD2 on different porcine immune cells, for example B-cell subsets (Sinkora et al. 1998), some TCR- $\gamma\delta$ T cells (Sedlak et al. 2014a), and all TCR- $\alpha\beta$ T cells and NK cells (Piriou-Guzylack and Salmon 2008) suggests importance of this molecule in the pig. This assumption is further supported by the fact that CD2 is generally a very conserved molecule in mammals (Davis and van der Merwe 1996).

1.4.2 CD4

CD4 is an essential monomeric coreceptor present on all Th cells. CD4 binds to MHC-II on APCs on a non-variable part and not in the region where the MHC-II presents the foreign antigen. CD4 is therefore stabilising the cell-cell contact (Alberts et al. 2017). Furthermore, CD4 partakes in T-cell activation, reducing the threshold of activation significantly (Vermeire, Elisa Claeys and Kurt 2019). T cells can normally start intracellular signalling after recognising only one agonistic pMHC. Blocking CD4 leads to a decreased sensitivity of the T cell, requiring recognition of 30 agonistic pMHC simultaneously to start intracellular signalling (Irvine et al. 2002). Porcine CD4⁺ cells were already described above.

1.4.3 LFA-1 (CD11a/CD18)

LFA-1 is a heterodimeric adhesion molecule, comprised of an CD11a and an CD18 molecule (Dustin 2014). It is considered an integrin and can be found on almost all white blood cells, interacting with a variety of immunoglobulin molecules. Intercellular adhesion molecule-1 (ICAM-1) is considered to be the main ligand of LFA-1. Nevertheless, other binding partners include ICAM-2 and ICAM-3 (CD50) (Dustin 2014). On T cells LFA-1 is present in three different affinity states. A low, intermediate, and high affinity state. An inactive T cell exhibits LFA-1 in the low affinity state (Shi and Shao 2023). As previously described, LFA-1/ICAM-1 interaction significantly facilitates TCR-pMHC binding, but this requires LFA-1 to be in a higher affinity state. This higher affinity state is achieved through chemokines in the environment of the T cell, or through selectin binding to the T cell, enabling to participate in APC scanning (Shi and Shao 2023, Shimaoka et al. 2006). If the T cell then detects the fitting antigen on the APC,

the LFA-1/ICAM-1 binding is further enhanced through inside-out signalling, forming a stable IS (Dustin 2014). LFA-1 not only brings T cells and APCs closer together, LFA-1 is also involved in actin remodelling and keeping inhibitory molecules out of the IS (Shi and Shao 2023).

In 2005, the sequence of porcine CD11a was compared to the sequence of other animals including humans. Minor changes were visible, but the main characteristics of CD11a were preserved (Vanden Bergh et al. 2005). A similar preservation was observed in CD18 (Lee et al. 1996). This suggests, that porcine LFA-1 should behave rather similar to human LFA-1 (Vanden Bergh et al. 2005).

1.4.4 CD50

In humans, CD50 (ICAM-3) can be found on almost all leukocytes. As already mentioned, ICAM-3 is one of the ligands of LFA-1. This bond shows only low affinity though. The main binding partners of CD50 are considered to be the dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) and the $\alpha_D\beta_2$ receptor (Guerra-Espinosa et al. 2024, Svajger et al. 2010). ICAM-3, a molecule belonging to the immunoglobulin superfamily, can therefore play an important role in the initial contact between DC and T cell, due to the fact that ICAM-1 is hardly expressed on human naïve T cells. ICAM-3 on T cells bind to LFA-1 or DC-SIGN on DCs, leading to LFA-1 activation in the T cell (shifts LFA-1 to a higher affinity state), enabling the T cell to reinforce cell-cell contact (Guerra-Espinosa et al. 2024).

The presence of porcine DC-SIGN has been shown in lymph node tissue on the surface of dendritic cells and macrophages, comparable to human tissue. Knowledge about its interaction with porcine ICAM-3 and about porcine ICAM-3 in general is very limited, but it has been shown, that porcine DC-SIGN cross-reacts with human ICAM-3, suggesting a similar role with porcine ICAM-3 (Huang et al. 2009).

1.3.5 MHC-II

The major histocompatibility complex presents antigens on the surface of APCs. After antigen phagocytosis, APCs degrade proteins in the endo-lysosome, producing small peptide fragments. MHC-II containing vesicles are produced in the endoplasmic reticulum and are transported to the late endosome. There, these peptide fragments can bind to the MHC-II, assuming that there is a strong attraction between these two molecules (Rocha and Neefjes 2008). Then, MHC-II molecules get transported to the cell membrane via vesicles or tubules

(Rocha and Neefjes 2008, Rock et al. 2016). TCR $\alpha\beta$ cells can only recognize foreign peptides, presented by the MHC, further demonstrating the importance of this molecule in immune response. This limitation is known as MHC-restriction (Rock et al. 2016). Besides the effect MHC-II binding has on T cells, APCs are also affected by the binding of their MHC-II molecule, causing for example maturation of DCs and chemokine secretion (Al-Daccak et al. 2004).

1.5 Aim of this study

During this study, we first wanted to characterize the components of the porcine immunological synapse in more detail and investigate the distribution of certain molecules on different cell types, with the focus on APCs and CD4⁺ T cells. We selected the markers CD2, CD4, CD11a, CD18, CD50 and MHC-II for this thesis. Another goal was to establish a functional *in-vitro* assay to study a potential blockage of the immunological synapse. For this purpose, peripheral blood mononuclear cells from animals immunised with PCV2 or OVA were used and mAbs directed against above mentioned molecules were added during an antigen-specific re-stimulation. Therefore, it was possible to investigate the effects of the used antibodies on CD4⁺ memory cell proliferation and other T-cell subsets.

Overall, these results will provide a more detailed species-specific understanding of the porcine immunological synapse and how CD2, CD4, CD11a, CD18, CD50 and MHC-II molecules affect T-cell reactivation.

2 Materials and methods

2.1 Material

2.1.1 Reagents

Table 1: Reagents used in this study

Product	Manufacturer
PBS (w/o Calcium, w/o Magnesium)	PAN-Biotech (Aidenbach, Germany)
Freezing medium: • 50 % RPMI 1640 • 40 % Fetal bovine serum (FBS) • 10 % Dimethyl sulfoxide (DMSO)	PAN-Biotech Sigma-Aldrich (St. Louis, USA) Sigma-Aldrich
Cell culture medium: • RPMI 1640 • 10 % FBS • 100 IU/ml penicillin • 0.1 mg/ml streptomycin	PAN-Biotech Sigma-Aldrich PAN-Biotech PAN-Biotech
Flow cytometry (FCM) buffer 1: • PBS • 10 % porcine plasma	PAN-Biotech In-house
Flow cytometry (FCM) Buffer 2: • PBS • 3 % FBS	PAN-Biotech Sigma-Aldrich

2.1.2 Antibodies used for integrin phenotyping by FCM

Table 2: Primary antibodies used for phenotype characterisation

Antibody target	Conjugate	Clone name	Isotype	Manufacturer
CD2	Brilliant Violet 605	RPA2.10	IgG1	BioLegend (San Diego, USA)
CD4	PerCP/Cyanine 5.5	74-12-4	IgG2b	BD Biosciences (Franklin Lakes, USA)
CD8 α	PE	76-2-11	IgG2a	BD Biosciences
CD11a	Alexa Fluor 488	3H2 1B12	IgG1	In-house
CD18	None	1C6 3H3	IgG2a	In-house
CD50	None	12F3 2F1	IgG2b	In-house
CD172a	Biotin	74-22-15	IgG1	In-house
MHC-II	None	MSA3	IgG2a	In-house

Table 3: Secondary antibodies used for phenotype characterisation

Antibody target	Conjugate	Target species	Host species	Manufacturer
IgG2a	Brilliant Violet 421	Mouse	Goat, polyclonal	Jackson ImmunoResearch (West Grove, USA)
IgG2b	Alexa Fluor 647	Mouse	Goat, polyclonal	Thermo Fisher Scientific (Waltham, USA)
Target	Conjugate	Binding agent		Manufacturer
Biotin	PE-Cyanine 7	Streptavidin		Thermo Fisher Scientific

Table 4: Blocking antibody used for phenotype characterisation

Isotype	Conjugate	Host species	Manufacturer
IgG, whole molecule	None	Mouse	Jackson ImmunoResearch

Table 5: Viability dye used for phenotype characterisation

Viability dye	Manufacturer
Fixable Viability Dye eFluor™ 780	Thermo Fisher Scientific

2.1.3 Antibodies used for proliferation assay and subsequent FCM staining**Table 6: Antibodies used in synapse blocking reaction**

Antibody target	Conjugate	Clone name	Isotype	Manufacturer
CD2	None	MSA4	IgG1	BioLegend
CD4	None	74-12-4	IgG2b	BD Biosciences
CD11a	None	3H2 1B12	IgG1	In-house
CD18	None	1C6 3H3	IgG2a	In-house
CD50	None	12F3 2F1	IgG2b	In-house
MHC-IIy	None	MSA3	IgG2a	In-house

Table 7: Isotype controls used in synapse blocking reaction

Isotype	Conjugate	Clone name	Manufacturer
IgG1	None	P3.6.2.8.1	Thermo Fisher Scientific
IgG2a	None	eBM2a	Thermo Fisher Scientific
IgG2b	None	eBMG2b	Thermo Fisher Scientific

Table 8: Primary antibodies used for proliferation FCM staining

Antibody target	Conjugate	Clone name	Isotype	Manufacturer
CD3	PerCP/Cyanine5.5	BB23-8E6-8C8	IgG2a	BD Biosciences
CD8 α	PE	76-2-11	IgG2a	BD Biosciences (Franklin Lakes, USA)
CD8 β	Biotin	PPT23	IgG1	In-house
TCR $\gamma\delta$	Alexa Fluor 647	PPT16	IgG2b	In-house

Table 9: Secondary antibodies used for proliferation FCM staining

Target	Conjugate	Binding agent	Manufacturer
Biotin	PE-Cyanine 7	Streptavidin	Thermo Fisher Scientific

Table 10: Viability dye used for proliferation FCM staining

Viability dye	Manufacturer
Fixable Viability Dye eFluor™ 780	Thermo Fisher Scientific

Table 11: Negative control used for PCV2 blocking experiments

Following negative control was used for PCV2 samples in accordance with literature (Pernold et al. 2022)

Substance
Baculovirus envelope fusion protein GP64

2.1.4 Scientific equipment

Table 12: Devices and software used during this study

Device name	Manufacturer
Heraeus Multifuge 1 S-R centrifuge	Thermo Fisher Scientific
Heraeus Megafuge 40R centrifuge	Thermo Fisher Scientific
Sysmex XP-300 haematology analyser	Sysmex (Kobe, Japan)
Heracell 150i CO ₂ incubator	Thermo Fisher Scientific
CytoFLEX LX flow cytometer	Beckman Coulter (Brea, USA)
FlowJo v10.10 software	BD Biosciences
GraphPad Prism v10.2.2 software	Dotmatics (Boston, USA)

2.1.5 Samples

2.1.5.1 OVA samples

The ovalbumin samples used during this experiment were gathered during a previous study in which healthy pigs were immunized using an ovalbumin formulation as antigen. Four pigs (six weeks of age) received an initial immunization of 100 µg ovalbumin formulation with complete Freund's adjuvant (study day 0). On study days 21 and 56 booster immunizations were performed using an ovalbumin formulation with a dosage of 100 µg ovalbumin with incomplete Freund's adjuvant. Vaccinations were given intramuscularly. Samples of this study were derived from blood of study day 78. Animals were anaesthetized by intramuscular injection of Ketaminhydrochlorid (Narketan®, V toquinol, Vienna, Austria, 10 mg/ kg body weight) and Azaperon (Stresnil®, Janssen Pharmaceutica, Beerse, Belgium, 1.3 mg/ kg body weight). Subsequently, animals were euthanized via intracardial injection of T61® (MSD Animal Health, Vienna, Austria, 1.0 ml/ 10 kg body weight). This study was approved by the institutional ethics committee and BMWF-68.205/0082-II/3b/2012.

2.1.5.2 PCV2 samples

Furthermore, samples from four pigs from a conventional Austrian pig farm were used, that received one vaccination against the porcine circovirus type 2 (PCV2, Ingelvac CircoFLEX®, Boehringer Ingelheim Vetmedica GmbH) at the farm. Animals were eight weeks of age and vaccinated at three weeks of age. Euthanasia of animals was performed as described above.

2.1.5.3 Further samples

For the integrin staining and preliminary experiments, samples from a slaughterhouse in Lower Austria were used. Animals were subjected to electric high-voltage anaesthesia followed by exsanguination in accordance with the Austrian Animal Welfare Slaughter Regulation.

During sample collections, whole blood was retrieved, and heparin was added to prevent coagulation. Peripheral blood mononuclear cells (PBMC) were extracted from heparinized blood, using density gradient centrifugation and PBMCs were stored at -150 °C in freezing medium (Table 1). Cells used in this study were already isolated and frozen.

2.2 Methods

2.2.1 Thawing of samples

Prior to assays, PBMCs needed to be thawed. Cryovials were warmed up in a water bath at 37 °C immediately after being removed from the -150 °C freezer. Once the samples were thawed, they were transferred to a 15 ml tube containing 10 ml pre-warmed FCM buffer consisting of PBS with 10 % porcine plasma (FCM buffer 1). The tube was then centrifuged (8 min, 470 g, 8 °C) and the supernatant was discarded. Two ml of PBS were added to resuspend the cell pellet. If large cell aggregates were detected, the cell suspension was filtered using a Partec CellTrics filter with a 50 µm filter size (Sysmex) and transferred to a new tube. For further calculations the cell number was assessed using the automated haematology analyser Sysmex XP-300 and 100 µl of cell suspension. For cell Violet™ staining and subsequent cell culture application, slight adaptations to the previously described thawing protocol had to be made. Two cryovials were thawed, therefore a 50 ml tube was prepared. As buffer, 20 ml PBS were used. Subsequent centrifugation was performed at room temperature (8 min, 470 g).

2.2.2 FCM staining

After thawing PBMCs (2.1.5.3), cells were transferred to a 96-well round-bottom microtiter plate (MTP) at a concentration of 4×10^6 cells per well. Next, the MTP was centrifuged (4 min, 470 g, 8 °C), which resulted in a cell-pellet formation at the bottom of the plate. The supernatant was discarded. Then the first staining step was conducted by applying the corresponding antibodies, followed by pulse-shaking the MTP and 20 min of incubation in the fridge at 4 °C to ensure adequate antibody binding to their epitopes. After the incubation a washing procedure was performed by adding 200 µl FCM buffer to the cells (FCM buffer 1 for integrin phenotyping, FCM buffer 2 for cultured cells), then the MTP was centrifuged (4 min, 470 g, 8 °C), the supernatant discarded, and the MTP pulse shaken. This washing procedure was repeated twice after each staining step, ensuring removal of excess and unbound antibodies. After the washing procedure, the next staining step was conducted. This procedure was repeated several times, depending on the desired staining panel as indicated below (Table 13) and in chapter 2.2.3 (Table 14). After this, cells were resuspended in 200 µl FCM buffer, transferred to tubes and samples were measured on a CytoFLEX LX flow cytometer. This

staining process was also conducted on violet stained PBMCs (OVA or PCV2) after four days of cell culture as described in 2.2.3.

Table 13: Staining panel for integrin staining

Sample ID	Staining step 1	Staining step 2	Staining step 3	Staining step 4
1	Unstained	Unstained	Unstained	Unstained
2	CD50 CD172a MHC-II	Anti-IgG2a Anti-IgG2b Streptavidin	Whole IgG	CD2 CD4 CD8 α CD11a VDeF780
3	CD18 CD50 CD172a	Anti-IgG2a Anti-IgG2b Streptavidin	Whole IgG	CD2 CD4 CD8 α CD11a VD eF780

Detailed information on these antibodies can be found in Tables 2-5.

2.2.3 Violet staining and proliferation assay

After thawing, the used OVA and PCV2 PBMCs had to be diluted with PBS to achieve a cell number of approximately 2×10^7 cells per ml. For the proliferation assay the Invitrogen CellTrace™ Violet Proliferation Kit was used (Thermo Fisher Scientific, USA). According to the instructions, the lyophilized dye was dissolved in 20 μ l DMSO and aliquoted in tubes with 5 μ l each. When used, 5 μ l of the dye stock solution were diluted with PBS to achieve a 5 μ M working solution. Equal amount of the working solution was added to the cell suspension and the tube was immediately vortexed. The tube was then incubated at 37 °C for 10 min, and vortexing was repeated every three minutes. Twice as much FBS as the initial cell solution was added, the suspension was vortexed again and incubated at room temperature for 15 minutes in the dark, interrupted by vortexing several times. After this, approximately 30 ml cell culture medium were added, the tube was centrifuged (8 min, 470 g, room temperature) and the supernatant discarded. This washing process was repeated two additional times. For cell count measurement (as described above), the cell pellet was resuspended in 2 ml cell culture medium and, if necessary, filtered using a Partec CellTrics filter with a 50 μ m filter size.

Violet stained cells were diluted with cell culture medium to achieve a cell concentration of 3×10^5 cells per 100 μl . Of this cell suspension, 100 μl per well were transferred into a 96-well round-bottom MTP. To the control group without stimulation, 90 μl RPMI medium with 10 μl PBS was added (to compensate for the fact that OVA/PCV2 was resolved in PBS). All other groups received 100 μl stimulus (either OVA or PCV2 virus like particles (VLPs)) to achieve a final OVA concentration of 100 $\mu\text{g/ml}$ (Sigma Aldrich) and a final PCV2 concentration of 4 $\mu\text{g/ml}$ per well (Pernold et al. 2022). As an additional negative control in PCV2 samples, the baculovirus protein GP64 was used, also at a final concentration of 4 $\mu\text{g/ml}$ per well. Furthermore, six groups received mAbs directed against either CD2, CD4, CD11a, CD18, CD50, or MHC-II. Another three groups were used as isotype controls, consisting of dialysed isotype-matched control antibodies (Thermo Fisher Scientific, USA). All antibodies were added to achieve a saturating final concentration of 2 μg per well. Cells were incubated for four days at a temperature of 37 °C and 5 % CO_2 in a Heracell 150i CO_2 incubator.

After this time period, cells were harvested, samples from the same group were pooled, washed and transferred to a new MTP. Cells were then stained for certain surface markers, as previously described. Samples were finally measured using the CytoFLEX LX.

Table 14: Staining panel after the proliferation assay

Sample ID	Staining step 1	Staining step 2
1	Unstained	Unstained
2	CD3 CD8 α CD8 β TCR $\gamma\delta$	Streptavidin VD eF780

Detailed information on these antibodies can be found in Tables 8-10.

2.2.4 Dialysis

Three controls corresponding to the isotypes of the mAbs used for the blocking assay were selected: IgG1, IgG2a, IgG2b (Thermo Fisher Scientific), with no specificity toward a target antigen. As the products contained 0.09 % sodium azide (NaN_3), antibodies were dialysed, to avoid cell death caused by NaN_3 . For dialysis, Slide-A-Lyzer™ MINI Dialysis Devices, 10K MWCO (Thermo Fisher Scientific) with a pore size of 10kDa were used according to manufacturer's protocol with PBS as dialysis buffer.

2.2.5 Data analysis

Experimental data received from flow cytometry was analysed using the FlowJo software. All samples were first gated for lymphocytes and monocytes by plotting the sideward scatter-area (SSC-A) against the forward scatter-area (FSC-A). Second, doublets were excluded, by plotting the FSC-height (FSC-H) against the FSC-A. Doublets are formed, when cells aggregate, whereby single-cell solution is not ensured, thus disturbing accurate measurement. Finally, dead cells were removed from further analysis by plotting the SSC-A against the viability dye and gating for viability dye negative cells (Figure 2).

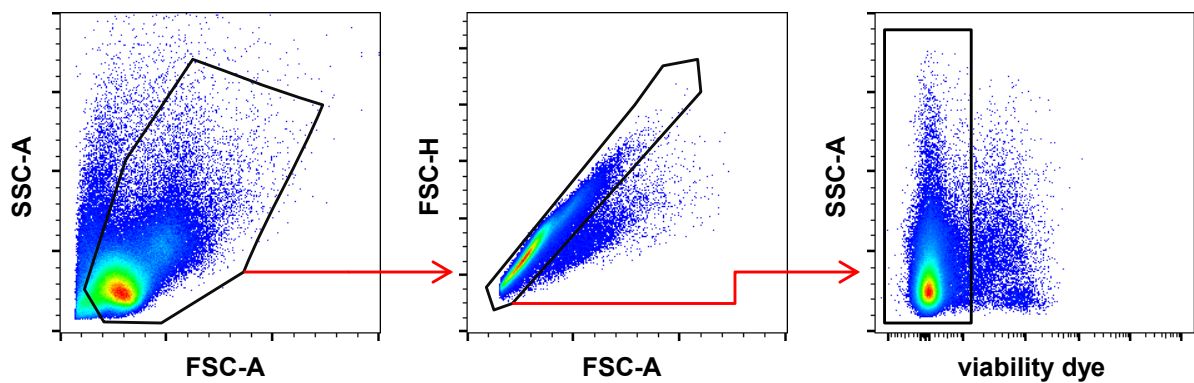


Figure 2: Gating strategy

Porcine PBMCs were analysed according to their SSC-A and FSC-A properties. Then, doublets that could interfere with further analysis were removed by plotting the FSC-H against the FSC-A. Finally, dead cells were excluded by gating on viability dye negative cells.

After further analysis were conducted, data was exported into GraphPad Prism for graphical representation. Due to overall low sample numbers, no statistical analysis has been performed. Further experiments will be conducted, to allow for statistical analysis.

3 Results

3.1 Integrin phenotyping

First, we addressed the question how the different components of the IS, namely CD2, CD11a, CD18, CD50, and MHC-II are distributed among CD4⁺ T cells, and myeloid cells. Samples of five different animals were used to investigate this distribution and the integrin staining was performed according to Table 13 with the use of monoclonal antibodies (mAbs) (Table 2-Table 5).

Cells were differentiated between myeloid cells by a CD172a⁺ phenotype and Th by a CD4⁺ phenotype. CD4⁺ T cells were further distinguished between naïve and memory cells by CD8 α expression (Figure 3A). All three cell populations were then investigated for their CD2, CD11a, CD18, CD50, and MHC-II expression. Frequencies of cells which were positive for the corresponding surface markers are represented by black gates in the scatter diagrams in Figure 3B-D. Finally, the data of all five animals is represented as bar charts (Figure 3E) for all three investigated cell populations. Each animal is represented by a unique symbol, allowing tracking across multiple diagrams. To compare different groups, median values were used. Comparing the three bar charts (Figure 3E), some differences in the expression of the investigated molecules were apparent. The relative number of CD2 expressing cells in naïve Th cells (91.5 %) matched the expression in memory Th cells (91.0 %). Myeloid cells on the other hand, showed a CD2 expression on only 2.9 % of cells. An obvious difference was therefore observed between Th cells and myeloid cells regarding CD2 expression. The amount of CD11a⁺ and CD18⁺ cells did not differ much between the three cell types. 90.7 % of naïve Th cells were CD11a⁺ and 87.8 % were CD18⁺, 97.2 % of Th memory cells showed a positive phenotype for CD11a and 91.2 % for CD18. Regarding myeloid cells, 97.2 % were CD11a⁺ and 96 % were CD18⁺. More pronounced differences were observed for CD50 and MHC-II. Naïve Th cells showed the lowest frequencies of CD50⁺ cells (9.0 %), memory Th cells expressed CD50 on 14.5% of cells and myeloid cells showed the highest number of CD50⁺ cells with 58.8 %. Naïve Th cells expressed MHC-II on 3.3 % of cells, differing from Th memory cells (46.5 %), and myeloid cells (54.5 %). On myeloid cells, the group of MHC-II⁺ cells represent APCs.

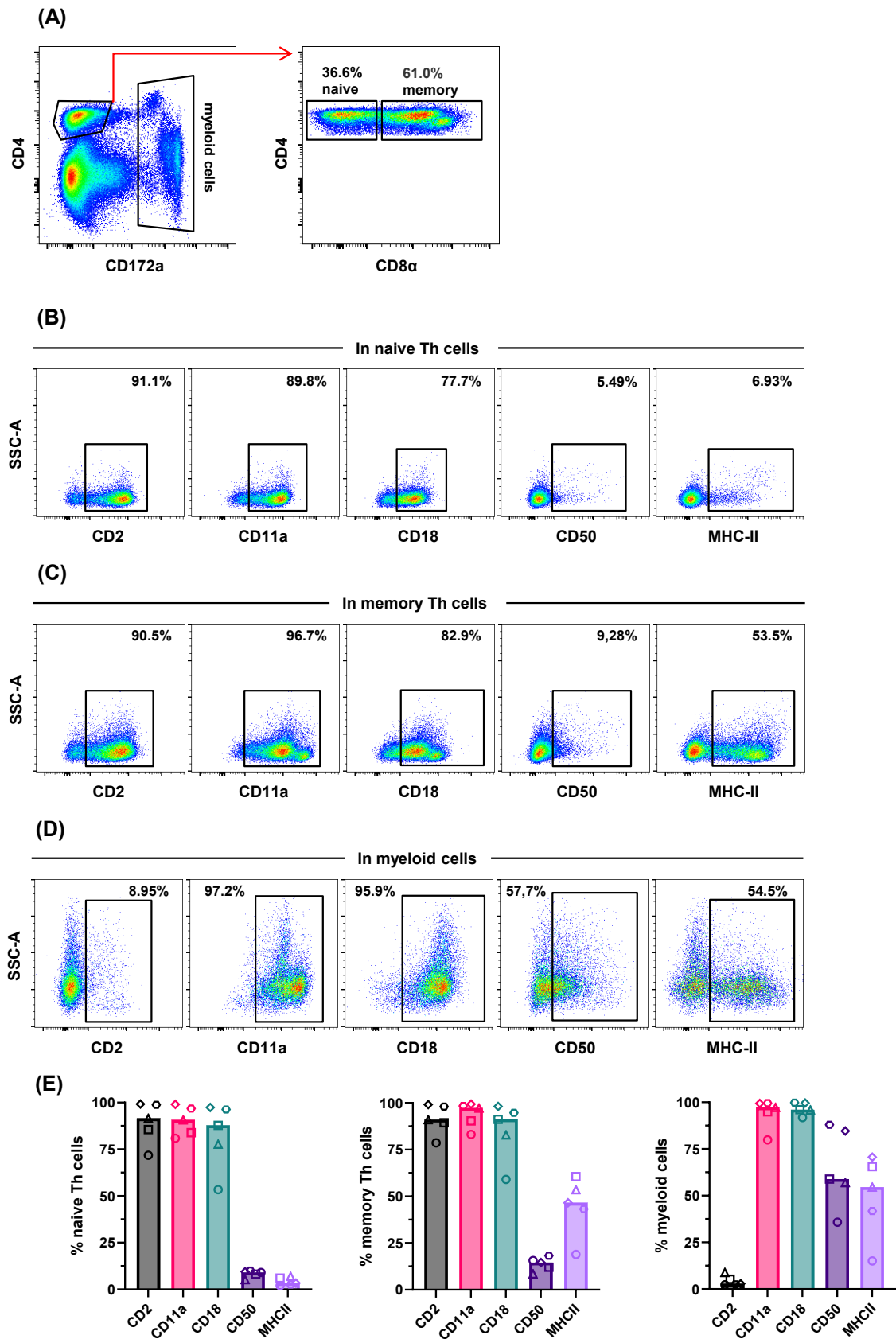


Figure 3: Integrin phenotyping

The distribution of CD2, CD11a, CD18, CD50 and MHC-II was investigated on naïve Th cells, memory Th cells and myeloid cells. Samples of five animals were used. CD4 was plotted against CD172a to distinguish myeloid cells and Th cells. Th cells were then further differentiated, using the CD8 α marker, into naïve Th cells (CD8 α ⁻) and memory Th cells (CD8 α ⁺) (**A**). The following graphs show the relative number of cells that express the corresponding molecule on their cell surface on naïve Th cells (**B**), memory Th cells (**C**) and myeloid cells (**D**). Frequencies are indicated in the corresponding gates. For demonstration purposes, data of one representative animal is shown. The three bar charts at the bottom summarize results from all five animals for the three cell populations. Bars show the median of all five animals. Each animal is represented by an individual symbol (**E**).

3.2 Proliferation assay

After investigating the distribution of the different molecules, we started the antigen-specific *in-vitro* re-stimulation assays using OVA and PCV2 responding cells. To make sure that cells respond specifically to the added stimulus, we compared the proliferation of T cells that did not receive any stimulus (medium/control group) with T cells that received their corresponding stimulus (either OVA or PCV2 VLPs). Cells were gated for CD3⁺ cells. T cells were then analysed for Violet proliferation dye and the relative number of proliferating T cells was represented as Violet^{dim} cells (Figure 4A, C). We also analysed the Th memory cell population (CD4⁺CD8 α ⁺), to identify stimulus-specific memory-cell reactivation. For this, samples were gated on CD3⁺ cells and the CD4⁺ cell population was identified indirectly by selecting CD8 β ⁻ and TCR γ δ ⁻ cells (Figure 4B, D). This approach was selected, as anti-CD4 mAbs that were also used for blocking may interfere with the Th staining procedure. Memory Th cells were further selected for CD8 α ⁺ proliferating cells to address memory reactivation. The data of a representative OVA animal simulated with OVA is depicted in Figure 4A and B. The data of a representative PCV2 animal stimulated with PCV2 VLPs is visible in Figure 4C and D. The scatter plots illustrate the results of all animals, where each animal is represented by an individual symbol to allow tracking across the graphs. The median values of all animals are depicted by a red line.

The OVA data (Figure 4A, B) show a substantial difference in the percentage of proliferating CD3⁺ cells between the unstimulated medium group (5.8 %) and the group stimulated with OVA (19.5 %). A relatively similar effect was seen when focussing on Th memory cells. The unstimulated group showed a proliferation of 0.4 %, whereas 2.1 % of cells proliferated in the OVA stimulated group.

PCV2 T cells displayed a low proliferation rate in the unstimulated groups medium (1.4 %) and PCV2 control (1.9 %). Proliferation rate was higher in cells, stimulated with PCV2 VLPs at 5.9 %. Regarding Th memory cells, medium and control group represented relatively similar proliferation values at 0.4 %, whereas the PCV2 stimulated group showed 2.4 % proliferating cells.

Overall, it was possible to demonstrate antigen-specific re-stimulation of the OVA and PCV2 groups, illustrated by the increased T-cell proliferation and Th memory cell proliferation after adding the corresponding stimulus, compared to the unstimulated groups.

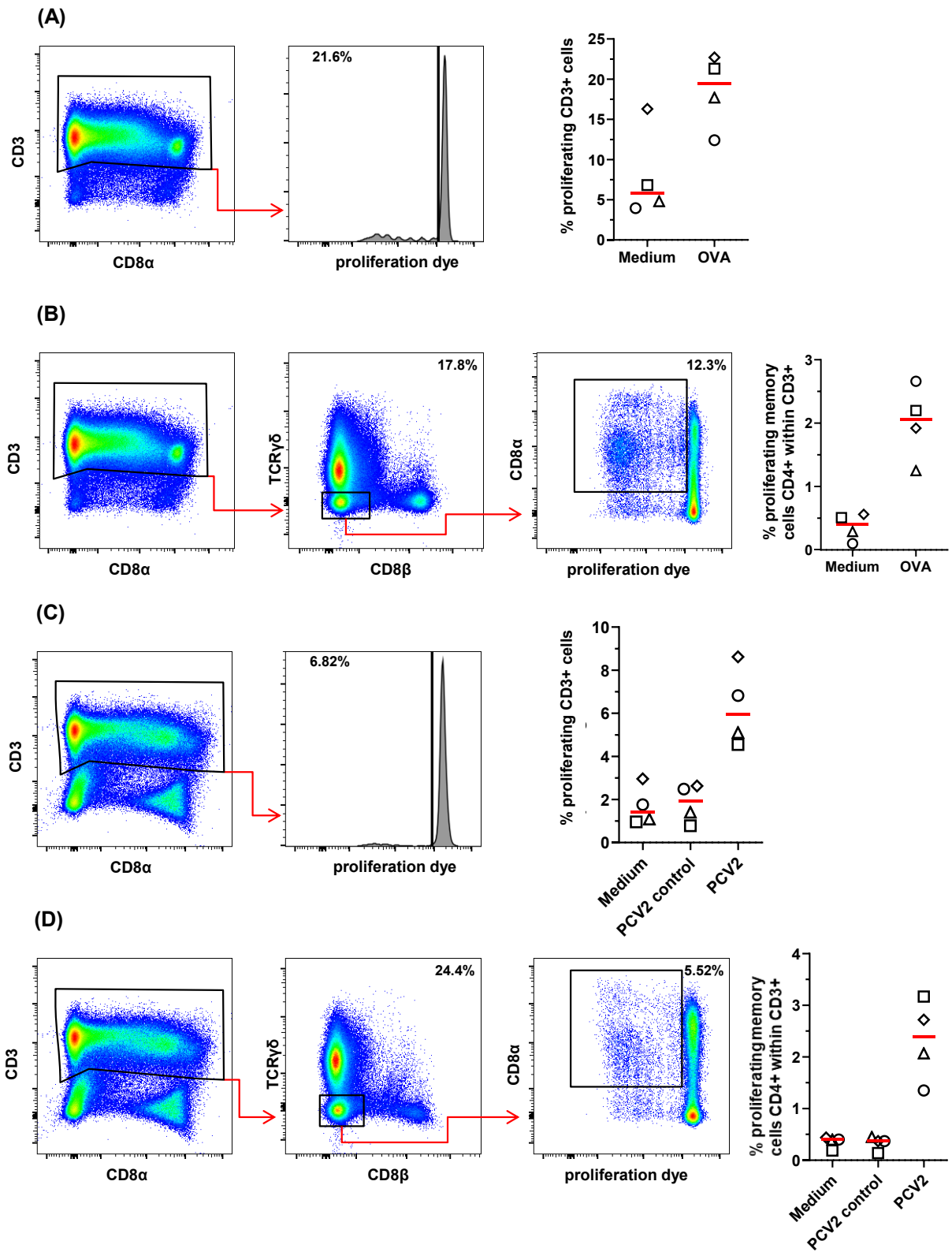


Figure 4: T-cell proliferation

The used samples were investigated for differences in the CD3⁺ cell proliferation between the medium/control group and the stimulated group for OVA (**A**) and PCV2 (**C**). Cells were gated on CD3, and histograms show the proliferation with indicated frequencies of proliferating cells (Violet^{dim}). Proliferation was also tested specifically for CD4⁺CD8 α ⁺ memory cells for OVA (**B**) and PCV2 (**D**). Gated CD3⁺ cells were used to select for CD4⁺ cells (indirectly by using the CD8 β -TCR- $\gamma\delta$ - phenotype) and subsequent gating for CD8 α ⁺ proliferating cells. The gating strategy is demonstrated for one representative animal of the OVA group (**A**, **B**) and one animal of the PCV2 group (**C**, **D**). Scatter diagrams on the right show the data of all four animals, with the median marked by a red line and each animal being represented by a different symbol.

3.3 Effects of antibody blocking on different T-cell subsets

Having established antigen-specific proliferation of T cells and Th memory cells, we now wanted to investigate the effects of the anti-CD2, CD4, CD11a, CD18, CD50 and MHC-II mAbs (Table 6) on reactivation of Th memory cells. During first analysis, we observed changes in the frequencies of different major T-cell subsets, namely the CD4⁺, CD8 β ⁺ (CTL), and TCR $\gamma\delta$ ⁺ populations. Figure 5 represents these changes in the different subsets. Gating is shown for one animal of the OVA group (Figure 5A) and one animal of the PCV2 group (Figure 5C), where the cells were stimulated by the corresponding antigen. First, cells were gated for T cells (CD3⁺). The further differentiation into the subsets was possible by plotting TCR $\gamma\delta$ against CD8 β , double-negative cells represent CD4⁺ T cells. The graphs below show the frequencies of these subsets within the overall CD3⁺ T-cell population of all four animals of the OVA group (Figure 5B) and of all four animals of the PCV2 group (Figure 5D). The median is represented by a red line and every animal by a different symbol.

In the first graph (Figure 5B), we can see slight differences in the relative CD4⁺ count. Blocking with anti-CD2 mAb led to a decrease in the CD4⁺ population (13.8 %) compared to the group that only received OVA stimulation (16.2 %), especially for one animal (triangle). This effect was more visible in the group that received anti-CD4 mAb, with a decrease of this population to 8.0 %. For CD11a, one animal (triangle) showed a decrease in CD4⁺ frequencies, but the median of the anti-CD11a mAb group was at 15.3 %, reasonably close to the OVA stimulation without antibody. In the middle graph, we see that anti-CD2 mAb led to a slight decrease in the CTL population to 5.0 %, whereas the OVA stimulated group without antibody showed a percentage of CTLs of 6.8 %. The graph to the right shows the frequencies of TCR $\gamma\delta$ T cells, where an increase is visible in the groups that received the anti-CD2 mAb (77.2 %) and the anti-CD4 mAb (82.2 %) compared to the group stimulated with OVA only (73.1 %). Control

isotype-matched antibodies did not show any obvious effect for all three cell subsets, similar to mAb treatment with anti-CD18, anti-CD50, and anti-MHC-II.

Looking at the PCV2 data (Figure 5D), we observed the same effects but considerably more pronounced. The frequencies of CD4⁺ cells for example dropped from 35.5 % in the PCV2 group, to 1.7 % in the group that received anti-CD2 mAb additionally. The group with anti-CD4 mAb dropped to 5.8 %. The groups that received anti-CD11a mAb (31.8 %) and anti-MHC-II mAb (32.4 %) show a slight decrease in their CD4⁺ cell subsets. In the middle graph representing CTLs, we can also observe anti-CD2 mAb treatment reducing the frequencies from 4.0 % in the PCV2 stimulated group, to 0.4 %. Anti-CD4 mAb on the other hand seemed to increase CTL counts to 5.3 %. In the group that received anti-MHC-II mAb, the CTL count was also reduced to 2.0 %. The treatment with anti-CD11a (3.0 %), anti-CD18 (3.5 %), and anti-CD50 (3.5 %) mAbs also showed minor effects on the CTL numbers, although isotype-matched controls also decreased the CTL count slightly (3.0 % - 3.4 %). In the graph on the right, the anti-CD2 mAb (94.0 %) and anti-CD4 mAb (85.3 %) groups showed an increase regarding the frequencies of TCRγδ cells compared to the PCV2 group without mAb treatment (54.0 %). No other obvious differences were seen in the other treatment groups.

Overall, main differences were observed, when using the anti-CD2 mAb, especially regarding the substantial decrease in CD4⁺ and CTL frequencies. The anti-CD4 mAb led to decreases primarily in the CD4⁺ population, without decreasing the CTL count. The decrease in the CD4⁺ and CTL population were accompanied by an increase in TCRγδ⁺ cells. All other tested mAbs did not seem to affect these T cell subsets, neither in OVA samples nor in PCV2 samples.

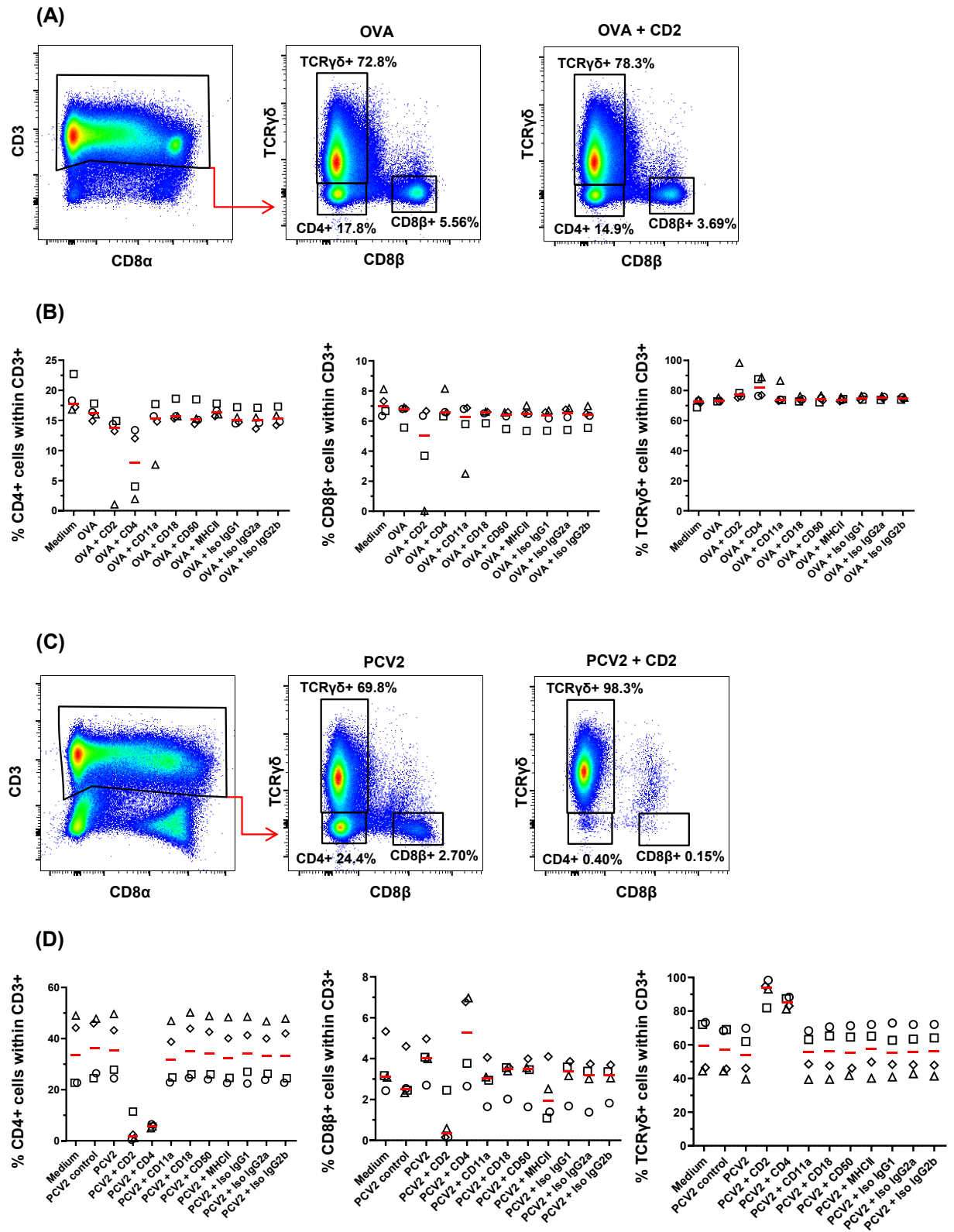


Figure 5: Effects of antibody treatment on T-cell subset frequencies

The effects of the used antibodies were tested by observing changes in frequencies of the major T-cell subsets. This experiment was conducted for OVA animal samples (**A, B**) and PCV2 animal samples (**C, D**). One representative animal is shown each, to demonstrate the gating strategy. First, CD3⁺ cells were gated and further differentiated in TCRγδ⁺, CD4⁺ and CD8β⁺ subsets by plotting TCRγδ against CD8β. An additional graph was added, where the gating is demonstrated for the group, that received anti-CD2 mAb (**A, C**). The frequencies of all groups within total T cells were then plotted in a scatter diagram for OVA (**B**) and for PCV2 (**D**), whereas the red lines demonstrate the median of all four animals and each animal is represented by an individual symbol.

3.4 Effects of antibody blocking on Th memory cells

To be able to investigate the antibody effects on the Th memory cells more profoundly, we investigated the frequencies of Th memory cells (CD4⁺CD8α⁺) within the overall CD3⁺ T-cell population.

Gating for the proliferating Th memory cells was performed as before (Figure 4B, D). The percentage of proliferating Th memory cells within CD3⁺ cells of all four OVA animals is illustrated in Figure 6A, as well as for all four PCV2 animals in Figure 6B. The red lines demonstrate the median and each animal is represented using a unique symbol.

Examining the graph representing the OVA data (Figure 6A), changes in the Th memory cell proliferation can be seen between the OVA stimulated group without antibody treatment (2.1 %) and the groups that received the isotype-matched control antibodies, IgG1 (1.4 %), IgG2a (1.6 %) and IgG2b (1.5 %). Due to this effect, it was difficult to interpret changes in Th memory cell proliferation in the other mAb groups. Nevertheless, a decrease in Th memory cell proliferation was observed when applying anti-CD2 mAb (0.9 %) compared to the sample without antibody. Anti-CD50 mAb (2 %) and anti-MHC-II mAb (2.2 %) did not seem to affect cell proliferation. Regarding the other groups, no clear conclusions could be made on the antibody-specific effects for anti-CD4 mAb (1.4 %), anti-CD11a mAb (1.8 %) and anti-CD18 mAb (1.3 %) due to the changes in the isotype control samples.

Upon analysis of the graph to the right representing PCV2 data (Figure 6B) a similar effect was observed between the PCV2 stimulated group without antibody treatment (2.4 %) and the isotype-matched control antibodies, IgG1 (1.9 %), IgG2a (2.1 %) and IgG2b (1.8 %). Some statements can be made though, due to the substantial differences compared to the PCV2 stimulated group without antibodies. Anti-CD2 mAb (0.1 %) decreased the Th memory cell

proliferation, resulting in even lower proliferation compared to the unstimulated groups with medium (0.4 %) and the PCV2 control group (0.4 %). Anti-CD4 mAb also led to a low count of proliferating memory cells (0.3 %), as well as the addition of anti-MHC-II mAb (0.8 %). Anti-CD11a mAb increased proliferation slightly (3.4 %), as well as anti-CD18 mAb (2.8 %). Using the anti-CD50 mAb (1.9 %), we cannot tell, if this slight decrease in proliferating cells is caused by the addition of the antibody, as similar values were obtained in the isotype control samples.

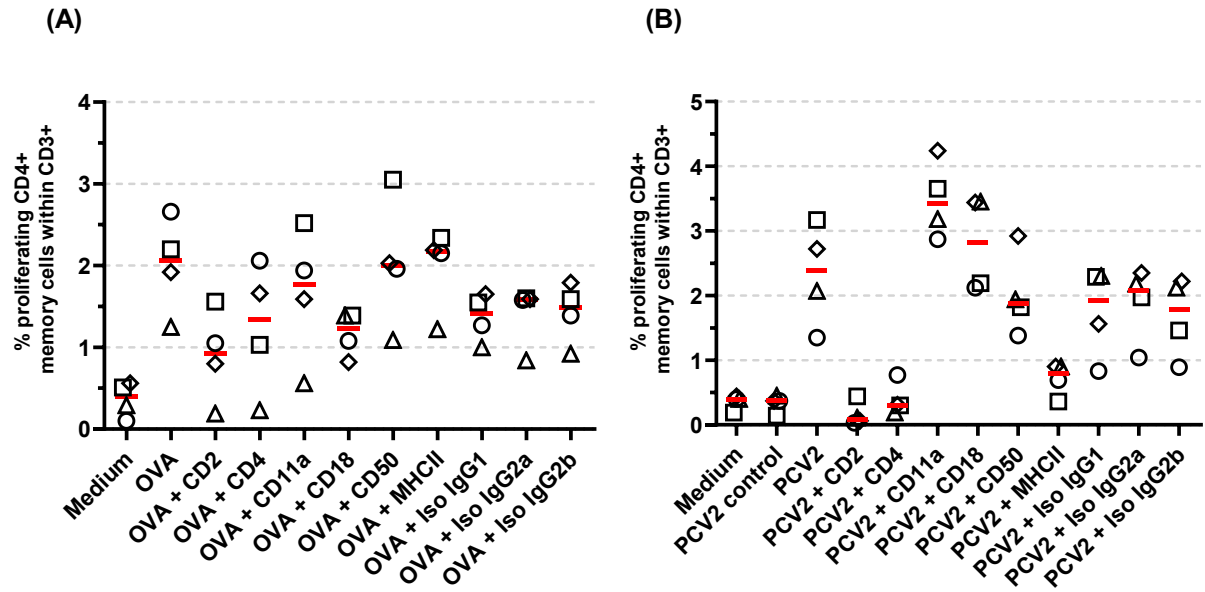


Figure 6: Effects of antibody treatment on Th memory cell proliferation

The effects of the used antibodies were tested by observing changes in frequencies of memory Th cell ($CD4^+CD8\alpha^+$) proliferation within the $CD3^+$ cell population. Data is shown for four animals of the OVA group (A) and four animals of the PCV2 group (B). Red lines demonstrate the median of all four animals and each animal is represented by a unique symbol.

4 Discussion

4.1 Summary of results

During our research, we first tried to characterise the porcine IS more thoroughly by analysing the distribution of CD2, CD11a, CD18, CD50, and MHC-II on naïve Th cells, Th memory cells and myeloid cells. Furthermore, *in-vitro* re-stimulation assays were used, to investigate the effects of anti-CD2, anti-CD4, anti-CD11a, anti-CD18, anti-CD50 and anti-MHC-II mAbs on Th memory cell proliferation.

First, it was shown in our first experiment that CD2 was primarily expressed on naïve and memory Th cells, as expected. CD11a and CD18 were expressed very homologous across the three cell types. MHC-II expression differed between naïve and memory Th cells. This result is not unexpected, as it has been shown, that a substantial number of activated Th cells (CD4⁺CD8α⁺) start expressing MHC-II on their surface, but the functional aspect of this expression is still not known (Gerner et al. 2022, Talker et al. 2013). Interestingly, CD50 was hardly expressed on porcine naïve and memory Th cells. This is of especial significance, considering that in humans, CD50 was described, as being highly expressed on resting lymphocytes (Fougerolles and Springer 1992). It is assumed that CD50 plays an important role in initial contact between APC and naïve Th cell (Guerra-Espinosa et al. 2024). In our experiments though, CD50 was primarily expressed on myeloid cells. Of note, investigation of the mentioned molecules on B cells is missing in this study. B cells are a further important population of APCs (Hong et al. 2018) and will therefore be analysed for the markers in the future.

Before we could investigate the effects, the antibodies may have on cell proliferation, we investigated if the PBMCs we used were responding to a re-stimulation in an antigen-specific manner. We were able to show increased proliferation of the overall T-cell population in groups that received their corresponding stimulus (OVA or PCV2 VPLs) in comparison to the non-stimulated groups. This difference in proliferation could also be seen in Th memory cells, suggesting that the used PBMCs showed antigen-specific proliferation in our further *in-vitro* assays. We saw substantially more proliferation of the overall T-cell population (CD3⁺) compared to the Th memory cell proliferation. This may be explained by a bystander effect. If Th memory cells detect the corresponding antigen via the TCR, cytokines are produced that may activate other T-cell subsets with the crucial difference that this activation is antigen-independent (Lee et al. 2020).

During our blocking experiments, we observed changes in different T-cell subsets and in Th memory cell proliferation. Generally speaking, anti-CD2 mAb seemed to decrease the numbers of CD4⁺ T cells, and CTLs (CD8 β ⁺). Corresponding to that, the numbers of TCR $\gamma\delta$ T cells increased. When looking at Th memory cell proliferation, a noticeable decrease in proliferation was detected when using anti-CD2 mAb. This effect was less distinct for the OVA population, but clearly visible for the PCV2 animals. Anti-CD4 mAb led to a decrease in CD4⁺ T cells without decreasing CTLs. Again, a slight increase in TCR $\gamma\delta$ T cells could be detected. A noticeable drop in Th memory cell proliferation could also be observed in PCV2 animals. All other mAbs did not seem to affect numbers of CD4⁺ T cells, CTLs and TCR $\gamma\delta$ T cells. Nevertheless, Th memory cells seemed to be affected by anti-MHC-II mAb, reducing proliferation substantially in PCV2 animals, which would be consistent with literature (Summerfield et al. 1996). Anti-CD11a and anti-CD18 seemed to increase Th memory cell proliferation slightly in PCV2 animals but this effect could not be replicated in OVA animals.

It has to be mentioned, that the used isotype-matched control antibodies also slightly decreased the Th memory cell proliferation but did not affect the overall frequencies of the other major T-cell subsets. Due to this, it is difficult to interpret decreases in Th memory cell proliferation. It is not possible to say if an antibody-specific effect can be observed, when relative proliferation numbers of other groups lie in between the simulated group and the isotype-matched control groups. Nevertheless, very strong changes in proliferation can still have validity.

4.2 Problem resolution

4.2.1 Sodium azide (NaN₃)

One explanation for the decrease in Th memory cell proliferation in the isotype controls could lie in the fact, that these antibodies contain 0.09 % of sodium azide in the buffer. Sodium azide is known for its toxic effects, inducing for example loss of mitochondrial membrane potential. Cells seem to vary in their sensitivity to sodium azide, but overall, it can be stated that even low mM amounts can induce cell death. Both, apoptosis and necrosis have been observed after NaN₃ exposure (Jones et al. 1980, Sato et al. 2008). Sodium azide is furthermore assumed to emit a gaseous product, affecting cells in adjacent wells (Kho et al. 2017).

Preliminary experiments conducted by us using OVA PBMCs have shown, that the used isotype controls lead to a substantial increase in dead cells by up to 79 % (Data not shown). Even though cells were only subjected to low amounts of NaN₃, they were exposed for four

days in cell culture, explaining the drastic increase in dead cells. This prompted us to perform dialysis of the isotype controls, which normalised the percentage of dead cells. However, it cannot be excluded that low remaining amounts of NaN_3 persisted and specifically affected Th memory cell proliferation.

4.2.2 Cell-dependent factors

Taking a closer look at our results, some differences can be detected between OVA and PCV2 samples. This may be caused by the different times of sample collection and the different lengths of storage. OVA PBMCs have been isolated approximately 13 years ago and have been stored at $-150\text{ }^{\circ}\text{C}$ until our analyses. PCV2 PBMCs on the other hand were isolated early in 2024 and have also been stored at $-150\text{ }^{\circ}\text{C}$. It has been demonstrated that cryopreservation can affect overall cell count and cell viability in PBMCs. But in a study conducted over six months, the cell viability remained relatively stable after an initial decrease. Nevertheless, cryopreservation can affect different cell subsets. For example, Li et al. have shown that CTL populations decreased significantly over long-term storage, but CD4^+ T-cell levels remained relatively stable. Especially monocytes and B cell counts dipped after cryopreservation (Li et al. 2022). Taking a closer look at our own data, a clear reduction of CD3^+ cells can be observed in OVA samples compared to the PCV2 animals. Due to this, it may be speculated, that PCV2 data represent more accurate, lifelike results.

4.2.3 Epitope binding

For the investigated antibodies, the exact epitopes on the corresponding molecules are not known. This can be important due to differing effects of the antibody depending on its binding site. For example, it has been described that some anti-CD2 mAbs could cause apoptosis in human T cells (Dumont et al. 1998). In a human erythroleukemia cell line, different effects of anti-CD18 mAbs have been described, where one antibody induced rapid proliferation in this cell line, whereas another anti-CD18 mAb was not capable of doing so (Tsuji et al. 1998). For CD11a other interesting aspects have been discovered. A certain anti-CD11a was speculated to keep LFA-1 in the high affinity conformation state, impeding with detachment of T cells from another cell (Dransfield et al. 1992). These results are intended to show that different antibodies can have different effects depending on their binding site, possibly leading to other results than shown in our experiments.

5 Summary

During activation of the adaptive immune system, antigen presenting cells get in contact with T or B cells. They form the immunological synapse, in which antigen recognising molecules, costimulatory molecules and adhesion molecules play an important role. Adhesion molecules close the distance between these two cells, to establish prolonged contact and to achieve antigen recognition by the adaptive immune system. The formation of the immunological synapse is also essential for memory cell reactivation.

Several molecules act in this synapse, of which six were investigated more closely in this thesis: CD2 acts as an adhesion molecule with powerful costimulatory properties. CD11a and CD18 form the lymphocyte function-associated antigen 1 (LFA-1), one of the most important adhesion molecules during synapse formation. CD50 on the other hand is assumed to be one of the first adhesion molecules involved in synapse assembly. CD4 and MHC-II are important molecules for antigen recognition and therefore form the core of the immunological synapse.

During our studies on porcine peripheral blood mononuclear cells, naïve T cells, memory T cells and myeloid cells were first phenotypically characterised using flow cytometry, to describe the distribution of the above-mentioned molecules on the different cell types. Our results show, that CD2 can be primarily found on naïve and memory T cells and only in very low amounts on myeloid cells. CD11a and CD18 are present on almost all myeloid cells, naïve T cells and memory T cells. CD50 can be found on approximately 60 % of myeloid cells, about 10 % of naïve T cells, and 15 % of memory T cells.

For our second experimental setup, cells from pigs that have been immunised with either an ovalbumin formulation (OVA) or porcine circovirus type 2 (PCV2) vaccine (Ingelvac CircoFLEX®, Boehringer Ingelheim Vetmedica GmbH) were used. Antigen-specific re-stimulation in *in-vitro* assays were performed to induce activation and proliferation of memory T cells. In addition, monoclonal antibodies (mAbs) directed against the above-mentioned molecules were added to the setup, to investigate whether activation can be blocked/reduced by interfering with one of these molecules. Using a proliferation dye (CellTrace™ Violet solution), the proliferative response to the stimulus could be assessed. We looked for changes in memory-cell proliferation within CD4 T cells, but also monitored changes in other T-cell subsets like cytolytic T cells and TCRγδ T cells.

Even though changes in memory cell proliferation were difficult to interpret, the percentage of CD4⁺ cells within all T cells proved to be a good indicator of a potential effect of the used

antibodies. The anti-CD2 mAb led to a considerable decrease in CD4⁺ T cells, cytolytic T cells and proliferating Th memory cells. Even though this effect was visible in both, animals immunised with OVA or with PCV2, the effect was more noticeable in PCV2 animals. The anti-CD4 mAb also resulted in a drop in CD4⁺ T cells and proliferating Th memory cells without decreasing the frequency of cytolytic T cells. Anti-MHC-II mAbs did not seem to influence frequencies of the investigated T cell subsets, but also led to a decrease in Th memory cell proliferation in PCV2 animals. These results suggest, that besides molecules involved in antigen recognition, especially CD2 plays an important role during memory cell reactivation. The other tested antibodies did not seem to obviously effect the reactivation of memory cells and overall T cell populations.

6 Zusammenfassung

Während der Aktivierung des adaptiven Immunsystems kommen Antigen-präsentierende Zellen in Kontakt mit T und B-Zellen. Sie bilden die immunologische Synapse, in welcher Antigen erkennende Moleküle, kostimulierende Moleküle und Adhäsionsmoleküle eine wichtige Rolle spielen. Adhäsionsmoleküle führen die zwei involvierten Zellen näher zueinander, um den Zell-Zell Kontakt zu verlängern und es den adaptiven Immunzellen zu ermöglichen, das Antigen zu erkennen. Diese immunologische Synapse ist darüber hinaus essenziell für die Reaktivierung von Gedächtniszellen.

Mehrere Moleküle spielen eine Rolle in diese Synapse, von welchen wir sechs näher untersucht haben: CD2 agiert dabei als Adhäsionsmolekül mit costimulatorischen Eigenschaften. CD11a und CD18 bilden gemeinsam das lymphocyte function-associated antigen 1 (LFA-1), eines der wichtigsten Moleküle während der Synapsenbildung. Eines der ersten Adhäsionsmoleküle, dass an der Synapsenbildung beteiligt sein soll, ist hingegen CD50. CD4 und MHC-II sind wichtige Moleküle für die Antigen-Erkennung, und bilden den Kern der immunologischen Synapse.

Bei unseren Untersuchungen an mononukleären Zellen aus dem peripheren Blut von Schweinen wurden naive T-Zellen, Gedächtnis-T-Zellen und myeloide Zellen zunächst phänotypisch mit Hilfe der Durchflusszytometrie charakterisiert, um die Verteilung der oben genannten Moleküle auf den verschiedenen Zelltypen zu beschreiben. Unsere Ergebnisse zeigen, dass CD2 primär auf naiven T-Zellen und Gedächtnis-T-Zellen präsent ist und kaum auf myeloiden Zellen. CD11a und CD18 kann auf den meisten naiven T-Zellen, Gedächtnis-T-Zellen und myeloiden Zellen gefunden werden. CD50 ist auf circa 60 % der myeloiden Zellen vertreten, zu ungefähr 15 % auf Gedächtnis-T-Zellen und zu 9 % auf naiven T-Zellen.

Für unser zweites Experiment haben wir Zellen von Schweinen verwendet, die entweder mit einer Ovalbumin Formulierung (OVA) immunisiert wurden oder mit einem Impfstoff (Ingelvac CircoFLEX®, Boehringer Ingelheim Vetmedica GmbH) gegen das porcine Circovirus 2 (PCV2). Wir versuchten *in-vitro* antigenspezifische Re-stimulationen durchzuführen, um T-Gedächtniszellen zu aktivieren und Zellteilung anzuregen. Zusätzlich wurden monoklonale Antikörper (mAk) gegen die vorhin erwähnten Moleküle hinzugegeben, um zu untersuchen, ob die Aktivierung der T-Gedächtniszellen reduziert/blockiert werden kann. Mit einem Proliferationsfarbstoff (CellTrace™ Violet solution) konnten wir die Proliferation der Zellen in Antwort auf den hinzugegebenen Stimulus näher untersuchen. Wir haben nach

Veränderungen bei der Proliferation von Gedächtniszellen innerhalb der CD4-T-Zellen gesucht, aber auch Veränderungen bei anderen T-Zell-Untergruppen wie zytolytischen T-Zellen und TCR $\gamma\delta$ -T-Zellen inspiziert.

Veränderungen in der Gedächtniszell-Proliferation waren schwierig zu interpretieren, der Prozentsatz der CD4⁺ T-Zellen innerhalb aller T-Zellen hat sich aber als guter Indikator eines möglichen Effekts der Antikörper erwiesen. Der anti-CD2 mAk hat die Anzahl an CD4⁺ T-Zellen deutlich reduziert, genauso wie die Zahl der zytolytischen T-Zellen und der proliferierenden Th-Gedächtniszellen. Dieser Effekt war in beiden Tieren sichtbar, immunisiert mit OVA oder PCV2, aber deutlich stärker in PCV2 Tieren. Der anti-CD4 mAk hat die Zahl der CD4⁺ T-Zellen sowie der proliferierenden T-Gedächtniszellen reduziert, ohne die Anzahl an zytolytischen T-Zellen zu verringern. Der Anti-MHC-II mAk hat die einzelnen T-Zell-Untergruppen nicht wesentlich beeinflusst, jedoch auch die Zahl der proliferierenden T-Gedächtniszellen in PCV2 Tieren reduziert. Diese Ergebnisse zeigen, dass neben den Molekülen, die für die Antigenerkennung essenziell sind, vor allem CD2 eine wichtige Rolle in der Reaktivierung von Gedächtniszellen spielt. Die anderen untersuchten Antikörper haben dagegen nur insignifikante Veränderungen in den T-Zell-Untergruppen und in der Reaktivierung von T-Gedächtniszellen hervorgerufen.

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8 Figures and tables

Figure 1: Graphic representation of the immunological synapse	8
Figure 2: Gating strategy	20
Figure 3: Integrin phenotyping	23
Figure 4: T-cell proliferation.....	26
Figure 5: Effects of antibody treatment on T-cell subset frequencies.....	29
Figure 6: Effects of antibody treatment on Th memory cell proliferation	30
Table 1: Reagents used in this study.....	12
Table 2: Primary antibodies used for phenotype characterisation.....	13
Table 3: Secondary antibodies used for phenotype characterisation	13
Table 4: Blocking antibody used for phenotype characterisation	13
Table 5: Viability dye used for phenotype characterisation	14
Table 6: Antibodies used in synapse blocking reaction.....	14
Table 7: Isotype controls used in synapse blocking reaction	14
Table 8: Primary antibodies used for proliferation FCM staining.....	14
Table 9: Secondary antibodies used for proliferation FCM staining	15
Table 10: Viability dye used for proliferation FCM staining	15
Table 11: Negative control used for PCV2 blocking experiments.....	15
Table 12: Devices and software used during this study	15
Table 13: Staining panel for integrin staining.....	18
Table 14: Staining panel after the proliferation assay	19