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Canine $CD4^+CD8^+$ double-positive T cells: unique cells with an activated phenotype, remarkable heterogeneity, different origins, and a high capacity of interferon-gamma production

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Meinen Eltern

Contents

3 Discussion

Abbreviations

APC	**antigen-presenting cells**
CD	**cluster of differentiation**
CDV	**Canine distemper virus**
ConA	**concanavalin A**
CFSE	**carboxyfluorescein succinimidyl ester**
dp	**double-positive**
ELISA	**enzyme-linked immunosorbent assay**
e.g.	**exempli gratia / for example**
FACS	**fluorescence-activated cell sorting**
FBS	**fetal bovine serum**
FoxP3	**forkhead box P3**
FSC-A	**forward scatter-area**
FSC-H	**forward scatter-height**
i.e.	**id est / that is to say**
IFN-γ	**interferon-gamma**
IL	**interleukin**
LN	**lymphonodus / lymph node**
MHC-I	**major histocompatibility complex class I**
MHC-II	**major histocompatibility complex class I**
mRNA	**messenger ribonucleic acid**
no.	**number**
PBMC	**peripheral blood mononuclear cells**
PBS	**phosphate buffered saline**

PFA	**paraformaldehyde**
PMA	**phorbol 12-myristate 13-acetate**
RT-qPCR	**reverse transcription quantitative polymerase chain reaction**
RTE	**recent thymic emigrants**
SEB	***Staphylococcos aureus* enterotoxin B**
sp	**single-positive**
TCR	**T cell receptor**
T_{CM}	**T central memory cell**
T_{EM}	**T effector memory cell**
Th1	**T helper cell type 1**
Th2	**T helper cell type 2**
TNF-α	**tumor necrosis factor-alpha**
Treg cell	**regulatory T cell**
vs	**versus**

1 Introduction

The dog as one of the most important companion animals in the world, just like us and all other species, is daily confronted with various pathogens. Elaborate mechanisms of defense are provided by the immune system, which is composed of a complex network of organs, cells, and soluble factors.

The immune system can be divided into the evolutionary older innate immunity and the adaptive immunity, which developed in vertebrates. Innate immunity represents the first line of defense that mediates a rapid immune response against a broad range of invading pathogens. One of the characteristics of delayed adaptive immunity is the use of antigen-specific receptors to drive targeted effector-responses with subsequent development of immunological memory. The adaptive immune system is built upon two major cell types, namely B and T lymphocytes. B cell-mediated humoral immune responses are characterized by production of antibodies that protect the body from extracellular pathogens. T cells, which are involved in a wide range of immunological processes, provide B cell help and are essential to control intracellular infections.

1.1 T cells in the adaptive immune response

T cell-mediated immunity is driven by T effector cells, which develop from naïve T cells in a primary immune response after first contact with a specific antigen in the lymph node. T cells can be further divided into distinct T cell subpopulations with specialized functions. The two major subsets are $CD8^+$ cytotoxic T cells and $CD4^+$ T helper cells. Naïve $CD8^+$ T cells differentiate into cytotoxic T cells after encountering their specific antigen presented by major histocompatibility complex class I (MHC-I) of an antigen-presenting cell (APC) and additional costimulatory signals. $CD8^+$ effector T cells have cytolytic function and are important, e.g. for clearance of viral infections (MESCHER et al. 2006).

Naïve $CD4^+$ T cells become activated by recognition of their specific antigen presented by MHC class II (MHC-II) in the presence of costimulatory signals. $CD4^+$ T cells can develop several functional properties by differentiation in either classical subsets such as T helper type 1 (Th1) and T helper type 2 (Th2) cells, and more recently discovered T helper type 17 (Th17) cells, follicular helper T cells (Tfh), induced regulatory T cells (iTreg), regulatory type 1 cells (Tr1) and T helper type 9 (Th9) cells (LUCKHEERAM et al. 2012). The main function of Th1 cells is clearance of intracellular pathogens, whereas Th2 cells are primarily involved in elimination of extracellular antigens, e.g. by providing B cell help (ROMAGNANI et al. 1998).

After a primary immune response some T cells develop into memory T cells. Memory T cells persist in the circulating T cell pool and are important for protection upon secondary antigen challenge. They sense low doses of antigen and are able to give a qualitatively different and quantitatively enhanced immune response compared with naïve T cells (ROGERS et al. 2000; SALLUSTO et al. 1999). Currently, two major types of memory cells are defined: T effector memory cells (T_{EM}) and T central memory cells (T_{CM}). T_{EM} home to peripheral tissues and secrete cytokines, such as interferon-gamma (IFN-γ), immediately after antigen encounter but show limited proliferative capacity. In contrary, T_{CM} recirculate together with naïve T cells between peripheral blood and lymphoid organs and show weaker effector functions but they can proliferate to give rise to effector cells (LANZAVECCHIA and SALLUSTO 2005).

The development of robust memory is of particular importance and the purpose of vaccination (ADA 2007). Besides vaccine-induced antibody production and formation of B memory cells, the induction of T cell memory is of great purpose, for example for the clearance of intracellular pathogens (HUSTER et al. 2006).

1.2 Cytokine production by T cells

During an immune response communication between different immune cells is necessary. Besides direct cell-cell-contact, cells can communicate using cytokines. Cytokines are small soluble peptides secreted by cells, which can change their own or other cells' properties. Cytokines have various biological effects. An important cytokine secreted by $CD8^+$ T effector cells is IFN-γ. IFN-γ can stop viral replication or eliminate virus from virus-infected cells without harming it (viral purging) (GUIDOTTI and CHISARI 1999). $CD4^+$ T cells secrete different sets of cytokines depending on their function. The two major $CD4^+$ T cell subsets Th1 and Th2 cells mainly secrete IFN-γ, interleukin-2 (IL-2), and tumor necrosis factor-alpha (TNF-α) or IL-4, IL-5, IL-10 and IL-13, respectively (SANTANA and ROSENSTEIN 2003). The cytokine milieu has an important impact on the fate and development of the above mentioned distinct T cell subpopulations (LUCKHEERAM et al. 2012).

1.3 Failure of the immune system

Besides pathogen control, the immune system is also responsible for resistance against body's own dangers, such as neoplasms. The term immune surveillance describes the fact that immune cells specifically can identify and eradicate tumor cells which express tumor antigens or stress-induced molecules (SWANN and SMYTH 2007). That $CD8^+$ and also $CD4^+$ T cells play a role in antitumor immunity has already been described for a long time (GREENBERG et

al. 1981; GREENBERG et al. 1985; PREHN and MAIN 1957). However, even in the presence of a functional immune system tumors can escape immune surveillance. Mechanisms of escape are often discussed but still not completely resolved (RESTIFO et al. 2002). In addition, the lymphatic system itself can be the source of tumors as in cases of lymphoma, which also in dog is a most commonly encountered neoplasm (JOHNSTON et al. 2014). Overall, tumors are a prevalent disease among dogs, since the tumor incidence rate is 213 cases per 100,000 dogs (KESSLER et al. 2012).

Another important mechanism of the immune system is self-tolerance. Immunological self-tolerance is ensured by regulation or elimination of self-reactive lymphocytes and is divided in central and peripheral tolerance. To achieve T cell tolerance, during thymic development immature self-reactive T cell clones are eliminated (central tolerance) (KAPPLER et al. 1987). Peripheral tolerance includes several aspects: (i) mature self-reactive T cells can fade into an anergic state due to missing costimulatory signals, which are required for a functional immune response, (ii) T cells can be deleted due to apoptosis induction, or (iii) T cells are suppressed by regulatory T (Treg) cells (GOODNOW 1996; MILLER and MORAHAN 1992; SAKAGUCHI 2004). If, however, the balance between immunity and tolerance is disturbed, the immune system is dysregulated and autoimmune diseases can be the result.

Also, an undesirable reaction of the immune system is hypersensitivity. It occurs when the immune system responds to usually harmless substances. Several cell types can be responsible for development of different types of hypersensitivity (type I - IV) (MURPHY et al. 2008). The most common type of hypersensitivity is allergy (type I), which is mediated by immunoglobulin E (IgE). Allergens, as small antigens, enter the body at low doses and are able to trigger a Th2 response. Allergen-specific Th2 cells induce a switch to IgE production in B cells by IL-4 and IL-13 secretion and costimulatory signals. In allergy, often a biphasic response occurs with the early reaction mediated by IgE and the late phase response mediated by e.g. eosinophil recruitment (O'HEHIR et al. 1991). Allergy can lead to chronic inflammation such as atopic dermatitis, which also occurs in dogs (SINKE et al. 2002). The delayed type hypersensitivity (type IV), such as contact dermatitis, is T cell-mediated and both, $CD4^+$ and $CD8^+$ T cells, play an important role in disease development (KIMBER and DEARMAN 2002). Contact dermatitis is less common in the canine species (LINEK 2014).

1.4 Molecular structure and function of CD4 and CD8 surface receptors

CD4 and CD8 coreceptors are surface molecules which are involved in thymic selection and T cell activation. They enhance adhesion and facilitate effective signaling through the T cell receptor (TCR). The CD4 receptor is a single chain molecule consisting of four

immunoglobulin-like domains (MADDON et al. 1985). It is assumed that CD4 receptors form homodimers on cell surfaces for ligand/MHC-II recognition (MOLDOVAN et al. 2002). The CD8 receptor is a dimeric molecule which can be differently expressed on the cell surface. Normally, it appears as a heterodimer of the two different α- and β-chains connected by a disulfide bridge. Each chain consists of one immunoglobulin-like domain (LITTMAN et al. 1985). In addition, the CD8 receptor can also be expressed as a CD8αα homodimer (MOEBIUS et al. 1991; TERRY et al. 1990). The CD8β chain mediates the participation of CD8αβ as a TCR coreceptor (WONG et al. 2003) and several studies show that the CD8αβ heterodimer is a more effective TCR coreceptor than CD8αα (WHEELER et al. 1998; WITTE et al. 1999; WONG et al. 2003). CD8αα T cells are the predominant intraepithelial lymphocyte population in murine small intestine (CHEROUTRE 2004).

1.5 The special case of mature peripheral blood $CD4^+CD8^+$ double-positive T cells

Several functional subtypes of T cells were already introduced in the above paragraphs. However, the two major precursor T cells are $CD4^+$ and $CD8^+$ T cells. This classification is based on the pathway of T cell development. Pluripotent hematopoietic stem cells leave the bone marrow and migrate via the blood to the thymus, the organ of T lymphocyte development (MILLER and OSOBA 1967). These $CD4^-CD8^-$ double-negative (dn) precursor cells pass four stages (DN1 - DN4) during T cell development. Double-negative cells can give rise to either αβ or γδ TCR expressing cells (ROBEY and FOWLKES 1998). With entry in the DN3 stage arrangement of the TCR β, γ, and δ gene loci begins and cells first expressing pre-TCR-α are committed to the TCRαβ lineage. Eventually, after further selection and re-arrangements of αβ TCR, $CD4^-CD8^-$dn cells become immature $CD3^+$ $CD4^+CD8^+$ double-positive (dp) αβ TCR^+ cells (HAYDAY et al. 1999). Consequently, during positive selection $CD3^+CD4^+CD8^+$ cells whose TCR efficiently interacts with MHC antigen on cortical epithelial cells survive and the remaining cells die (VON BOEHMER et al. 1989). Moreover, at this point of development the decision whether $CD4^+CD8^+$dp cells become $CD4^+$ or $CD8^+$ single-positive (sp) is made. Nowadays, two theses exist on thymic CD4 or CD8 commitment: either the ligation of MHC-I or MHC-II or the extent/duration of signaling that results from TCR/self-peptide–MHC ligand engagement is crucial for CD4 or CD8 decision (GERMAIN 2002). Thus, thymic immature $CD4^+CD8^+$dp T cells ultimately become either $CD4^+$ or $CD8^+$sp. Finally, during negative selection cells with a high affinity for self-antigens are eliminated via apoptosis (NOSSAL 1994). Overall, only $CD4^+$ or $CD8^+$ sp T cells should emigrate from the thymus to enter the periphery. This has led to the assumption that the expression of either CD4 or CD8 is mutually exclusive.

However, in some species, such as swine, monkey, rat, mouse, chicken, and human, peripheral mature T cells were found which express both coreceptors, $CD4^+$ and $CD8^+$, simultaneously (ZUCKERMANN 1999). Maturity of these T cells in swine, monkey, and human was confirmed by absence of either thymic marker CD1, CD1b, or CD1a (AKARI et al. 1996; ORTOLANI et al. 1993; PESCOVITZ et al. 1990; SAALMÜLLER et al. 1989). In rat, maturity of $CD4^+CD8^+$dp T cells was shown by a high expression level of αβ TCR (KENNY et al. 2000). This is in contrast to the long believed paradigm of CD4 or CD8 T cell lineage commitment. More recently, a $CD4^+CD8^+$dp T cell subpopulation was also described for dogs (ALEXANDRE-PIRES et al. 2010; HOSHINO et al. 2008; OTANI et al. 2008; SCHÜTZE et al. 2009).

1.6 Canine peripheral blood $CD4^+CD8^+$ double-positive T cells

Canine $CD4^+CD8^+$dp T cells were found among blood leukocytes in a study on canine leishmaniosis (ALEXANDRE-PIRES et al. 2010) and also by comparison of umbilical cord to dam's peripheral blood (OTANI et al. 2008). Their proportion increases after cultivation of peripheral blood mononuclear cells (PBMC) with IL-2 and concomitant stimulation (HOSHINO et al. 2008; KATO et al. 2007) or after restimulation with *Parapoxvirus ovis* (SCHÜTZE et al. 2009). Further characterization of canine $CD4^+CD8^+$dp T cells remained to be conducted.

1.7 Peripheral blood $CD4^+CD8^+$ double-positive T cells in other species

Very well known in the field of veterinary medicine are porcine $CD4^+CD8^+$dp T cells. In swine, the $CD4^+CD8^+$dp T cell population is prominent and can comprise up to about 60% of the peripheral blood T lymphocytes (PESCOVITZ et al. 1985; SAALMÜLLER et al. 1989). Porcine $CD4^+CD8^+$dp T cells develop during a primary immune response after $CD4^+$ T helper cell activation. Upon priming porcine $CD4^+CD8^+$dp T cells can reside as memory T cells, increase strongly with age, and provide protection against secondary infections (SAALMÜLLER et al. 2002; ZUCKERMANN and HUSMANN 1996).

Several monkey species are also known to have a prominent $CD4^+CD8^+$dp T cell subpopulation (5 - 25%) in peripheral blood (AKARI et al. 1996; DEAN et al. 1996; MURAYAMA et al. 1997; NAM et al. 1998; REIMANN et al. 1994). Cynomolgus monkeys also show a gradual increase of double-positive T cells with age and a memory related phenotype was described (AKARI et al. 1997; NAM et al. 1998).

In contrast, human peripheral blood $CD4^+CD8^+$dp T cells show a low frequency (depending on report 3% up to < 6% of T lymphocytes) (BLUE et al. 1985; COLOMBATTI et al. 1998). An

increase of the $CD4^+CD8^+$dp T cell population can be seen during some viral infections (FLAMAND et al. 1998; KITCHEN et al. 1998; ORTOLANI et al. 1993), several immune disorders (BANG et al. 2001; DE MARIA et al. 1987; HIRAO and SUGITA 1998; IWATANI et al. 1993; MUNSCHAUER et al. 1993; QUANDT et al. 2014), neoplastic conditions (BAGOT et al. 1998; BERNARD et al. 1981; DESFRANCOIS et al. 2009; ORTOLANI et al. 1993), and allograft rejection (PREFFER et al. 1986). Besides, in a few cases even in healthy individuals high numbers of peripheral blood $CD4^+CD8^+$dp T cells (>20%) can be found (KAY et al. 1990).

1.8 Importance of canine $CD4^+CD8^+$ double-positive T cells

As shown, $CD4^+$ and $CD8^+$sp T cells have an outstanding role during the adaptive immune response and thus are able to be protective against pathogens and neoplasms. On the other hand, their dysregulated action can cause diseases such as tumors and autoimmune disorders. $CD4^+CD8^+$dp T cells in particular seem to be connected with T cell memory in swine, monkey, and human (AKARI et al. 1997; NASCIMBENI et al. 2004; SAALMÜLLER et al. 2002). However, human $CD4^+CD8^+$dp T cells also occur during disease. Distinct properties of double-positive T cells differ between species, which makes it difficult to draw conclusions for the dog.

Nowadays, with the dog as a companion animal having a great acceptance in our society, small animal medicine is constantly emerging. Vaccination of dogs is daily routine in veterinary praxis, neoplasms are common diseases, some with race disposition (KESSLER et al. 2012), and allergies are gaining ground. Research of the immune system has to keep pace and is necessary to build the basis for prevention/prophylaxis and therapy, with regard to pathogen clearance, vaccination, and immune diseases.

Therefore, an in-depth characterization of canine $CD4^+CD8^+$dp T cells, which could play a role in health and disease, is of great importance and provides new insights in the understanding of the canine immune system.

1.9 Aims and focuses of this study

The aim of this study was a first characterization of canine $CD4^+CD8^+$dp T cells of healthy dogs; regarding their phenotype, origin, occurrence in lymph nodes, and function (i.e. IFN-γ production).

The first part of this work (BISMARCK et al. 2012) addresses phenotypical analysis and activation potential of canine $CD4^+CD8^+$dp T cells. In particular, maturity, TCR utilization (αβ vs γδ), and CD8 receptor composition of $CD4^+CD8^+$dp T cells was of interest. The activation

state, which can be determined by expression of the surface markers CD25 (α chain of IL-2 receptor) and CD62L (L-selectin), and the activation potential of $CD4^+CD8^+$dp T cells, as measured by stimulation-induced proliferation, and expression of surface markers was analyzed.

The second part of this work (BISMARCK et al. 2014) focuses on the progenitors of canine peripheral blood $CD4^+CD8^+$dp T cells and cellular conditions for $CD4^+CD8^+$dp T cell development. Since only single-positive T cells are supposed to emigrate from the thymus, the origin of peripheral blood $CD4^+CD8\alpha^+$dp T cells needed to be identified.

The third part (unpublished work) covers several topics: (i) a more detailed view of canine $CD4^+CD8^+$dp T cell subsets (ii) the distribution of canine $CD4^+CD8^+$dp T cells and their subsets in secondary lymphatic organs, and (iii) a precise functional characterization of IFN-γ production of canine $CD4^+CD8^+$dp T cells.

2 Results

2.1 1st publication:

Canine $CD4^+CD8^+$ double-positive T cells in peripheral blood have features of activated T cells

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- practical experimental work
- data acquisition
- data analysis and interpretation
- manuscript writing

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- practical experimental work at beginning of the study

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- experimental design
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- manuscript writing

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Research paper

Canine CD4$^+$CD8$^+$ double positive T cells in peripheral blood have features of activated T cells

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ABSTRACT

In dogs a CD4$^+$CD8$^+$ double positive T cell subpopulation exists that has not been phenotypically defined yet. We demonstrate that canine CD4$^+$CD8$^+$ T cells are mature CD1a$^-$ and TCRαβ$^+$ T cells. To analyse the activation potential of CD4$^+$CD8$^+$ T cells, PBMC from dogs vaccinated against canine distemper virus (CDV) were re-stimulated with CDV. Upon antigen-specific stimulation, the CD4$^+$CD8$^+$ T cell fraction increases and consists nearly exclusively of proliferated cells. Similarly, other features of activated effector/memory T cells such as up-regulation of CD25 and MHC-II as well as down-regulation of CD62L (L-selectin) were observed in CD4$^+$CD8$^+$ T cells after stimulation. Canine CD4$^+$CD8$^+$ T cells are less abundant, but more heterogeneous than porcine ones, comprising a small proportion expressing the β chain of CD8 in addition to the CD8α chain, like human CD4$^+$CD8$^+$ T cells. In summary, this analysis provides the basis for functional characterisation of the in vivo relevance of CD4$^+$CD8$^+$ T cells in T-cell mediated immunity.

1. Introduction

T cells, as one essential part of the immune system, are divided into cytotoxic T cells (CTL) that, in addition to their TCRαβ, express the co-receptor CD8 and T helper cells with co-receptor CD4 on their surface. During thymic development T cells are present in a CD4$^+$CD8$^+$ double positive interim state. Moreover, in several species such as rat, macaque, chicken, man and especially the swine even in peripheral blood mature T cells expressing CD4 as well as CD8 have been characterised (reviewed in (Zuckermann, 1999)). In most of these species these cells were defined to be part of the memory T cell pool increasing with age and antigen contact (Akari et al., 1997; Pescovitz et al., 1994; Zuckermann, 1999). In swine most of them lack CD8β expression resulting in CD8αα homodimer positive cells (Saalmuller et al., 2002). Porcine double positive T cells were shown to be activated T helper cells depending on MHC-II antigen presentation as shown by CD4 and MHC-II blocking experiments (Summerfield et al., 1996). In addition to MHC-II-mediated antigen presentation, human CD4$^+$CD8$^+$ T cells can also be activated by MHC-I restricted epitopes, consistent with their expression of perforin and granzyme A as typical features of CTL (Nascimbeni et al., 2004). In humans an increase of cells expressing the CD4$^+$CD8$^+$ phenotype has been observed after vaccination as well as acute, chronic or persistent viral infections (Nascimbeni et al., 2004), Chagas disease

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(Giraldo et al., 2011) and cancer (Desfrancois et al., 2009, 2010; Sarrabayrouse et al., 2011). Analysing the immunostimulatory properties of parapoxvirus ovis (PPVO) on naïve canine peripheral blood mononuclear cells (PBMC) we also observed a $CD4^+CD8^+$ T cell population after in vitro stimulation (Schutze et al., 2009). This population was also found in freshly isolated non-stimulated canine blood leukocytes (Alexandre-Pires et al., 2010; Otani et al., 2008) but not in umbilical cord blood (Otani et al., 2008). Moreover, the proportion of $CD4^+CD8^+$ T cells was reported to increase after cultivation with IL-2 and concomitant stimulation with concanavalin A or CD3 stimulation of canine PBMC (Hoshino et al., 2008; Kato et al., 2007). However, so far canine $CD4^+CD8^+$ T cells have not been analysed for their phenotype and function. Here we present a phenotypic analysis of canine $CD4^+CD8^+$ T cells with respect to maturity, activation status, and expression of CD8α and CD8β. We show that about one third of $CD4^+CD8^+$ T cells are already constitutively activated and after stimulation these cells show a pronounced activation phenotype. In contrast to swine, canine double positive T cells are less abundant and appear more heterogeneously with a subpopulation expressing CD8αβ, pointing to different subtypes and potentially to distinct progenitors.

2. Materials and methods

2.1. Animals, blood

Blood was collected from 19 healthy dogs by venipuncture into heparinised vacutainer tubes (lithium-heparin S-Monovette, Sarstedt AG & Co., Nümbrecht, Germany). 12 dogs were of Beagle breed (6 females and 6 males, 2.5–6 years, all castrated) and belong to the College of Veterinary Medicine, University of Leipzig. The other 7 dogs (3 females, 1 castrated and 4 males, age range: 2.75 months–8 years) belong to private owners. 6 were of pure breed (2 Shiba Inus, 1 Labrador Retriever, 1 Miniature Australian Shepherd, 1 German Wirehaired Pointer, 1 Collie) and 1 was of mixed breed. All dogs were regularly vaccinated against canine distemper, rabies, canine infectious hepatitis, parvovirus infection, parainfluenza, and leptospirosis.

2.2. Virus preparations and reagents

Canine distemper virus (CDV), strain Onderstepoort, was grown in Vero cells. When 80% cythopathic effect (cpE) (syncytium formation) was reached, CDV infected Vero cells were freeze-thawed three times and cell debris was removed by low speed centrifugation. Afterwards CDV containing supernatant (10^6 $TCID_{50}$/ml) was heat-exposed at 95 °C for 30 min as described (Ghosh et al., 2001). For the mock control non-infected Vero cells were treated in the same way.

2.3. CDV-specific virus neutralisation assay

Canine plasma was heat-inactivated for 30 min at 56 °C. Plasma was diluted in DMEM medium (PAA Laboratories GmbH, Coelbe Germany) in two-fold dilution steps ranging from 1:20 to 1:2560. To each well 100 $TCID_{50}$ CDV was added. After 2 h of incubation Vero cells were added. CpE was detected by light microscopy after 4 days of incubation at 37 °C in a humidified atmosphere containing 5% CO_2. Neutralisation titres are given as the highest serum dilution still leading to 50% cpE free cultures.

2.4. Isolation and stimulation of PBMC

Peripheral blood mononuclear cells (PBMC) were isolated from heparinised canine whole blood by density gradient centrifugation using lymphocyte separation medium LSM 1077 (PAA Laboratories GmbH, Coelbe Germany). Subsequently, cells were washed once with phosphate buffered saline (PBS) and treated with erythrocyte lysis buffer (150 mM NH_4Cl, 8 mM $KHCO_3$, 2 mM EDTA; pH 7) for 10 min. PBMC were resuspended in cell culture medium (RPMI 1640; PAA Laboratories GmbH, Coelbe, Germany) supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin). Cells were stimulated and cultured in 96 well polystyrene flat-bottom plates (Beckton Dickinson, Heidelberg, Germany) at a concentration of 1×10^6 cells per 200 μl in complete culture medium for 7 days at 37 °C in humidified atmosphere containing 5% CO_2. Oligoclonal stimulation of cells was done with *Staphylococcus aureus* enterotoxin B (5 μg/ml; Sigma–Aldrich, Taufkirchen, Germany) or polyclonal stimulation with concanavalin A (5 μg/ml; Biochrom AG, Berlin, Germany). Viral re-stimulation was done with heat-inactivated CDV (MOI 0.025). Medium and Vero mock preparation served as controls.

2.5. CFSE proliferation assay

PBMC (1×10^7 cells/ml PBS) were stained with 5 μM carboxy fluorescein diacetate succinimidyl ester (CFSE; ebioscience, Frankfurt, Germany) at room temperature protected from light for 20 min. Unbound dye was captured by adding one half of the volume FBS to the stained cell suspension for 5 min. Afterwards, cells were washed twice with cell culture medium (RPMI 1640; PAA Laboratories GmbH, Coelbe, Germany) supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin) and stimulated with indicated stimuli for 7 days.

2.6. Flow cytometry

PBMC were stained before and after 7 days of stimulation. After staining with fixable viability dye eFluor780® (ebioscience, Frankfurt, Germany) according to manufacturer's description cells were incubated with a 1% dilution of a mixture of heat-inactivated rat/mouse/dog/rabbit normal serum (rat/mouse/dog: 5% each, rabbit: 10% in PBS) to block Fc receptors. The following antibodies were used for specific staining and as isotype controls: FITC-mouse IgG1 κ isotype control (clone P3.6.2.8.1) (ebioscience, Frankfurt, Germany), FITC- or PE- or APC-ratIgG2a κ isotype control (clone eBR2a) (all three: ebioscience, Frankfurt, Germany), PE- or APC-rat IgG1 κ isotype control (clone BRG1) (both: ebioscience, Frankfurt, Germany), PE-mouse IgG1 isotype control (MCA928PE) (Serotec, Morphosys AbD GmbH, Düsseldorf, Germany), PE-mouse IgG2b

κ isotype control (clone eBMG2b) (ebioscience, Frankfurt, Germany), APC-mouse IgG1 κ isotype control (clone P3.6.2.1) (ebioscience, Frankfurt, Germany), biotinylated mouse IgG1 isotype control (clone IS5-21F5) (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), FITC-mouse IgG1anti-dog CD3 (clone CA17.2A12) (Serotec, Morphosys AbD GmbH, Düsseldorf, Germany), FITC-or PE-rat IgG2a κ anti-canine CD4 (clone YKIX302.9) (first: ebioscience, Frankfurt, Germany; second: Serotec, Morphosys AbD GmbH, Düsseldorf, Germany), PE- or APC-rat IgG1 anti-canine CD8α (clone YCATE55.9) (first: Serotec, Morphosys AbD GmbH, Düsseldorf, Germany; second: ebioscience, Frankfurt, Germany), biotinylated mouse IgG1 anti-canine CD8β (clone CA 15.4G2) (Sonea et al., 2000), PE- or eFluor® 660-anti-canine CD25 (clone P4A10) (both: ebioscience, Frankfurt, Germany), Alexa Fluor® 647-rat IgG2a anti-canine MHCII (clone YKIX334.2) (ebioscience, Frankfurt, Germany), cross reactive PE-mouse IgG2b anti-human CD62L (clone FMC46) (Santa Cruz Biotechnology, Santa Cruz, California) (Schuberth et al., 2007). Mouse IgG1 anti-canine CD1a (clone CA13.9H11) (Looringh van Beeck et al., 2008), mouse IgG1 anti-canine TCRαβ (clone CA158G7) and mouse IgG2a anti-canine TCRγδ (clone CA20.6A3) (McDonough and Moore, 2000) were secondary labelled with FITC-anti mouse IgG (polyclonal antibody; manufacturer's designation: STAR9b) (Serotec, Morphosys AbD GmbH, Düsseldorf, Germany).

Acquisition of cell samples was done with BD FACS Canto II™ (Beckton Dickinson, Heidelberg, Germany) flow cytometer and analysed with BD FACS Diva™ 6.1.3. Living cells were gated using fixable viability dye eFluor780® and lymphocytes with respect to their size and granularity. Afterwards, doublet exclusion was performed using FSC-A vs. FSC-H. Only non-doublet, viable lymphocytes were included into subsequent analysis.

2.7. Statistical analysis

The two-tailed Mann–Whitney *U*-test was performed to determine the significance of differences in surface marker expression between T cell subpopulations. Data are presented as the mean ± SEM. The level of confidence for significance is depicted in figure legend. Correlation analysis was done calculating Spearman's rank correlation co-efficient.

3. Results

3.1. $CD4^+CD8^+$ T cells in canine peripheral blood are mature αβ T cells

Recently, we identified a population of $CD4^+CD8^+$ T cells by flow cytometry in ex vivo proliferating canine PBMC after stimulation with inactivated parapoxvirus ovis (iPPVO) (Schutze et al., 2009). Next, we looked whether this phenotype could also be found in freshly isolated PBMC. Among living cells lymphocytes were gated based on their forward and side scattering properties. After exclusion of cellular doublets in a FSC-A vs. FSC-H plot we focused on $CD3^+$ T cells and found an average of 2.44% (minimum: 1.4%; maximum: 4.2%; $n = 19$) $CD4^+CD8^+$ T cells (Fig. 1A). Most blood donors belonged to the Beagle breed ($n = 12$), but also in all individuals of other breeds studied ($n = 7$) the $CD4^+CD8^+$ T cell fraction was present at a similar rate. In Fig. 1B the percentage of $CD4^+CD8^+$ cells among T cells relative to age is depicted for the group of the 12 Beagle dogs. A correlation between the proportion of the double positive T cells and the age of the animals was found (Spearman's co-efficient $r_s = 0.4410$) looking at our study cohort consisting of the 12 adult Beagle dogs (Fig. 1B).

The $CD4^+CD8^+$ phenotype is well known for immature T cells during thymic maturation. Thus, after confirming CD1a as a marker for canine $CD4^+CD8^+$ thymocytes (Looringh van Beeck et al., 2008) (data not shown), we determined its expression on canine peripheral blood T cells. As depicted in Fig. 2B, $CD4^+CD8^+$ T cells in canine peripheral blood do not express the CD1a marker in contrast to monocytes used as internal positive control (data not shown). Two distinct T cell receptors can be found on canine T cells. In the peripheral blood TCRαβ is predominantly found on $CD3^+$ cells, however, especially in the gut also TCRγδ T cells are present. We analysed the T cell receptor chains expressed by canine $CD4^+CD8^+$ T cells. Comparable to $CD4^+$ and $CD8^+$ single positive T cells, $CD4^+CD8^+$ double positive T cells homogenously express TCRαβ on their surface, whereas TCRγδ was not present on blood derived $CD4^+CD8^+$ T cells (Fig. 2B). Taken together, $CD4^+CD8^+$ T cells in canine peripheral blood are mature T cells expressing exclusively TCRαβ.

3.2. $CD4^+CD8^+$ T cells have an activated phenotype

To investigate the activation potential of canine $CD4^+CD8^+$ T cells proliferation was studied by CFSE dilution assays. CFSE labelled PBMC were incubated for 7 days with medium or Vero mock, having only minor effects on the proportions of the T cell subpopulations (Fig. 3A (I)). Whereas only a marginal proportion of $CD4^+$ and $CD8^+$ single positive T cells have proliferated during the 7 day period of incubation with medium or Vero mock, a substantial proportion of the $CD4^+CD8^+$ T cells analysed after 7 days have proliferated pointing to constitutive activation of canine $CD4^+CD8^+$ T cells (Fig. 3A (IV)). Following oligoclonal stimulation with SEB, the proportion of $CD4^+CD8^+$ T cells increased (Fig. 3B (I)). While only a minority of $CD4^+$ and $CD8^+$ single positive T cells underwent proliferation (Fig. 3B (II) and (III)), about two thirds among the $CD4^+CD8^+$ T cell fraction have proliferated after 7 days of oligoclonal stimulation (Fig. 3B (IV)).

Moreover, we analysed the behaviour of canine $CD4^+CD8^+$ T cells upon recall antigen stimulation. To this end CDV was chosen as antigen, because vaccine-induced protection is conferred by cell-mediated immunity (Appel et al., 1984; Beauverger et al., 1996). Successful immunisation against CDV was confirmed by the detection of high titres (up to 6400) of CDV-neutralising antibodies (data not shown). PBMC of CDV immune dogs were re-stimulated with heat-inactivated CDV. Similar to the SEB stimulation the $CD4^+CD8^+$ T cell subpopulation increases after recall antigen stimulation (Fig. 3C (I)). In contrast to single positive T cells where only a portion of about 50% underwent proliferation in response to viral recall antigen stimulation

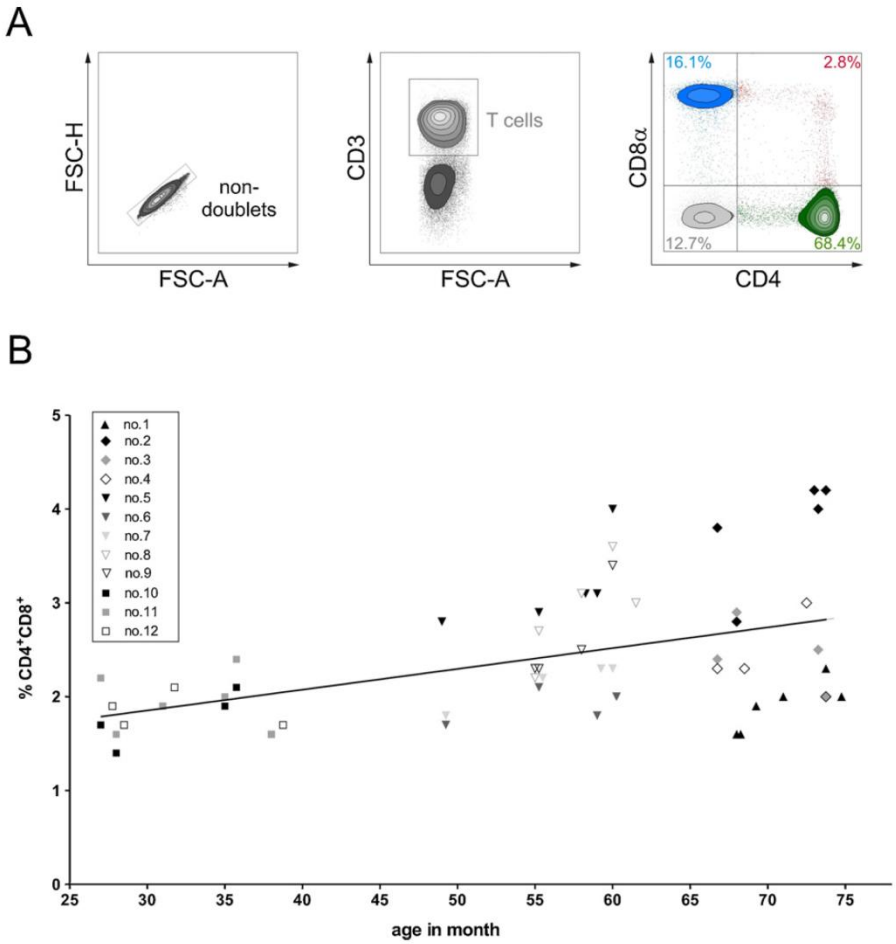

Fig. 1. Dogs possess a $CD4^+CD8^+$ T cell fraction in peripheral blood
PBMC were purified from blood samples (12 blood donors, beagle breed) and stained using antibodies against canine CD3, CD4 and CD8α. Living lymphocytes were gated with respect to their size and granularity. (A) After exclusion of doublets (left panel) $CD3^+$ T cells were gated (middle panel) and analysed for their CD4 and CD8α expression (right panel). Percentages of T cell subpopulations are depicted in each quadrant corner. (B) Relative abundance of $CD4^+CD8^+$ T cells in relation to age on day of blood sampling is presented for beagle breed blood donors (Mean: 2.44%; $n = 12$). Spearman's rank co-efficient ($r_s = 0.4410$).

(Fig. 3C (II) and (III)), nearly all cells within the increased $CD4^+CD8^+$ T cell fraction have proliferated after 7 days of stimulation (Fig. 3C (IV)). In conclusion, this shows that the double positive phenotype of canine T cells is acquired after in vitro antigen stimulation especially on proliferating T cells.

In addition to proliferation, activation markers CD25, CD62L and MHC-II were determined before and at the end of the 7 day in vitro stimulation period. As seen in Fig. 4 only on minor proportions of single positive T cells CD25 can be detected ex vivo and after medium or Vero mock control incubation. In contrast, one third of $CD4^+CD8^+$ T cells expresses CD25 directly after isolation as well as after medium or Vero mock incubation again suggesting a constitutive activation status (Fig. 4A). After SEB stimulation and CDV re-stimulation the proportion of CD25 expressing cells increases in all 3 T cell subpopulations (Fig. 4A). Especially the majority of $CD4^+CD8^+$ T cells (up to 90%) expresses CD25 on its surface (Fig. 4A). Taken together among $CD4^+CD8^+$ T cells a significant higher proportion expresses CD25 under all conditions tested.

Upon activation T effector cells down-regulate the expression of CD62L. After oligoclonal activation as well as viral re-stimulation CD62L expression at the single-cell level declines in all T cell subpopulations as shown by the mean fluorescence intensity (MFI) for CD62L (Fig. 4B). $CD4^+CD8^+$ T cells reach the lowest expression of CD62L after oligoclonal and viral recall antigen stimulation as compared with $CD4^+$ or $CD8^+$ single positive T cells emphasising a particularly high activation and effector status of canine $CD4^+CD8^+$ T cells.

Activated canine T cells increase expression of MHC-II on their surface (Doveren et al., 1985). In line with the expression of the other activation markers, although not statistically significant, a trend to higher expression of MHC-II on $CD4^+CD8^+$ double positive T cells than on $CD4^+$ or $CD8^+$ single positive T cells after stimulation can be seen. With SEB, MHC-II expression is reduced on $CD8^+$ T cells whereas $CD4^+$ and $CD4^+CD8^+$ T cells show an increase of MHC-II MFI. Stimulation with viral recall antigen showed a tendency of enhanced MHC-II expression in all 3 T cell subpopulations with the highest expression

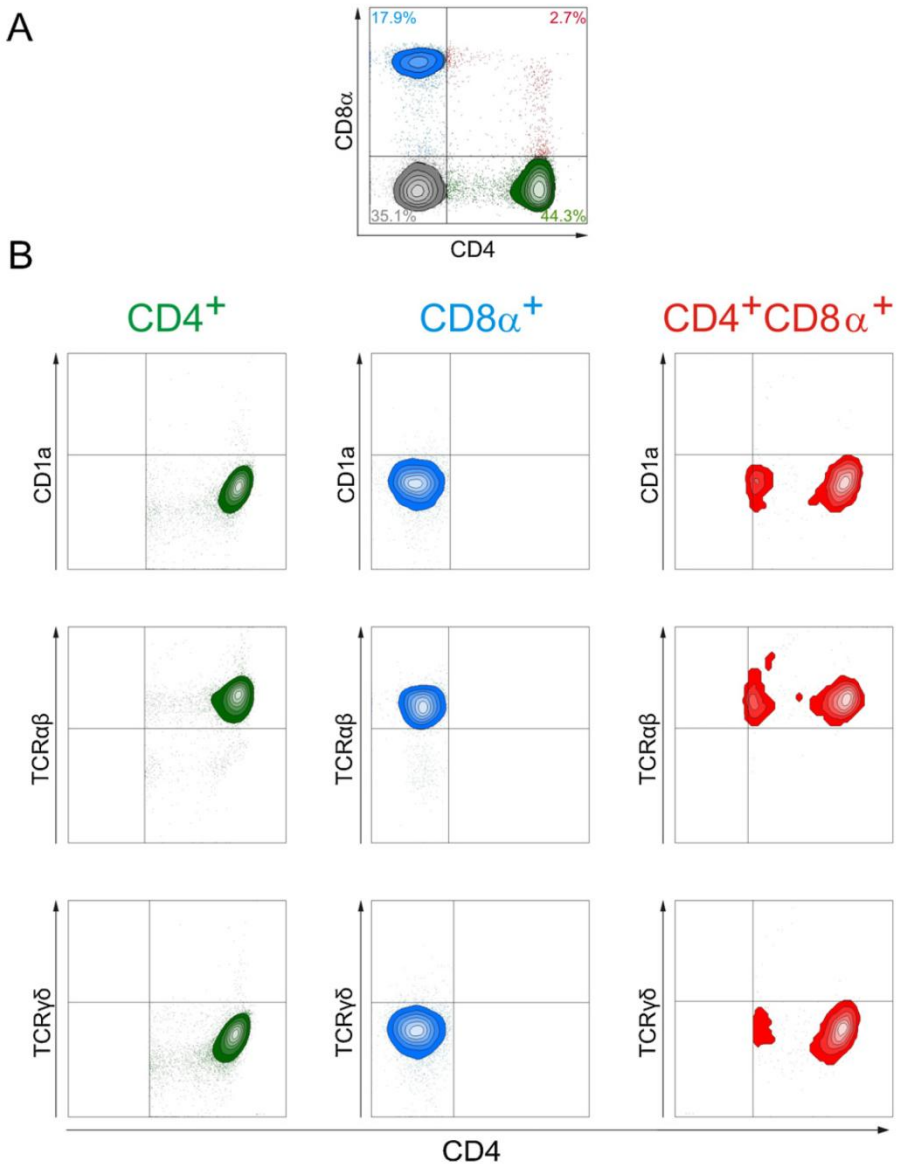

Fig. 2. CD4$^+$CD8$^+$ T cells have the phenotype of mature αβ T cells (CD3$^+$; CD1a$^-$; TCRαβ$^+$; TCRγδ$^-$)
Canine PBMC were gated for living cells. Doublets were excluded from cells in lymphocyte gate via FSC-H vs. FSC-A comparison. In (A) CD4 vs. CD8α plot is presented with defined quadrants for CD4$^+$ and CD8α$^+$ single positive cells and CD4$^+$CD8α$^+$ cells. Numbers in quadrants represent relative abundance of subpopulations. (B) Expression of thymic marker CD1a and TCRαβ and TCRγδ for the CD4$^+$ and CD8$^+$ single positive cells as well as CD4$^+$CD8α$^+$ cells. Results from a representative donor out of a total of 19 dogs are shown.

on CD4$^+$CD8$^+$ T cells. Thus, features of activation (CD25$^+$, CD62L^{dim}, MHC-II^{bright}) can be observed on freshly isolated and ex vivo stimulated CD4$^+$CD8$^+$ T cells. Oligoclonal and recall antigen-induced proliferation of T cells leads to a higher proportion of CD4$^+$CD8$^+$ T cells with a pronounced activation profile.

3.3. *CD4$^+$CD8$^+$ T cells consist of different subpopulations with CD4$^+$CD8α$^+$ and CD4$^+$CD8αβ$^+$ expressing T cells*

Canine CD3$^+$CD4$^+$CD8$^+$ T cells shown in Figs. 1–4 were characterised by anti-CD4 and anti-CD8α staining. The double positive T cells presented above, appeared rather heterogeneously regarding their CD4 and CD8α expression levels (see Figs. 1A, 2A, and especially Fig. 3A). Thus, we individually gated 3 major subpopulations of CD4$^+$CD8α$^+$ T cells, namely the CD4^{dim}CD8α^{bright}, CD4^{bright}CD8α^{bright} and CD4^{bright}CD8α^{dim}, as shown in Fig. 5A. We additionally analysed surface expression of CD8β on these three gated subpopulations of CD4$^+$CD8α$^+$ T cells and, for comparison, on the single positive helper and cytotoxic T cells. CD8β is not expressed on classical CD4$^+$CD8α$^-$ T helper cells (Fig. 5A). Interestingly, canine CD4$^-$CD8α$^+$ cytotoxic T cells show a heterogeneous expression of CD8β. A major proportion expresses CD8αβ, but a small proportion of CD8α$^+$ T cells is CD8β$^-$. This minor proportion

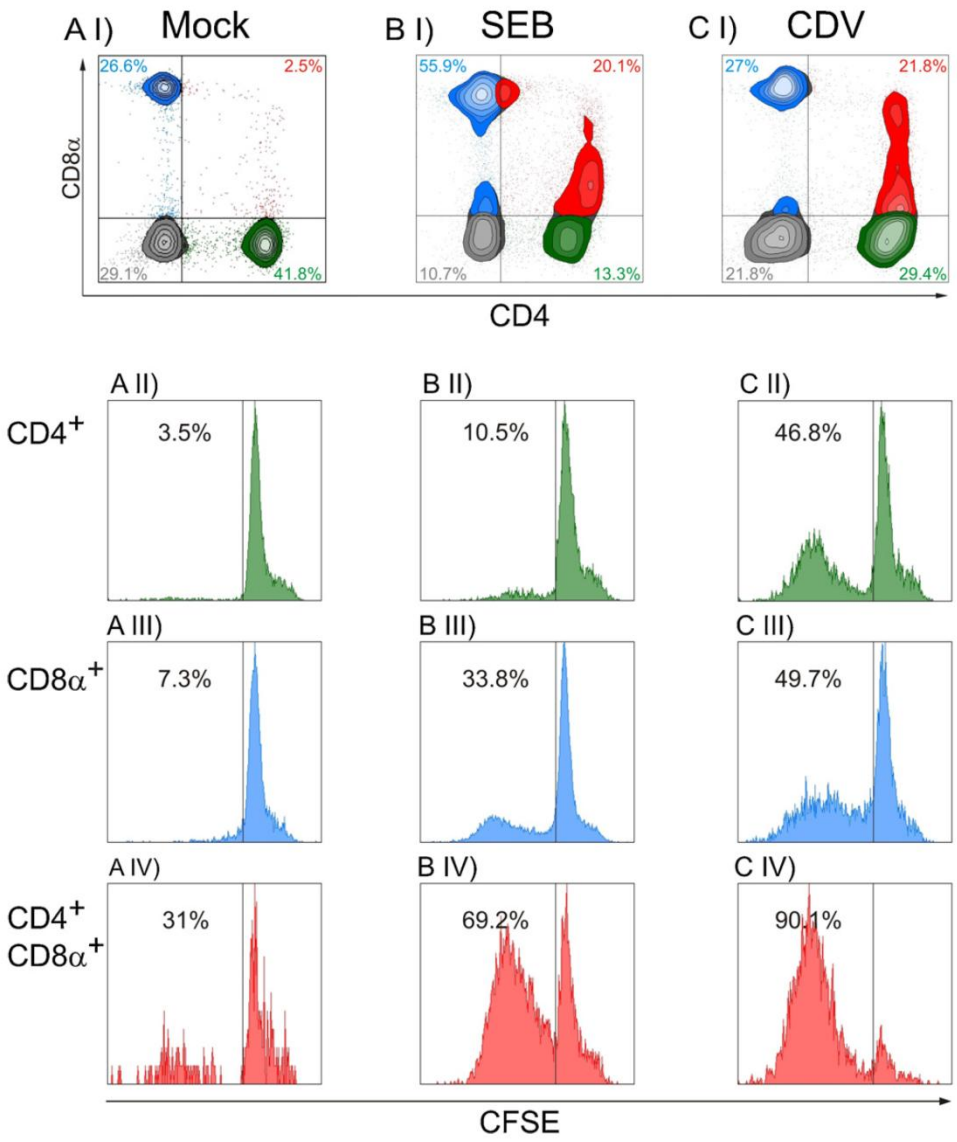

Fig. 3. Stimulation-induced canine T cell proliferation results in increased $CD4^+CD8^+$ proportions
Canine PBMC were stained with CFSE and stimulated for 7 days with oligoclonal stimulus SEB, viral recall antigen (CDV), or incubated with Vero mock control. In (A) CD4 vs. CD8α expression on lymphocytes after stimulation is depicted. Percentages of subpopulations are indicated in the quadrant corners. In (B) proliferation, indicated by CFSE dilution, is presented for $CD4^+$ and $CD8^+$ single positive T cells and $CD4^+CD8^+$ T cells. Proportion of $CFSE^{dim}$ cells (left) is indicated. Results from a representative donor out of a total of 19 dogs are shown.

of $CD4^-CD8\alpha^+CD8\beta^-$ T cells was found in 7 out of 7 analysed dogs (Fig. 5B) and was independent of the antibody dosing and of the preparation of the antibody used (i.e. purified biotinylated anti-CD8β vs. hybridoma supernatant; data not shown). Regarding $CD4^+CD8\alpha^+$ T cells we found that CD8β is present on the majority (about 2/3) of $CD4^{dim}CD8\alpha^{bright}$ T cells reaching nearly the percentage of CD8β expression in their $CD4^-CD8\alpha^+$ single positive counterparts. This high proportion can also be seen when only the cells showing the highest CD4 expression in this subpopulation are gated for the analysis excluding contamination with classical CTL. Both of the other $CD4^+CD8\alpha^+$ T cell subtypes ($CD4^{bright}CD8\alpha^{bright}$ and $CD4^{bright}CD8\alpha^{dim}$) show only minor CD8β chain expression (Fig. 5). In conclusion, canine $CD4^+CD8^+$ T cells are less homogeneous than their porcine counterparts. Whereas in the swine nearly all $CD4^+CD8^+$ T cells express only the α chain of CD8 (Saalmuller et al., 2002), canine $CD4^+CD8^+$ T cells partially consist of $CD4^+CD8\alpha\beta^+$ T cells. This places canine $CD4^+CD8^+$ T cells closer to human $CD4^+CD8^+$ T cells which partially also express the CD8αβ heterodimer (Parel and Chizzolini, 2004).

4. Discussion

T cells

are central players of the adaptive immune response. They are divided functionally in T helper

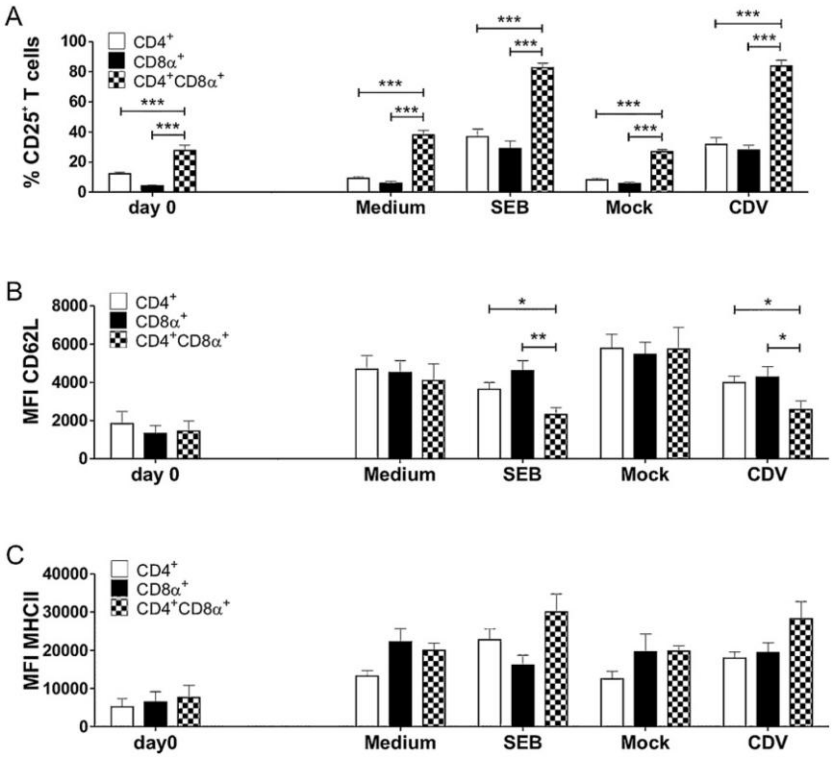

Fig. 4. Activation of $CD4^+CD8^+$ T cells induces elevated CD25 and decreased CD62L expression
PBMC were stimulated for 7 days with indicated stimuli. (A) Percentage of CD25 expressing cells within $CD4^+$ and $CD8^+$ single positive as well as $CD4^+CD8\alpha^+$ T cell fraction before and after stimulation is plotted ($n = 12$). Mean fluorescence intensity (MFI) indicating expression of (B) CD62L ($n = 12$) and (C) MHC-II ($n = 6$) for $CD4^+$ and $CD8^+$ single positive as well as $CD4^+CD8\alpha^+$ T cells is depicted. Statistical significant differences analysed by two-tailed Mann–Whitney *U*-test are indicated with arteriscs ($^*p < 0.05$; $^{**}p < 0.005$; $^{***}p < 0.001$).

cells and cytotoxic T lymphocytes (CTL). T helper cells phenotypically characterised by their co-receptor CD4 regulate other cells of the immune system by cytokine production and cell–cell interaction. On the other hand co-receptor CD8 expressing CTL are able to kill cells presenting non-self epitopes on MHC-I. We have analysed a third subpopulation expressing both CD4 and CD8, i.e. $CD4^+CD8^+$ double positive T cells, and identified them for the first time in dogs to be mature TCRαβ T cells ($CD1a^-CD3^+TCR\alpha\beta^+TCR\gamma\delta^-$). In canine blood we found a proportion of about 2.5% $CD4^+CD8^+$ T cells within the total T cell population which resembles rates described in healthy human volunteers (2–9%) (Blue et al., 1985; Nascimbeni et al., 2004). These cells have been described in other species such as rat, chicken, macaque, man and especially the swine, in the latter the population is most abundant (up to 60%) (Zuckermann, 1999). The proportion of $CD4^+CD8^+$ T cells correlates with age (Fig. 1B) as it was seen in humans, monkeys and especially the swine (Zuckermann, 1999; Zuckermann and Husmann, 1996). Gender does not seem to influence the $CD4^+CD8^+$ T cell proportion in dogs (data not shown).

$CD4^+CD8^+$ T cells were compared with their single positive counterparts for activation marker expression. Although MHC-II is constitutively expressed on canine T cells the higher expression of MHC-II on freshly isolated $CD4^+CD8^+$ T cells is indicative for their activated status (Cobbold and Metcalfe, 1994). This was also reported for porcine $CD4^+CD8^+$ T cells (Saalmuller et al., 2002). Even after oligoclonal as well as viral recall antigen stimulation, when MHC-II is up-regulated on all three major T cell subpopulations, $CD4^+CD8^+$ T cells still show a trend to higher expression of this activation marker. CD62L, mediating T cell homing into secondary lymphatic organs, is a marker for naïve and central memory T cells (T_{CM}) (Beverley, 2008). Thus, $CD4^+CD8^+$ T cells are characterised as effector cells (T effector or T memory/effector) by their low CD62L after SEB or recall antigen stimulation with CDV. A further characterisation as T effector or T effector/memory cells was impossible due to the lack of an established memory marker in the canine system (Lebrec et al., 2012). Another feature of T cell activation is the induction of CD25 expression to provide the high affinity receptor for IL-2, the cytokine especially essential for T cell proliferation. This antigen is detected on the vast majority of $CD4^+CD8^+$ T cells stimulated by SEB as well as viral recall antigen, whereas stimulated $CD4^+$ and $CD8^+$ single positive T cells are only partially $CD25^+$. Moreover, about one third of $CD4^+CD8^+$ T cells even show constitutive CD25 expression demonstrated in freshly isolated $CD4^+CD8^+$ T cells. This is maintained during 7 days of incubation in medium or Vero mock. In conclusion, the double positive phenotype in canine T cells is related to activation similar as it was reported for other species, e.g. rat, man and especially the swine (Zuckermann, 1999).

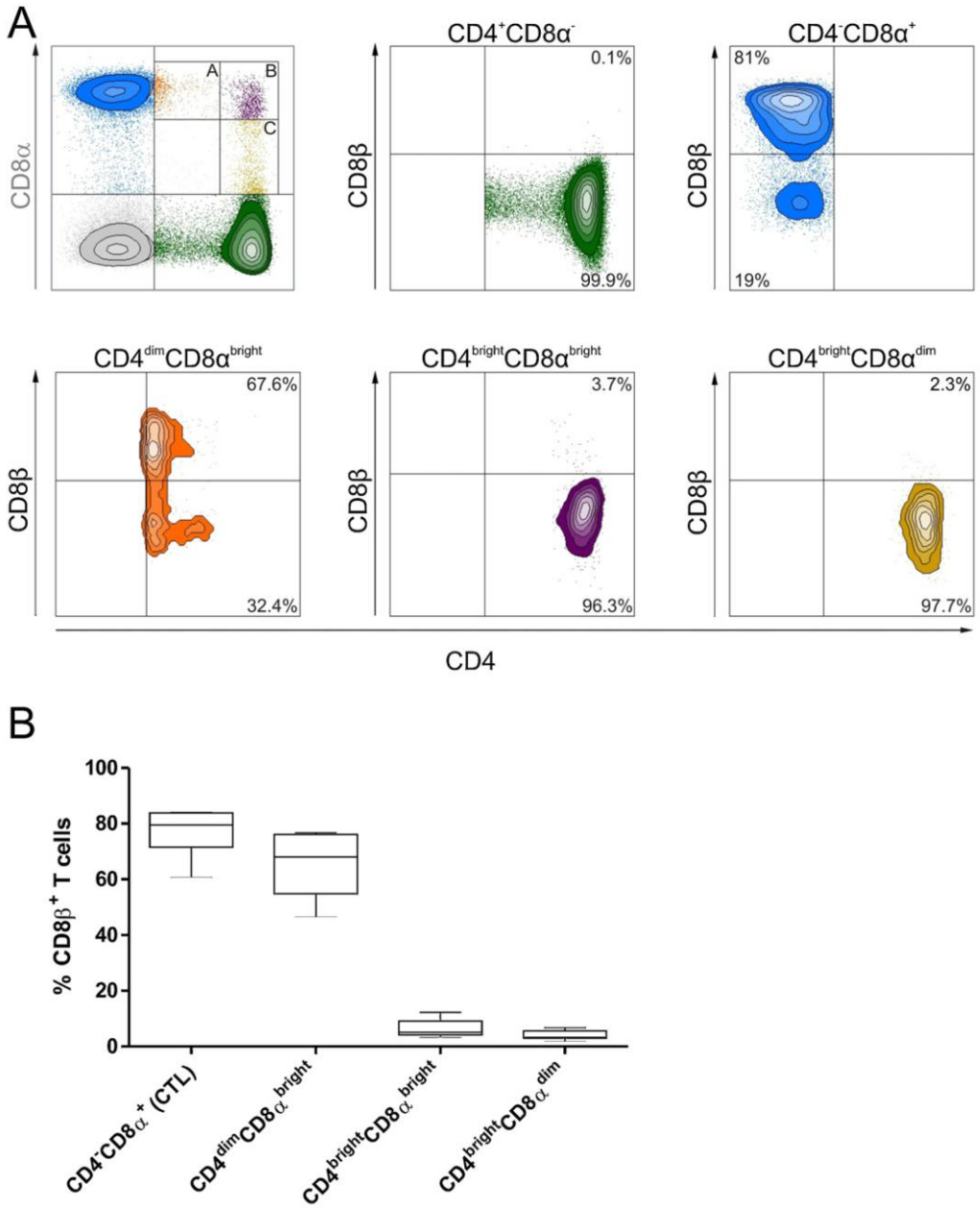

Fig. 5. Subpopulations of canine $CD4^+CD8^+$ T cells differ in the level of CD8β chain expression
(A) Canine PBMC were gated for living cells. Doublets were excluded from cells in lymphocyte gate via FSC-H vs. FSC-A comparison. Then $CD3^+$ T cells were analysed according to their CD4 vs. CD8α expression. Presented is the CD8β expression of $CD4^+CD8\alpha^-$ (i.e. $CD4^+$ single positive T helper cells), $CD4^-CD8\alpha^+$ (i.e. $CD8^+$ single positive cytotoxic T cells), and of the three subpopulations of double positive $CD4^+CD8\alpha^+$ T cells (i.e., (A) $CD4^{dim}CD8\alpha^{bright}$ (B) $CD4^{bright}CD8\alpha^{bright}$ and (C) $CD4^{bright}CD8\alpha^{dim}$). In (B) relative abundance of CD8β expressing cells is depicted for CTL and the three subpopulations of $CD4^+CD8^+$ T cells ($n = 7$).

To have a closer look on their functional properties we analysed proliferative responses of canine T cells upon oligoclonal stimulation with SEB or antigen-specific recall with CDV. The high proportion of proliferating cells among $CD4^+CD8^+$ T cells after 7 days of medium or mock control incubation was unexpected (Fig. 3A (IV)). It might be explained by the constitutive expression of CD25 on about 30% of freshly isolated $CD4^+CD8^+$ T cells (see Fig. 4A) which might respond to IL-2 constitutively expressed by PBMC (Scheffold et al., 2005). However, after SEB stimulation about two thirds and after viral recall antigen stimulation nearly all of the $CD4^+CD8^+$ T cells underwent proliferation. This suggests that $CD4^+CD8^+$ T cell development is induced during memory cell activation and proliferation. In the porcine system only the $CD4^+CD8^+$ T cells compose the memory T helper cell pool (Saalmuller et al., 2002). It

is noteworthy that, in contrast, canine memory T helper cells are present not only within $CD4^+CD8^+$ T cells but also within the $CD4^+$ single positive fraction, demonstrated by their proliferation in response to viral recall antigen stimulation (Fig. 3C (II)). Even using heat-inactivated CDV a substantial proportion of $CD8^+$ T cells has been activated, demonstrated by its proliferation and activation marker expression (Figs. 3 and 4). This might be due to cross presentation of viral antigens as it was shown for measles virus in humans (van der Vlist et al., 2011).

Whether canine memory cells reside in the $CD4^+CD8^+$ fraction itself or can develop from the $CD4^+$ and/or $CD8^+$ single positive T cell population during stimulation or both is interesting and will be a subject of future research. A considerable portion of the $CD4^+CD8^+$ T cells shows a $CD4^{bright}CD8\alpha^{dim}$ phenotype upon CDV stimulation suggesting that the $CD4^+CD8^{dim}$ double positive T cells develop from $CD4^+$ single positive T cells (Fig. 3C (I)). This would be consistent with the phenotype of human and porcine $CD4^+CD8\alpha\alpha^+$ activated T helper cells (Colombatti et al., 1998; Saalmuller et al., 2002). Analysing the CD8β expression we found a small proportion of $CD8\beta^-$ CTL which is reminiscent of feline CTL (Bucci et al., 1998) and especially canine intraepithelial T lymphocytes (Luckschander et al., 2009). A small proportion of $CD4^{dim}CD8\alpha^{bright}$ T cells seen after SEB stimulation (Fig. 3B (I)) that is even more prominent when the cells are treated with ConA (not shown) might indicate CD4 co-expression upon activation of CTL. This is supported by the finding that the majority of $CD4^{dim}CD8\alpha^{bright}$ cells expresses the β chain of CD8 (similar to the majority of canine CTL expressing $CD8\alpha\beta$). This can also be seen even when only cells with the highest expression of CD4 in this subpopulation are gated (not shown). In contrast, the other $CD4^+CD8^+$ subpopulations ($CD4^{bright}CD8^{dim}$ and $CD4^{bright}CD8^{bright}$) mainly consist of cells bearing CD8α only. Thus, further analysis of the individual subpopulations of canine $CD4^+CD8^+$ T cells should elucidate their functional properties and progenitors.

Taken together, our data link the phenotype of canine $CD4^+CD8^+$ T cells to activated effector/memory cells. Besides the possibility that $CD4^+$ T cells acquire the $CD4^+CD8^+$ phenotype during activation as it is known for porcine T cells (Saalmuller et al., 2002), also $CD8^+$ T cells might be the progenitors of some $CD4^+CD8^+$ T cells as reported in humans (Nascimbeni et al., 2004; Parel and Chizzolini, 2004; Schenkel et al., 2010). The elucidation of the progenitor cell(s) of the canine $CD4^+CD8^+$ T cells will be in the focus of future work. It is conceivable that CD8α bearing $CD4^+CD8^+$ T cells and $CD8\alpha\beta$ positive $CD4^+CD8^+$ T cells originate from different progenitors. In addition, functional characterisation of canine T cell subpopulations such as their cytokine profiles and/or cytotoxic activity as well as regulatory function is required to learn more about their in vivo relevance in T cell mediated immunity.

Conflict of interest

None of the authors has any conflict of interest that could influence (bias) the presented work.

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Research paper

Canine CD4$^+$CD8$^+$ double-positive T cells can develop from CD4$^+$ and CD8$^+$ T cells

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ABSTRACT

For a long time the expression of the CD4 and CD8 receptor on peripheral blood T cells was thought to be mutually exclusive. However, in canine peripheral blood, similar to other species as swine or human for example, mature CD4$^+$CD8$^+$ double-positive (dp) T cells exist which simultaneously express both surface receptors and have features of activated T cells. Canine CD4$^+$CD8$^+$dp T cells are heterogeneous and can be divided into three subpopulations by their intensity of CD4 and CD8α expression: CD4^{bright}CD8α^{bright}, CD4^{dim}CD8α^{bright} and CD4^{dim}CD8α^{dim}. The number of CD4$^+$CD8α$^+$dp T cells increases after *in vitro* stimulation of canine peripheral blood mononuclear cells (PBMC) raising the question of their progenitor(s). Thus, the aim of our study was to characterize the progenitor(s) of canine CD4$^+$CD8α$^+$dp T cells. By cell tracing experiments we identified both CD4$^+$ single-positive (sp) and also CD8α$^+$sp T cells as progenitors of canine CD4$^+$CD8α$^+$dp T cells after *in vitro* stimulation. CD4$^+$sp T cells almost exclusively upregulate a CD8αα homodimer, whereas CD8α$^+$sp T cells can become CD4$^+$CD8αβ$^+$ or CD4$^+$CD8αα$^+$. Even in the absence of other cells, highly purified CD4$^+$sp T cells can become double-positive upon *in vitro* stimulation, whereas highly purified CD8α$^+$sp T cells fail to do so. However, CD8α$^+$sp T cells can additionally express CD4 when stimulated in the presence of CD4$^-$CD8α$^-$ double-negative (dn) cells or more efficiently when stimulated in the presence of CD4$^+$sp T cells. Soluble factors secreted by CD4$^+$sp T cells are sufficient for the upregulation of CD4 on CD8α$^+$sp T cells, but direct cell–cell contact between CD4$^+$sp and CD8α$^+$sp T cells is more efficient. mRNA analysis shows that additional CD4 expression on CD8α$^+$sp T cells results from *de novo* synthesis. Thus, uptake of soluble CD4 or trogocytosis is less likely as mechanism for generation of canine double-positive T cells. CD4$^+$CD8α$^+$dp T cells are highly activated independent of their origin except when generated in coculture of CD8α$^+$sp T cells with CD4$^-$CD8α$^-$dn cells. Overall, in dog, CD4$^+$sp T cells are the more potent progenitors of CD4$^+$CD8α$^+$dp T cells compared to CD8α$^+$sp T cells.

1. Introduction

It is well known, that during thymic development immature CD4$^+$CD8$^+$ double-positive (dp) T cells either become mature CD4$^+$ or CD8$^+$ single-positive (sp). Therefore, it has been assumed for a long time that in peripheral blood mature T lymphocytes are either CD4$^+$sp or CD8$^+$sp.

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However, more recently it has been shown that in several species such as human, monkey, swine, rat, mouse and chicken (Zuckermann, 1999) also mature T lymphocytes exist, which simultaneously express both coreceptors on their surface and are designated $CD4^+CD8^+$ double-positive T cells.

In the canine species these double-positive T cells were also found in blood leukocytes (Alexandre-Pires et al., 2010; Otani et al., 2008). Their proportion increases after cultivation of peripheral blood mononuclear cells (PBMC) with IL-2 and concomitant stimulation with concanavalin A (ConA) or anti-CD3 (Hoshino et al., 2008; Kato et al., 2007). Our group observed an increase of the double-positive T cell fraction while analyzing the immune-stimulatory effect of *parapoxvirus ovis* on canine PBMC (Schütze et al., 2009). Subsequently, we demonstrated that also other stimuli such as antigen-specific activation of PBMC with canine distemper virus or oligoclonal and polyclonal stimulation using *Staphylococcus aureus* enterotoxin B (SEB) or ConA lead to an increase of the $CD4^+CD8^+$dp T cell fraction. We have shown that canine $CD4^+CD8^+$dp T cells in peripheral blood are mature TCRαβ T cells, which can be divided in three subpopulations by their intensity of CD8α and CD4 expression: $CD4^{bright}CD8\alpha^{bright}$, $CD4^{dim}CD8\alpha^{bright}$, and $CD4^{bright}CD8\alpha^{dim}$. Moreover, canine $CD4^+CD8\alpha^+$dp T cells differ in their CD8 receptor composition, which can consist of a CD8αα homodimer or a CD8αβ heterodimer similarly as $CD8\alpha^+$sp T cells. The CD8αα homodimer is expressed on all three subpopulations, whereas the CD8αβ heterodimer mainly can be found on the $CD4^{dim}CD8\alpha^{bright}$ subpopulation. Canine peripheral blood $CD4^+CD8\alpha^+$dp T cells are more activated compared to $CD4^+$sp or $CD8\alpha^+$sp T cells as shown by CD25 expression. The activation level of $CD4^+CD8\alpha^+$dp T cells increases further after antigen-specific, oligoclonal or polyclonal *in vitro* stimulation (Bismarck et al., 2012).

The increase of highly activated $CD4^+CD8\alpha^+$dp T cells after stimulation raises the question about the progenitor(s) of these canine double-positive T cells. Drawing conclusions from other species is not straightforward since there appear to be major differences in their features. Porcine $CD4^+CD8^+$dp T cells for example are known to be activated $CD4^+$ T helper cells, which become double-positive by upregulation of a CD8αα homodimer (Saalmüller et al., 2002). Later on they form the memory T helper cell pool and thus increase with age (Zuckermann and Husmann, 1996). In contrast to the more homogeneous porcine double-positive T cells, human $CD4^+CD8^+$dp T cells can be subdivided in three subpopulations similarly as canine double-positive T cells. Human double-positive T cells though express the CD8αβ heterodimer on both $CD8\alpha^{bright}$ subpopulations (*i.e.* $CD8\alpha^{bright}CD4^{dim}$ and $CD8\alpha^{bright}CD4^{bright}$) (Parel and Chizzolini, 2004). For human $CD4^+CD8^+$dp T cells only a small increase of $CD4^+CD8^+$dp T cells with age has been observed (Ghia et al., 2007). However, during autoimmune diseases human double-positive T cells occur in elevated frequency (Parel and Chizzolini, 2004). Both $CD4^+$sp and $CD8^+$sp T cells can be progenitors of human double-positive T cells (Blue et al., 1986).

The aim of our study was to determine whether canine $CD4^+$sp and/or $CD8^+$sp cells can develop to $CD4^+CD8^+$dp T cells. Furthermore, it was of interest to characterize the impact of the type of progenitor cell on the resulting CD8 receptor composition and activation status. In case of different progenitors, we wished to investigate their potentially distinct requirements for differentiation into each of the three subpopulations of canine $CD4^+CD8\alpha^+$dp T cells.

2. Materials and methods

2.1. Animals and blood

Blood was obtained repeatedly from 12 healthy dogs by venipuncture into heparinized vacutainer tubes (BD Vacutainer®, 10 ml, Li-Heparin 17 IU/ml, Beckton Dickinson, Heidelberg, Germany). All dogs had received routine vaccinations against canine distemper, rabies, canine infectious hepatitis, parvovirus infection, parainfluenza, and leptospirosis. 11 dogs were of Beagle breed (5 female, 6 male, all castrated, age range: 4.0–8.5 years) and belong to the College of Veterinary Medicine, University of Leipzig, Germany. The other dog (1 female mix breed; approximately 6 years) belongs to a private owner. The protocol for this study was approved by the Animal Care and Usage Committee of the Landesdirektion (state office) Sachsen in Leipzig, Germany (permit number: A 10/14).

2.2. Isolation of peripheral blood mononuclear cells

PBMC were isolated from heparinized canine whole blood by two consecutive density gradient centrifugations. For the first gradient a separation medium with a density of 1077 g/l (LSM 1077; PAA Laboratories GmbH, Coelbe, Germany/Bicoll Seperating Solution; Biochrom AG, Berlin, Germany) was used to eliminate granulocytes and erythrocytes. Subsequently, cells were washed once with phosphate buffered saline (PBS) and treated with erythrocyte lysis buffer (150 mM NH_4Cl, 8 mM $KHCO_3$, 2 mM EDTA; pH 7). Again, cells were washed with PBS and the second density gradient centrifugation was performed with a separation medium adjusted with PBS to a density of 1054 g/l to eliminate mainly thrombocytes. Finally, cells were washed twice with PBS containing 3% fetal bovine serum (FBS) (Invitrogen, Darmstadt, Germany).

2.3. Fluorescence activated cell sorting of freshly isolated PBMC for stimulation

After incubation of cells with a 3% dilution of a mixture of heat-inactivated rat/mouse/dog/goat normal serum to block Fc receptors, cells were stained with anti-canine CD4 and CD8α antibodies (for antibodies used see: flow cytometry). Cells were washed twice and resuspended in PBS containing 3% FBS (Invitrogen, Darmstadt, Germany). Sorts were performed with BD FACS Aria™ III (Becton Dickinson, Heidelberg, Germany) and cells were collected in complete cell culture medium (RPMI 1640 (PAA Laboratories GmbH, Coelbe, Germany/Biochrom AG, Berlin, Germany) supplemented with 10% FBS (Invitrogen, Darmstadt, Germany), 100 units/ml penicillin and 100 μg/ml

streptomycin). Three different lymphocyte subpopulations, after doublet exclusion, were sorted: (1) $CD4^+$ single-positive (sp), (2) $CD8\alpha^+$sp and (3) $CD4^-CD8\alpha^-$ double-negative (dn) cells. Purity of sorted cell populations was at least 99.1%. After sorting, cells were washed twice with complete cell culture medium.

2.4. Further processing and stimulation of sorted cells

Depending on the experimental setup some of the sorted cell populations were stained with a cell proliferation dye (Cell proliferation Dye eFluor® 450 or eFluor® 670; both: ebioscience, Frankfurt, Germany) according to manufacturer's description. Combinations of cell populations were resuspended in complete cell culture medium and cultured in 96-well polystyrene flat-bottom plates (Becton Dickinson, Heidelberg, Germany) ($CD4^+$sp cell tracer-positive (21.9–44.2% of total coculture) + $CD8\alpha^+$sp (21.9–44.3% of total coculture) + $CD4^-CD8\alpha^-$dn (24.6–49.7% of total coculture), or $CD8\alpha^+$sp cell tracer-positive (9.9–40.8% of total coculture) + $CD4^+$sp (32.6–57.1% of total coculture) + $CD4^-CD8\alpha^-$dn (22.9–33% of total coculture), or $CD8\alpha^+$sp cell tracer-positive (10.2–46.2% of total coculture) + $CD4^+$sp (53.2–89.8% of total coculture), or $CD4^+$sp cell tracer-positive (44.8–76.3% of total coculture) + $CD4^-CD8\alpha^-$dn (23.7–55.2% of total coculture), or $CD8\alpha^+$sp cell tracer-positive (39.9–68.5% of total coculture) + $CD4^-CD8\alpha^-$dn (31.5–60.1% of total coculture), or $CD4^+$sp cell tracer-positive (100% of total coculture), or $CD8\alpha^+$sp cell tracer-positive (100% of total coculture)) or in polycarbonate 96-transwell plates with 0.4 μm pore size (Corning, Lowell, MA, USA). Cells were seeded at a concentration ranging from 0.5×10^6 up to 1.0×10^6 cells per 200 μl and incubated for 4 or 7 days at 37 °C in humidified atmosphere containing 5% CO_2. Thereafter, stimulation of cells was done with *S. aureus* enterotoxin B (5 μg/ml; Sigma–Aldrich, Taufkirchen, Germany) or concanavalin A (5 μg/ml; Biochrom AG, Berlin, Germany). Medium incubation served as control.

2.5. Flow cytometry

Freshly isolated cells or cells after stimulation were stained with a live/dead dye (fixable viability dye eFluor780®; ebioscience, Frankfurt, Germany or LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit; life technologies, Darmstadt, Germany) according to manufacturer's description. Afterwards, they were incubated in a 3% dilution of a mixture of heat-inactivated rat/mouse/dog/goat normal serum to block Fc receptors. For flow cytometry staining the following antibodies were used: anti-canine CD4 (clone YKIX302.9) (ebioscience, Frankfurt, Germany), APC- or eFluor®-450- anti-canine CD8α (clone YCATE55.9) (ebioscience, Frankfurt, Germany), PE-anti-canine CD5 (clone: YKIX322.3) (ebioscience, Frankfurt, Germany), and PE-anti-canine CD25 (clone P4A10) (ebioscience, Frankfurt, Germany). Mouse IgG1 anti-dog CD8β (clone CA 15.4G2) (Serotec, Morphosys AbD GmbH, Düsseldorf, Germany) was detected using PerCP/Cy5.5-goat anti-mouse IgG (clone: poly 4053) (BioLegend, San Diego, CA, USA). As corresponding isotype controls FITC- or PE-ratIgG2a κ isotype control (clone eBR2a) (both: ebioscience, Frankfurt, Germany), APC-rat IgG1 κ isotype control (clone BRG1) (ebioscience, Frankfurt, Germany), PE-mouse IgG1 κ isotype control (clone P3.6.2.8.1) (ebioscience, Frankfurt, Germany), PE-mouse IgG2b κ isotype control (clone eBMG2b) (ebioscience, Frankfurt, Germany), and mouse IgG1 isotype control (BD biosciences Pharmingen™, San Diego, CA) were used.

Flow cytometric analyses of cell samples were done with a BD LSR Fortessa™ flow cytometer (Beckton Dickinson, Heidelberg, Germany) and analyzed with BD FACS Diva™ 6.1.3. Only living lymphocytes and lymphoblasts after doublet exclusion were included into subsequent analysis. Gates were set after the fluorescence minus one controls (FMO), which include all specific antibodies of the staining panel, except the one of interest, which is replaced by its isotype control.

2.6. Fluorescence activated cell sorting for PCR

For PCR analysis of CD4 and CD8α transcription, three different $CD4^+CD8\alpha^+$dp subpopulations (*i.e.* $CD4^{bright}CD8\alpha^{bright}$, $CD4^{dim}CD8\alpha^{bright}$, $CD4^{bright}CD8\alpha^{dim}$ cells) derived from freshly isolated PBMC were sorted. Sorts had a purity of at least 99.7%.

For PCR of $CD4^+CD8\alpha^+$dp cells, which developed from $CD8\alpha^+$sp cells after 4 days of ConA-stimulation, cells were first stained with a live/dead dye (fixable viability dye eFluor780®; ebioscience, Frankfurt, Germany) according to manufacturer's description. Staining of cells was done as mentioned above (see fluorescence activated cell sorting for stimulation). From *in vitro* ConA-stimulated and medium-incubated cells after exclusion of dead cells and doublets two or three subpopulations were sorted: (1) cell tracer-positive $CD8\alpha^+$sp cells, (2) cell tracer-positive $CD4^+CD8\alpha^+$dp cells, and (3) cell tracer-negative $CD4^+$sp cells. Sorts had a purity of at least 99.5%.

2.7. RNA isolation and real-time RT-qPCR

RNA isolation was done with RNeasy® Mini Kit or RNeasy® Micro Kit (Qiagen, Hilden, Germany) according to manufacturer's description. Homogenized cell lysates were stored in RLT buffer (Qiagen, Hilden, Germany) at −70 °C. For the subsequent column purification samples were thawed at 37 °C in a water bath. Afterwards, eluted mRNA was treated with DNase (1 unit per sample) (DNase I, RNase-free; Thermo Fisher Scientific GmbH, Schwerte, Germany) to eliminate genomic DNA. To protect the mRNA from RNases, a RNase inhibitor was added (20 units per sample) (RiboLock RNase Inhibitor; Thermo Fisher Scientific GmbH, Schwerte, Germany). Then, the mRNA was again purified with RNeasy® Mini Kit or RNeasy® Micro Kit (Qiagen, Hilden, Germany) to clear it from the DNase.

The isolated RNA was transcribed to cDNA with High capacity cDNA Reverse Transcription Kit (Applied Biosystems, Darmstadt, Germany) using the included random primer according to manufacturer's instruction.

PCR assays were performed with the iTaq TM Universal SYBR® Green Supermix (BIO-Rad, München, Germany) in

a Rotor-Gene® Q (Qiagen, Hilden, Germany) with a 5 min polymerase activation step and continued with 40 cycles of a three step PCR: denaturation at 95 °C for 15 s, annealing at 62 °C for 15 s and elongation at 72 °C for 30 s. PCR products were run on a 2% agarose gel and stained with ethidium bromide (Roth, Karlsruhe, Germany).

Primers were designed for Hprt1, CD4 and CD8α using Primer3 software (Untergasser et al., 2012) (Hprt1 (product size: 195 bp): forward CTACTGTAATGACCAGTCAACAGGG, reverse AGGGGTCCTTTTCACCAGCA; CD4 (product size: 290 bp): forward AAAACCTGGTCGGGGAACT, reverse GCACCTCAGGTCAGATTG; CD8α (product size: 191 bp): forward TCTGCAACCACAGGAACAGAA, rev CAAAAGGGAGGGACTGTTGC). CD4 mRNA and CD8α mRNA were quantified relative to Hprt1. Quantification was performed using relative quantification software of Rotor-Gene® Q (Qiagen, Hilden, Germany).

2.8. Statistical analysis

Statistical analysis was done based on the closed test principle. As global test the Kruskal–Wallis-*H*-test was used to examine significant differences between the three groups of medium incubation, ConA and SEB stimulation respective $CD4^+CD8\alpha^+$dp T cell development and CD25 expression for all coculture setups. Significance in the Kruskal–Wallis-*H*-test excluded multiplicity problems and allowed subsequent pairwise comparison with two-tailed Mann–Whitney-*U*-test. Data are presented with the mean. The level of confidence for significance is depicted in figure legends.

3. Results

3.1. Among peripheral blood $CD4^+CD8\alpha^+$dp T cells three distinct subpopulations can be defined by different methods

As already described in canine peripheral blood three different $CD4^+CD8\alpha^+$dp T cell subpopulations can be found on the basis of flow cytometric analysis which includes doublet exclusion: $CD4^{bright}CD8\alpha^{bright}$, $CD4^{dim}CD8\alpha^{bright}$, and $CD4^{bright}CD8\alpha^{dim}$ (Bismarck et al., 2012). Compared to the $CD4^{bright}CD8\alpha^{bright}$ subpopulation especially the $CD4^{dim}CD8\alpha^{bright}$ and the $CD4^{bright}CD8\alpha^{dim}$ subpopulations do not appear as distinct populations and one could easily mistake them for single-positive T cells (Fig. 1A). But, following a more rigorous gating strategy based on Fluorescence Minus One controls (FMO controls) already cells which express the additional coreceptor in a low intensity should count as double-positive cells (Fig. 1B). The FMO controls used contain every specific antibody of the staining panel except the one of interest which is substituted by its isotype control. To further verify these three subpopulations, they were sorted and analyzed with real-time RT-qPCR for CD4 mRNA and CD8α mRNA expression. Sorts had a purity of at least 99.7%. In $CD4^+$sp T cells CD4 mRNA and in $CD8\alpha^+$sp T cells CD8α mRNA could be detected (CD4 expression 7.26 relative to Hprt1; CD8α expression14.4 relative to Hprt1 from one preliminary experiment). In all three double-positive subpopulations CD4 and CD8α mRNA is present (Fig. 1A) ($CD4^{bright}CD8\alpha^{bright}$: CD4 mRNA 2.92 and CD8α mRNA 5.40 relative to Hprt1; $CD4^{dim}CD8\alpha^{bright}$: CD4 mRNA 0.22 and CD8α mRNA 10.41 relative to Hprt1; $CD4^{bright}CD8\alpha^{dim}$: CD4 mRNA 4.05 and CD8α mRNA 0.14 relative to Hprt1; preliminary median data from two experiments). It needs to be mentioned that also in highly purified $CD4^+$sp cells a very small amount of CD8α mRNA could be detected (Fig. 1A) (CD8α mRNA in $CD4^+$sp T cells: 0.01 relative to Hprt1) similarly as observed by others for transcribed, but instable CD8α mRNA precursor forms in human $CD4^+$sp T cells (Gao et al., 1996). However, the expression of CD8α mRNA in canine $CD4^{bright}CD8\alpha^{dim}$dp T cells is higher compared to the low expression in $CD4^+$sp T cells (Fig. 1A) (CD8α mRNA in $CD4^{bright}CD8\alpha^{dim}$dp T cells: 0.15 relative to Hprt1; *i.e.* 15.3-fold increase of CD8α mRNA in $CD4^{bright}CD8\alpha^{dim}$dp T cells compared to CD8α mRNA expression by $CD4^+$sp T cells; data from one preliminary experiment).

3.2. Cell proliferation dye can be used as a cell tracer to identify progenitors of $CD4^+CD8\alpha^+$dp T cells

As shown recently the number of canine $CD4^+CD8\alpha^+$dp T cells *in vitro* increases after antigen-specific restimulation and after polyclonal or oligoclonal stimulation with ConA or SEB, respectively (Bismarck et al., 2012). In this study, stimulation with ConA or SEB was chosen to identify the progenitor(s) of $CD4^+CD8\alpha^+$dp T cells because of their larger responder pool. Following *in vitro* stimulation, cells were analyzed by flow cytometry. After exclusion of dead cells, lymphocytes were gated based on their forward and side scattering properties. Cellular doublets were excluded and the remaining cells analyzed for their CD4 *versus* CD8α expression. To set proper gates for CD4 *versus* CD8α, FMO controls were used (Fig. 1B). The same gating strategy was used for other surface markers such as CD8β and CD25.

To investigate whether or not $CD4^+$sp or $CD8\alpha^+$sp T cells can be progenitors of the newly developed $CD4^+CD8\alpha^+$dp cells after *in vitro* stimulation we performed sort experiments. All sorted cell fractions had a purity of at least 99.1%. Gradient-purified PBMC were used to sort $CD4^+$sp, $CD8\alpha^+$sp, and as a source for antigen-presenting cells (APC) $CD4^-CD8\alpha^-$ double-negative (dn) lymphocytes. The $CD4^-CD8\alpha^-$dn cells include as APC B cells, some monocytes, dendritic cells, but they also include NK cells, $CD4^-CD8^-\alpha\beta$ T cells and γδ T cells.

We used a cell proliferation dye as a cell tracer. For example, highly purified $CD4^+$sp cells were stained with proliferation dye and afterwards added to proliferation dye-negative $CD4^-CD8\alpha^-$dn cells (Fig. 1C, left histogram). After 4 days of *in vitro* stimulation previously $CD4^+$sp cells still could be traced with the help of the proliferation dye (*i.e.* cell tracer). Cell tracer-positive, previously $CD4^+$sp T cells could be clearly distinguished from cell tracer-negative $CD4^-CD8\alpha^-$dn cells, even in case of proliferation (Fig. 1C, right histogram). Subsequently, we gated only on these cell tracer-positive $CD4^+$sp T cells and analyzed their CD8α expression *versus* cell tracer

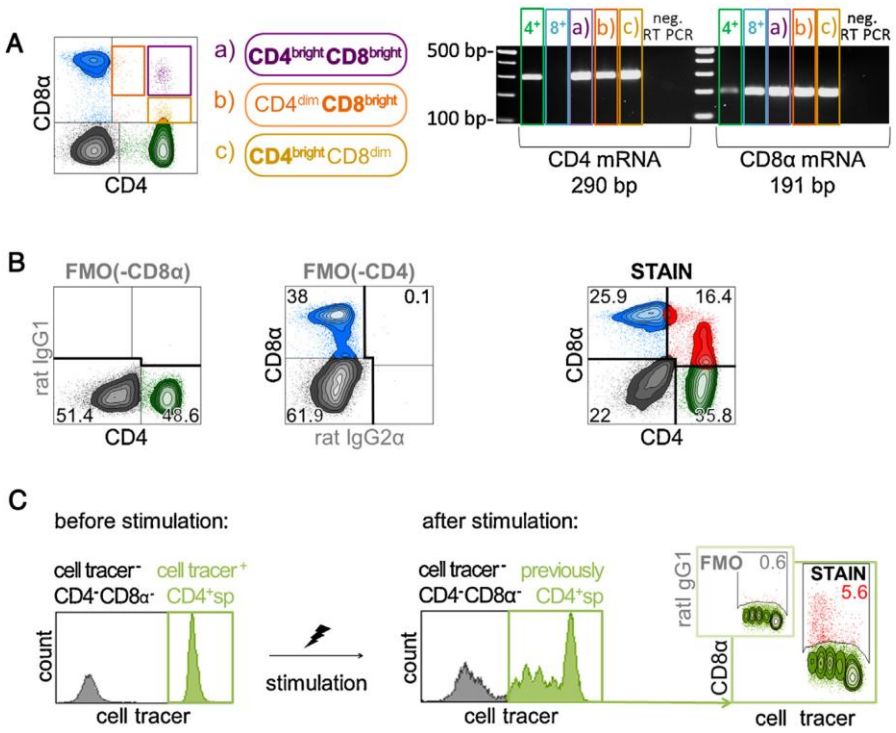

Fig. 1. Gating strategy for definitive analysis of CD4$^+$CD8α$^+$ double-positive T cell subpopulations and experimental setup for progenitor analysis. (A) CD4 and CD8α mRNA expression on CD4$^+$CD8α$^+$ double-positive (dp) T cell subpopulations: The FACS plot shows canine PBMC gated on lymphocytes after doublet exclusion (FSC-H *versus* FSC-W) for their CD4 *versus* CD8α expression. Among the CD4$^+$CD8α$^+$dp T cells three subpopulations can be defined: CD4^{bright}CD8α^{bright}, CD4^{dim}CD8α^{bright}, and CD4^{bright}CD8α^{dim}. Each of these three subpopulations, CD4$^+$sp, and CD8α$^+$sp cells were sorted (purity at least 99.7%) and analyzed for CD4 and CD8α mRNA. For the experiment shown the double-positive subpopulations were pooled from three dogs to reach sufficient cell numbers. Analysis of the gel shows the presence of CD4 and CD8α mRNA in the corresponding single-positive T cell subpopulation and in all three double-positive subsets. CD4$^+$sp T cells unexpectedly also show some CD8α mRNA expression as observed by others for human CD4$^+$sp T cells (Gao et al., 1996). Negative controls which contained water instead of RNA for the reverse transcription (RT) or water instead of cDNA for the PCR reaction were included. Shown is one of two experiments for mRNA analysis of CD4$^+$CD8α$^+$dp T cells (n = 2, 4 different dogs) and one experiment for mRNA analysis of CD4$^+$ and CD8α$^+$sp T cells. (B) Gates are set after Fluorescence Minus One controls (FMO controls): SEB-stimulated canine PBMC were stained using antibodies against canine CD4 and CD8α. Shown are living lymphocytes and lymphoblasts after doublet exclusion for their CD8α *versus* CD4 expression (right FACS plot). The gates were set after FMO controls (left and middle FACS plot), which include all antibodies of the staining panel, except the one of interest, which is substituted by its isotype control. Numbers in FACS plots represent percentages. (C) Cell proliferation dye was used as a cell tracer: Canine peripheral blood CD4$^+$ single-positive (sp) and CD8α$^+$sp T cells and CD4$^-$CD8α$^-$ double-negative (dn) cells in the lymphocyte gate, after doublet exclusion, were sorted (purity >99.1%). Shown is one representative experiment with CD4$^+$sp T cells stained with proliferation dye for cell tracing. Afterwards, these CD4$^+$sp cell tracer-positive cells were added to cell tracer-negative CD4$^-$CD8α$^-$dn cells (left histogram) and *in vitro* stimulated. After 4 days of stimulation with SEB cells were again stained with antibodies against canine CD4, CD8α and other antibodies of interest. Cell tracer-positive cells which were CD4$^+$sp before stimulation can be clearly identified (shown in green; right histogram) even when they proliferated. Next, we gated only on those previously CD4$^+$sp T cells for their CD8α expression *versus* cell tracer (FACS plot: STAIN). This way we were able to detect CD4$^+$CD8α$^+$dp cells developing from CD4$^+$sp T cells. Gates were set after the FMO controls (FACS plot: FMO). Likewise, this gating strategy was used for the other cell combinations with CD8α$^+$sp T cells cell tracer marked. Numbers in FACS plots represent percentages.

(Fig. 1C big contour plot). In cases where cells strongly proliferated and already became cell tracer-negative, we gated only on clearly cell tracer-positive cells and always left a safety distance to the cell tracer-negative peak. As described above, gates for surface antigens were set based on FMO controls (Fig. 1C small contour plot). This gating strategy also excludes false-positive signals from proliferating lymphoblasts with possibly high auto-fluorescence. Thus, using this experimental setup, we demonstrate that newly developed CD4$^+$CD8α$^+$dp T cells derive from CD4$^+$sp T cells. *Vice versa*, we also used the proliferation dye as a cell tracer for CD8α$^+$sp T cells. The time of stimulation (*i.e.* 4 or and 7 days) was adapted as a compromise of a good separation of cell tracer-positive/negative cells and the efficiency of generation of CD4$^+$CD8α$^+$dp T cells.

3.3. CD4$^+$sp and CD8α$^+$sp T cells can be progenitors of CD4$^+$CD8α$^+$dp T cells

To determine the progenitor(s) of *in vitro* developed CD4$^+$CD8α$^+$dp T cells highly purified CD4$^+$sp, CD8α$^+$sp, and APC-containing CD4$^-$CD8α$^-$dn cells were added together, thus representing the cellular composition of PBMC without CD4$^+$CD8α$^+$dp T cells. Either the CD4$^+$sp cells or the CD8α$^+$sp cells were cell tracer stained (coculture

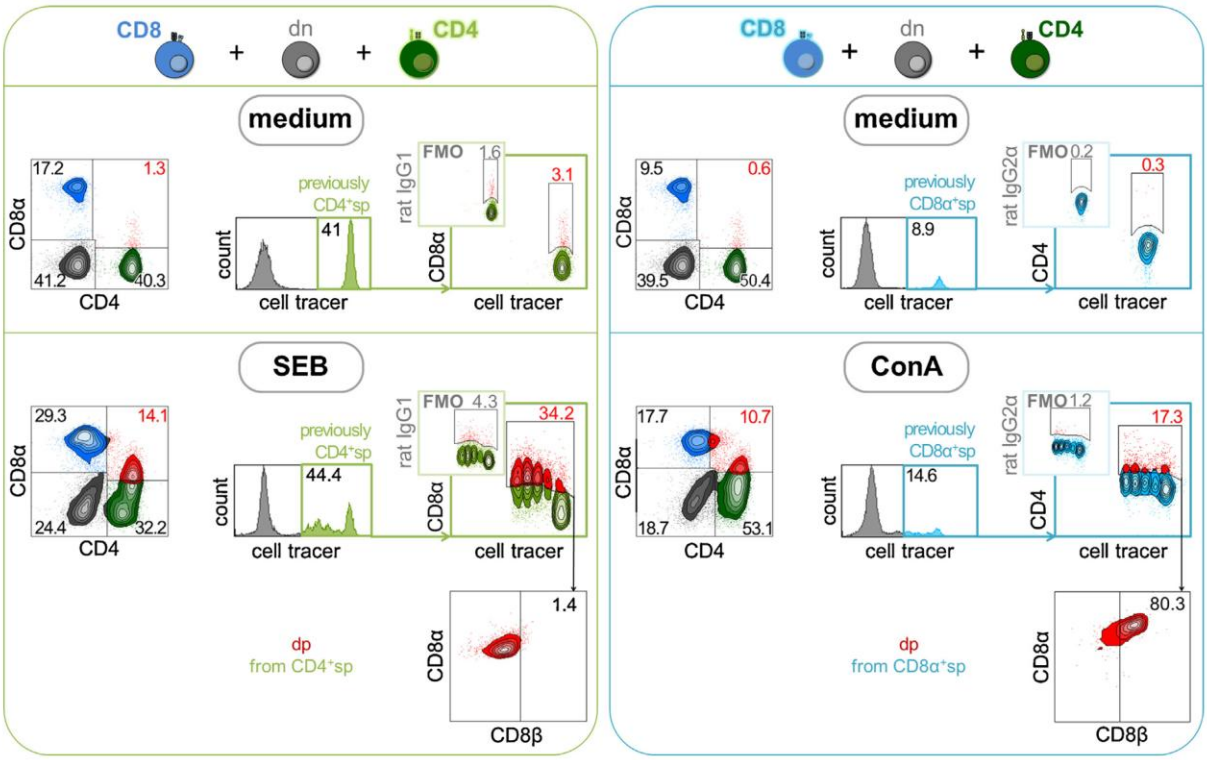

Fig. 2. Both $CD4^+$sp and $CD8\alpha^+$sp T cells can be progenitors of $CD4^+CD8\alpha^+$dp T cells. A mixture of sorted $CD4^+$sp, $CD8\alpha^+$sp and $CD4^-CD8\alpha^-$, excluding $CD4^+CD8\alpha^+$dp T cells, was *in vitro* stimulated with SEB (*Staphylococcus aureus* enterotoxin B) or ConA (concanavalin A) or medium-incubated for 4 days. To identify the progenitor of $CD4^+CD8\alpha^+$dp T cells (using the gating strategy shown in Fig. 1C) either $CD4^+$sp T cells (left panel) or $CD8\alpha^+$sp T cells (right panel) were stained with cell tracer. Shown is one representative experiment ($n = 5$, 5 different dogs). Compared to medium incubation, *in vitro* stimulation with SEB or ConA of these cocultures leads to the generation of new $CD4^+CD8\alpha^+$dp T cells. Only one stimulus for each setup is shown, but both stimuli (ConA and SEB) lead to the development of $CD4^+CD8\alpha^+$dp T cells from both progenitors. SEB is a better inducer of double-positive generation from $CD4^+$sp T cells and ConA from $CD8\alpha^+$sp T cells. Gating only on the previously $CD4^+$sp or $CD8\alpha^+$sp still clearly cell tracer-positive cells shows that $CD4^+$sp T cells can acquire an additional CD8α receptor. *Vice versa*, $CD8\alpha^+$sp T cells can upregulate an additional CD4 receptor. $CD4^+$sp T cells mainly generate $CD4^+CD8\alpha^+$dp T cells with $CD8\alpha\alpha^+$ (left panel, bottom row), whereas $CD8\alpha^+$sp T cells (consisting of $CD8\alpha\alpha^+$sp and $CD8\alpha\beta^+$sp) lead to both, $CD4^+CD8\alpha\alpha^+$ and $CD4^+CD8\alpha\beta^+$ (right panel, bottom row) ($n = 4$, 4 different dogs). Numbers in FACS plots represent percentages.

with cell tracer-positive $CD4^+$sp cells: Fig. 2, left panel; coculture with cell tracer-positive $CD8\alpha^+$sp cells: Fig. 2, right panel). These triple-cocultures were polyclonally (ConA) or oligoclonally (SEB) *in vitro* stimulated for 4 days. Upon stimulation a substantial portion of $CD4^+CD8\alpha^+$dp T cells develops compared to medium incubation which only shows little spontaneous development of $CD4^+CD8\alpha^+$dp T cells (Fig. 2). In the triple-coculture both, $CD4^+$sp and $CD8\alpha^+$sp T cells, can be progenitors of double-positive T cells. Shown is only one stimulus for each setup, but either stimulus (ConA or SEB) leads to the development of double-positive T cells from each progenitor. We found that SEB is a better inducer for $CD4^+$sp cells and ConA a more efficient inducer for $CD8\alpha^+$sp cells to become double-positive. $CD4^+CD8\alpha^+$dp T cells reproducibly develop upon stimulation of cells from different dogs (supplementary figure 1). The number of newly developed $CD4^+CD8\alpha^+$dp T cells derived from either progenitor after stimulation varies between dogs but remains significantly elevated compared with medium incubation.

Supplementary Fig. 1 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetimm.2014.09.008.

3.4. $CD4^+$sp T cells can become $CD4^+CD8\alpha^+$dp in the absence of other cells, in contrast to $CD8\alpha^+$sp T cells which remain single-positive

Next, we wished to analyze whether $CD4^+$sp or $CD8\alpha^+$sp T cells need the presence of other cells to become $CD4^+CD8\alpha^+$ double-positive. Thus, cell tracer-positive $CD4^+$sp or $CD8\alpha^+$sp T cells were *in vitro* stimulated in the absence of other cells for 7 days. $CD4^+$sp T cells upregulate an additional CD8 receptor, even in the absence of other cells, especially after SEB (Fig. 3, left panel) but also at a lower degree after ConA stimulation (data not shown). In comparison only very few double-positive T cells develop spontaneously after 7 days of medium incubation. In contrast to $CD4^+$sp T cells, $CD8\alpha^+$sp T cells remain single-positive after 7 days of *in vitro* stimulation (Fig. 3, right

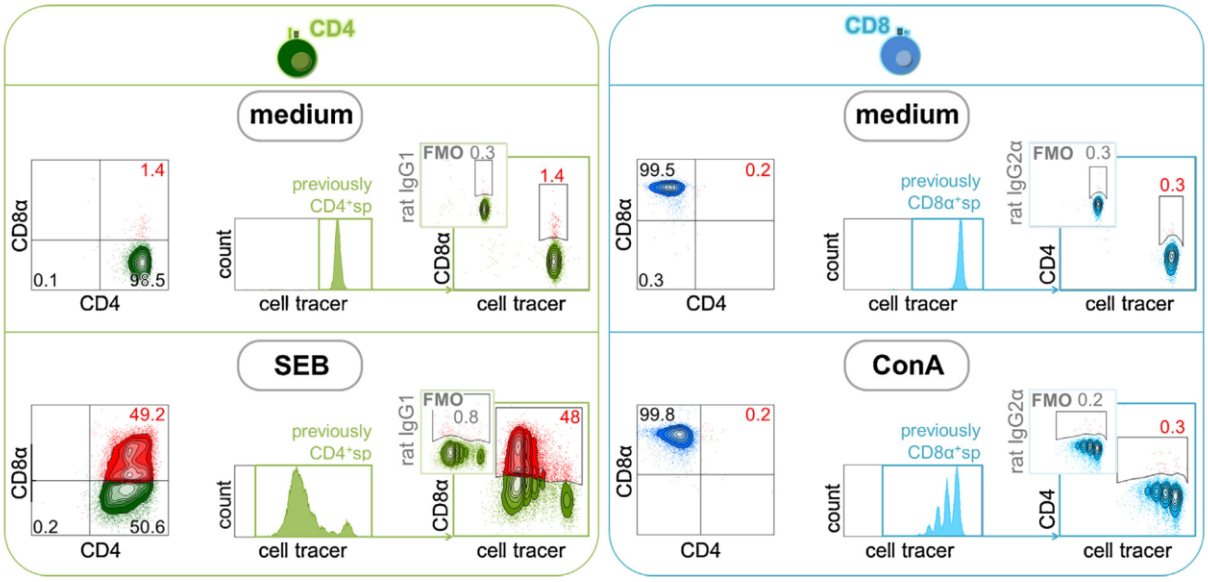

Fig. 3. CD4⁺sp, but not CD8α⁺sp T cells, can become double-positive after *in vitro* stimulation without the help of other cells. CD4⁺sp or CD8α⁺sp T cells were cell tracer-stained and stimulated for 7 days. CD4⁺sp T cells can become double-positive after *in vitro* stimulation with SEB (left panel) and ConA (data not shown). In contrary, CD8α⁺sp T cells do not become double-positive after ConA (right panel) or SEB (data not shown) stimulation. Shown is one representative example (CD4⁺sp: $n = 5$, 4 different dogs/CD8⁺sp: $n = 2$, 2 different dogs). Numbers in FACS plots represent percentages.

panel). However, after ConA stimulation, but not after SEB stimulation (data not shown), CD8α⁺sp T cells proliferate indicating their activation by the stimulus.

We also stimulated a coculture of cell tracer-positive CD4⁺sp T cells with cell tracer-negative CD4⁻CD8α⁻dn cells for 4 days. Not surprisingly, also in this combination CD4⁺CD8α⁺dp T cells develop (data not shown).

3.5. CD8α⁺sp T cells can become CD4⁺CD8α⁺dp with the help of CD4⁺sp T cells or CD4⁻CD8α⁻dn cells

Potentially, CD8α⁺sp progenitor cells need the help of CD4⁺sp T cells or CD4⁻CD8α⁻dn cells to give rise to CD4⁺CD8α⁺dp T cells. To clarify this, cell tracer-positive CD8α⁺sp T cells were *in vitro* stimulated either in the presence of CD4⁺sp T cells for 4 days (Fig. 4A, left panel) or in the presence of CD4⁻CD8α⁻dn cells for 7 days (Fig. 4A, right panel). In both cocultures, compared to some spontaneously developing double-positive T cells after medium incubation, a substantial proportion of CD8α⁺sp T cells upregulates the additional CD4 receptor in response to ConA (Fig. 4A) and SEB (data not shown). This shows that CD8α⁺sp T cells differ from CD4⁺sp T cells in their requirements for development of CD4⁺CD8α⁺dp T cells. In the presence of CD4⁺sp T cells the generation of CD4⁺CD8α⁺dp T cells from CD8α⁺sp T cells is more efficient than in the presence of CD4⁻CD8α⁻dn T cells.

3.6. Soluble factors of activated CD4⁺ T cells provide enough help for CD8α⁺sp T cells to become double-positive

To clarify the mechanism(s) of the CD4⁺sp T cell-mediated help in development of CD4⁺CD8α⁺dp T cells from CD8α⁺sp T cells, CD4⁺sp and CD8α⁺sp T cells were cultured together in a transwell system. Thereby cell–cell contacts are blocked by a membrane which is impermeable for cells but permeable for soluble factors which can be exchanged between the chambers. CD4⁺sp cell tracer-negative T cells were seeded in the lower chamber and CD8α⁺sp cell tracer-positive T cells in the upper chamber. Not surprisingly, CD4⁺sp T cells located in the lower chamber can become double-positive (data not shown), since singly stimulated they already can co-express CD8α (as shown above in Fig. 3, left panel). Interestingly, after SEB stimulation in this transwell system CD8α⁺sp T cells located in the upper chamber can also upregulate the additional CD4 coreceptor albeit at a smaller rate (Fig. 4B). SEB-induced double-positive T cell development in the transwell system is probably due to soluble factors secreted from activated CD4⁺sp T cells or their double-positive descendants. In contrast, highly purified CD8α⁺sp T cells stimulated with SEB in the absence of other cells remain single-positive, and neither proliferate nor get activated (data not shown). Different from SEB, ConA stimulation in the transwell system does not lead to the upregulation of CD4 on CD8α⁺sp T cells ($n = 2$, 2 different dogs, data not shown).

3.7. The CD4⁺coreceptor on CD8α⁺sp T cells is de novo synthesized and not the effect of receptor transfer

The appearance of the CD4 receptor on CD8α⁺sp T cells might be an effect of transfer of the CD4 receptor from CD4⁺sp to CD8α⁺sp T cells (trogocytosis, *i.e.* contact-dependent intercellular transfer of membrane fragments) (Rabinowitz et al., 1997; Eisenberg et al., 2013) or uptake of soluble CD4 receptor. To analyze for *de novo* synthesis of CD4 in CD8α⁺sp T cells we performed a second sort after

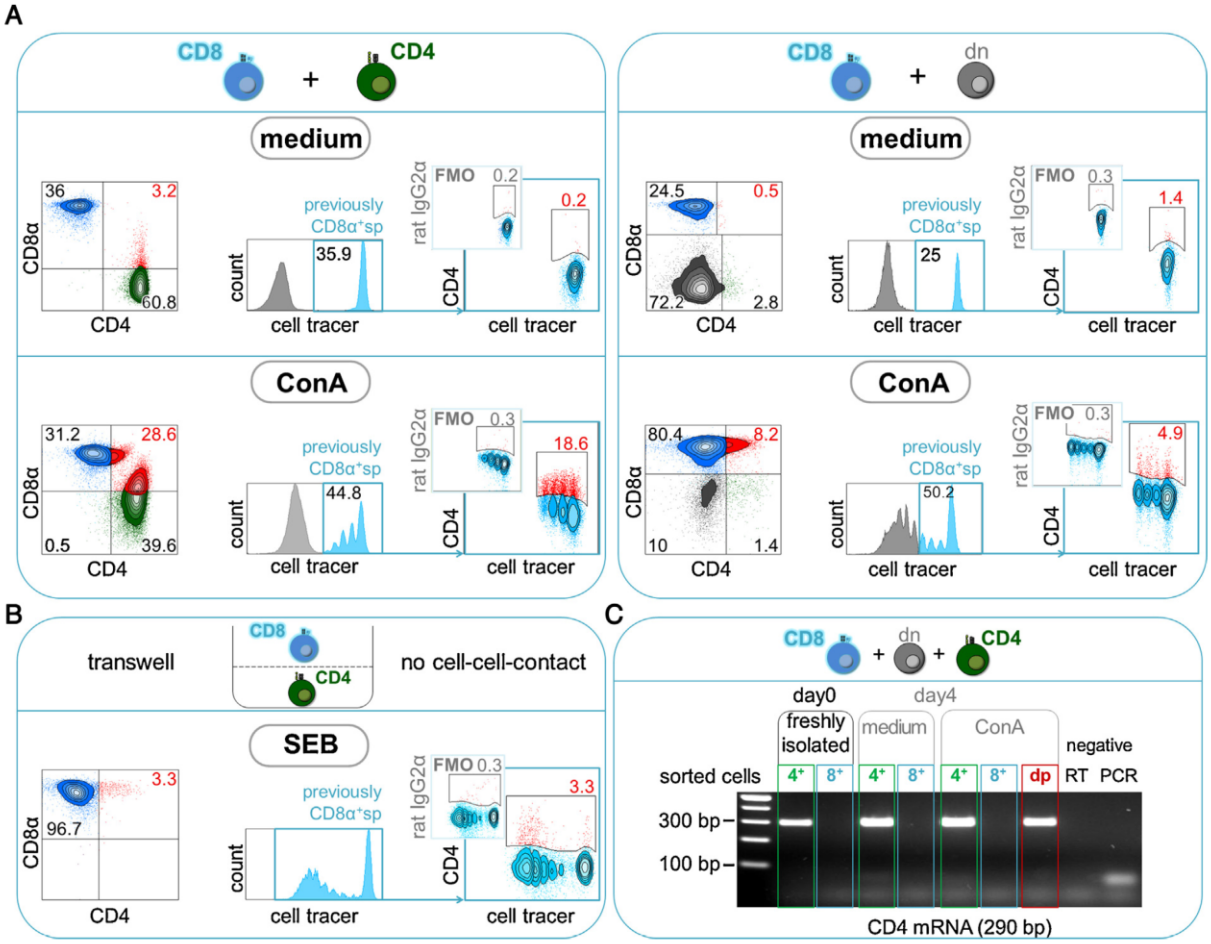

Fig. 4. CD8α⁺sp can become double-positive with help of CD4⁺sp or CD4⁻CD8α⁻dn cells. (A) CD8α⁺sp T cells were stained with cell tracer and either added to cell tracer-negative CD4⁺sp T cells (left panel) or cell tracer-negative CD4⁻CD8α⁻dn cells (right panel) and stimulated for 4 days (setup with CD4⁺sp) or 7 days (setup with CD4⁻CD8α⁻dn cells). With the help of CD4⁺sp or CD4⁻CD8α⁻dn cells CD8α⁺sp cells can upregulate an additional CD4 receptor. Shown is one representative experiment (CD8α⁺sp + CD4⁺sp: $n = 8$, 6 different dogs/CD8α⁺sp + CD4⁻CD8α⁻dn: $n = 5$, 5 different dogs). Numbers in FACS plots represent percentages. (B) CD4⁺sp T cells can secrete soluble factors which help CD8α⁺sp cells to become double-positive: Cell tracer-positive CD8α⁺sp cells were *in vitro* stimulated together with CD4⁺sp cell tracer-negative cells in a transwell system. Hence, cells are separated by a membrane non permissive for cells but for soluble factors. After 7 days of SEB stimulation, but not after ConA stimulation (data not shown), CD8α⁺sp T cells become double-positive. Shown is one representative experiment ($n = 3$, 3 different dogs). Numbers in FACS plots represent percentages. (C) The CD4 coreceptor on CD8α⁺sp T cells is *de novo* synthesized: The same experimental setup as in Fig. 2 (right panel) was used. Freshly isolated CD4⁺sp and CD8α⁺sp T cells were analyzed by RT-PCR. A second sort was performed after 4 days of medium incubation or ConA stimulation of a mixture of CD8α⁺sp cell tracer-positive and cell tracer-negative CD4⁺sp and CD4⁻CD8α⁻dn cells. Here, after medium incubation and ConA stimulation CD8α⁺sp cell tracer-positive and cell tracer-negative CD4⁺sp T cells were sorted. Additionally, in case of ConA stimulation, cell tracer-positive CD4⁺CD8α⁺dp T cells were sorted, which represent double-positive cells originating from CD8α⁺sp T cells. Sorts had a purity of at least 99.7%. RT-PCR analysis of these sorted cells revealed the presence of CD4 mRNA in CD4⁺CD8α⁺dp T cells, which develop from CD8α⁺sp T cells. Negative controls were included for the reverse transcription (RT) and the PCR reaction. Shown is one of three experiments of medium incubation and ConA stimulation ($n = 3$, 3 different dogs) and one experiment for analysis of freshly isolated CD4⁺ and CD8α⁺sp T cells.

4 days of ConA stimulation of the triple-coculture with CD8α⁺sp T cells cell tracer marked (experimental conditions shown in Fig. 2, right panel). CD8α⁺sp T cells, CD4⁺sp T cells, and double-positive T cells generated from CD8α⁺sp T cells were sorted. RT-PCR analysis of these sorted cells reveals CD4 mRNA in CD4⁺CD8α⁺dp T cells which developed from CD8α⁺sp T cells (Fig. 4C). Relative quantification in these CD4⁺CD8α⁺dp T cells shows detectable CD4 mRNA levels which are about 47-fold lower than in CD4⁺sp T cells as expected from the flow cytometry data (Figs. 1A and 2) (CD4 mRNA expression in sorted CD4⁺CD8α⁺dp T cells generated from CD8α⁺sp T cells: 0.0952 relative to Hprt1 compared to no CD4 mRNA expression in sorted CD8α⁺sp T cells after medium incubation or ConA stimulation; CD4 mRNA expression in sorted CD4⁺sp T cells after medium incubation 6.17 relative to Hprt1, in sorted CD4⁺sp T cells after ConA stimulation 4.47 relative to Hprt1; median from 3 experiments; 3 different dogs). CD8α mRNA expression ranged from 8.5 to 10.2 relative to Hprt1 in sorted CD4⁺CD8α⁺dp T cells and CD8α⁺sp T cells. These data indicate that CD8α⁺sp T cells *de novo* synthesize the additional CD4 coreceptor.

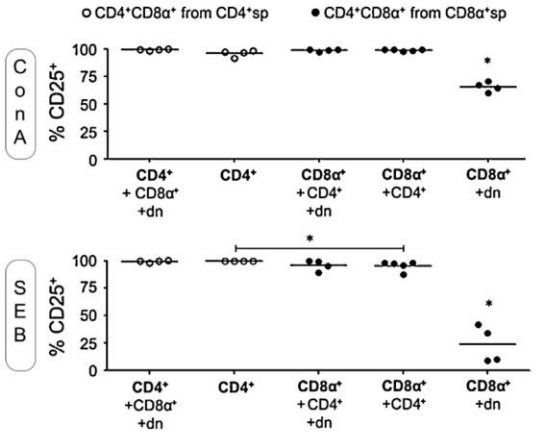

Fig. 5. Activation status of *in vitro* developed CD4$^+$CD8α$^+$dp T cells. Shown is the CD25 expression of CD4$^+$CD8α$^+$dp T cells which either developed from CD4$^+$sp or CD8α$^+$sp T cells after ConA or SEB stimulation under the different coculture conditions. Data are for cell tracer-positive cells only. The stimulation-induced CD4$^+$CD8α$^+$dp T cells show high CD25 expression independent of their origin except in the coculture of CD8α$^+$sp T cells with CD4$^-$CD8α$^-$dn cells. Statistical significant differences analyzed by two-tailed Mann–Whitney-*U*-test are indicated with asterisks ($^*p < 0.05$).

3.8. CD4$^+$sp T cells give rise to double-positive T cells expressing the CD8αα homodimer and CD8α$^+$sp T cells to double-positive T cells expressing the CD8αβ heterodimer or the CD8αα homodimer

Since canine CD4$^+$CD8α$^+$dp T cells can acquire a CD8αα homodimer or a CD8αβ heterodimer (Bismarck et al., 2012), we analyzed CD4$^+$CD8α$^+$dp T cells originating either from CD4$^+$sp T cells or from CD8α$^+$sp T cells for their CD8β expression. In all different coculture conditions CD4$^+$sp T cells almost exclusively upregulate a CD8αα homodimer (exemplarily shown for triple-coculture: Fig. 2, left panel, bottom row). The majority of CD4$^+$CD8α$^+$dp cells evolving from CD8α$^+$sp T cells though expresses a CD8αβ heterodimer, but a minor portion of CD4$^+$CD8α$^+$dp cells also expresses the CD8αα homodimer (exemplarily shown for triple-coculture: Fig. 2, right panel, bottom row) similarly as their CD8α$^+$ single-positive progenitors.

3.9. Activation state of in vitro developed CD4$^+$CD8α$^+$dp T cells

Canine CD4$^+$CD8α$^+$dp T cells display an activated phenotype, especially after *in vitro* stimulation (Bismarck et al., 2012). To investigate possible differences in the activation status of CD4$^+$CD8α$^+$dp T cells originating either from CD4$^+$sp or CD8α$^+$sp T cells we compared the expression of CD25. CD25 is equally expressed on all CD4$^+$CD8α$^+$dp T cells independent of their origin from either CD4$^+$ or CD8α$^+$sp T cells, except in the coculture setup of CD8α$^+$sp T cells with CD4$^-$CD8α$^-$dn T cells (Fig. 5). It is noticeable that CD8α$^+$sp T cells stimulated in the absence of CD4$^+$sp cells and only in the presence of CD4$^-$CD8α$^-$dn cells give rise to CD4$^+$CD8α$^+$dp T cells comprising a smaller portion expressing CD25 compared to all other coculture setups emphasizing again the importance of help by CD4$^+$sp T cells for proper CD8α$^+$sp T cell activation.

4. Discussion

In this study we have shown that canine CD4$^+$CD8α$^+$dp T cells can develop *in vitro* from both, CD4$^+$sp and CD8α$^+$sp T cells. Each single-positive T cell subpopulation shows distinct requirements for the upregulation of the additional coreceptor: *in vitro* stimulated CD4$^+$sp T cells are able to become double-positive in the absence of other cells (Fig. 3, left panel), whereas CD8α$^+$sp T cells fail to do so (Fig. 3, right panel). CD8α$^+$sp T cells either need the presence of CD4$^-$CD8α$^-$dn cells or even more efficiently upregulate the additional CD4 receptor in the presence of CD4$^+$sp T cells (Fig. 4A). Thus, clearly, canine CD4$^+$sp T cells are more potent progenitors of CD4$^+$CD8α$^+$dp T cells than CD8α$^+$sp T cells.

After *in vitro* stimulation the newly developed canine CD4$^+$CD8α$^+$dp T cells show a heterogeneity in their CD4 and CD8α expression. It is interesting that most of the double-positive T cells express the additional coreceptor at a low level (*e.g.* CD4^{bright}CD8α^{dim} and CD4^{dim}CD8α^{bright}) (Fig. 2). However, especially in the absence of other cells highly purified CD4$^+$sp T cells are also capable of a high CD8α expression after a longer stimulation period (7 days) (Fig. 3, left panel). In contrast, only few CD8α$^+$sp T cells in coculture with CD4$^-$CD8α$^-$dn cells reach a high level of CD4 receptor expression (Fig. 4, right panel).

Furthermore, newly developed CD4$^+$CD8α$^+$dp T cells show differences in their CD8 receptor composition. The CD8 receptor can occur as a CD8αα homodimer or as a CD8αβ heterodimer on canine CD8α$^+$sp and on CD4$^+$CD8α$^+$dp T cells (Bismarck et al., 2012). In all our coculture setups with CD4$^+$sp T cells as progenitors, CD4$^+$sp T cells almost exclusively upregulate the CD8αα homodimer (*e.g.* Fig. 2, left panel, bottom row). In contrast, CD4$^+$CD8α$^+$dp T cells evolving from CD8α$^+$sp T cells express the CD8αα homodimer and at a higher frequency the CD8αβ heterodimer (*e.g.* Fig. 2, right panel, bottom row). As can be seen in Fig. 2 (bottom row) discrimination between different intensities of CD8β expression (bright or dim) is not possible with the commercially available antibody.

The above mentioned different levels of CD4 and CD8α expression and the CD8 receptor structure of *in vitro* newly developed double-positive T cells enable us to draw conclusions for the development of the three subpopulations of CD4$^+$CD8α$^+$dp T cells in canine peripheral blood. For now, we can assume that the CD4^{dim}CD8α^{bright}dp subpopulation evolves from CD8$^+$sp T cells (*i.e.* CD8αβ$^+$sp and CD8αα$^+$sp T cells) (Bismarck et al., 2012) and thus expresses both CD8 receptor dimers; the CD4^{bright}CD8α^{dim}dp subpopulation evolves from CD4$^+$sp T cells and expresses almost exclusively the CD8αα homodimer. More diverse could be the origin of the CD4^{bright}CD8α^{bright} subpopulation. They could originate from CD4$^+$sp T cells, because of the potential high CD8αα expression after stimulation (Fig. 3, left panel). Nevertheless, there are also some CD8α$^+$sp cells which show high expression of CD4 after stimulation in the presence of CD4$^-$CD8α$^-$dn cells (Fig. 4A, right panel). Moreover, very few of CD8αβ$^+$ cells can be found in flow cytometric analysis among the CD4^{bright}CD8^{bright}dp subpopulation

(Bismarck et al., 2012). Therefore, at this point we cannot exclude that a minor portion of the $CD4^{bright}CD8\alpha^{bright}$ cells also originates from $CD8\alpha^+$sp cells. Finally, another possibility remains to be tested: the potential ability of canine $CD4^+CD8\alpha^+$dp cells to differentiate from one double-positive subpopulation into another.

In summary, in dog, $CD4^+$sp cells seem to have a higher potential to become double-positive in comparison to $CD8\alpha^+$sp cells. This somehow places the dog in between human and swine. For swine it is known that $CD4^+$sp cells can upregulate the additional CD8αα coreceptor in response to activation (Saalmüller et al., 2002). Porcine $CD8^+$ T cells do not upregulate a CD4 receptor after *in vitro* stimulation (Zuckermann and Husmann, 1996). In human, similar to dog, both single-positive subpopulations can become double-positive, but $CD8^+$ T cells are the more potent progenitors, at least after ConA stimulation (Blue et al., 1986). Also contrary to canine, in which $CD4^{bright}CD8\alpha^{bright}$ cells mainly express the CD8αα homodimer, the human equivalent expresses a CD8αβ heterodimer (Parel and Chizzolini, 2004). The progenitor of these cells in human was never analyzed and they are only present in very low frequencies, but increase in several autoimmune diseases (Parel and Chizzolini, 2004).

$CD8\alpha^+$sp T cells only become double-positive after stimulation in the presence of other cells (Fig. 2; left panel, Fig. 4A and B). $CD4^+CD8\alpha^+$dp T cell development from $CD8\alpha^+$sp T cells is more efficient in the presence of $CD4^+$sp T cells than in the presence of $CD4^-CD8\alpha^-$dn cells. In contrast to the coculture of $CD8\alpha^+$sp T cells with $CD4^-CD8\alpha^-$dn cells, in the coculture setup of $CD8\alpha^+$sp with $CD4^+$sp T cells double-positive T cell development could be a result of trogocytosis (contact-dependent intercellular transfer of membrane fragments). Trogocytosis has been shown to occur between melanoma antigen-specific cytotoxic T cells and their tumor target (Eisenberg et al., 2013). Similarly, $CD8\alpha^+$sp T cells could acquire their additional CD4 coreceptor from $CD4^+$sp T cells. Therefore, $CD8\alpha^+$sp T cells were stimulated together with $CD4^+$sp T cells in the transwell system where direct cell–cell contact between $CD4^+$sp and $CD8\alpha^+$sp T cells – and thus trogocytosis – is impossible. Still, after SEB stimulation in the transwell system, $CD8\alpha^+$sp T cells can upregulate an additional CD4 receptor. After ConA stimulation in the transwell system though no $CD4^+CD8\alpha^+$dp T cells develop. Probably, SEB as an oligoclonal stimulus activates $CD4^+$sp T cells in a more distinct manner than ConA leading to secretion of a cytokine profile required for the generation of $CD4^+CD8\alpha^+$dp T cells from $CD8\alpha^+$sp T cells. Taken together, if trogocytosis was involved in $CD4^+CD8\alpha^+$dp T cell development then it is clearly an additional mechanism. Nevertheless, it is noticeable that direct cell–cell contact between $CD4^+$sp and $CD8\alpha^+$sp T cells is more efficient in inducing $CD4^+CD8\alpha^+$dp T cell development from $CD8\alpha^+$sp T cells. In contrary to the transwell conditions, direct cell–cell contact between $CD4^+$sp and $CD8\alpha^+$sp T cells enables $CD4^+CD8\alpha^+$dp T cell development after ConA stimulation.

Another mechanism of passive receptor transfer could be uptake of soluble CD4. It is known that the CD4 receptor occurs not only cell-surface-bound on $CD4^+$sp cells but also soluble due to receptor shedding (Tseng et al., 2013). Soluble CD4 receptor could pass the transwell membrane and be integrated in the cell membrane of $CD8\alpha^+$sp T cells. However, in our experiments no double-positive T cells appear after ConA stimulation in the transwell system. Similarly, for human dp T cell development PHA stimulation was found ineffective under transwell conditions suggesting that soluble CD4 is less likely to play a role (Rabinowitz et al., 1997). Finally, to examine *de novo* synthesis of CD4 in $CD8\alpha^+$sp T cells we performed RT-PCR of sorted $CD4^+CD8\alpha^+$sp T cells which developed from $CD8\alpha^+$sp T cells after ConA stimulation of the triple-coculture (Fig. 4C). Indeed, we found CD4 mRNA in the sorted $CD4^+CD8\alpha^+$dp T cells. In conclusion, our results from the transwell setup combined with the PCR analysis demonstrate that CD4 on $CD8\alpha^+$sp T cells after ConA and after SEB stimulation can be *de novo* synthesized.

In human and swine the expression of an additional coreceptor on single-positive cells is associated with activation (Saalmüller et al., 2002; Sullivan et al., 2001). *In vitro* developed canine $CD4^+CD8\alpha^+$dp cells also highly express CD25, independent of their origin from $CD4^+$sp or $CD8\alpha^+$sp T cells with one exception (Fig. 5). It is only in the coculture of $CD8\alpha^+$sp T cells with $CD4^-CD8\alpha^-$dn cells that newly developed $CD4^+CD8\alpha^+$dp T cells are not highly activated (Fig. 5), but still they show a higher upregulation of CD25 compared to cells which remain single-positive (data not shown). Possibly, due to $CD4^-CD8^-$dn cells with suppressive function (D'Acquisto and Crompton, 2011) and/or lacking activation from $CD4^+$sp T cells in this coculture conditions newly developed $CD4^+CD8\alpha^+$dp T cells are not as strongly activated as in the other setups. $CD4^+$ T cell-mediated help for high activation of $CD8^+$sp T cells has been reported in several murine infection models, *e.g.* in herpes simplex virus 1 (HSV-1) (Smith et al., 2004) or lymphocytic choriomeningitis virus infection (LCMV) (Trautmann et al., 2014). $CD4^+$ T cell help beneficial for *ex vivo* expansion of human $CD8^+$ T cells, is at least partially provided by soluble factors (Butler et al., 2012) consistent with our findings in the transwell assay. Another marker of activation is proliferation. Analyzing cell tracer-positive cells *versus* CD4 or CD8α expression (Figs. 2, 3 (left panel), and 4), it is noteworthy that the additional coreceptor-upregulation is not necessarily associated with proliferation. In all conditions of double-positive T cell development used in this study, there is a fraction of nonproliferating cells which is able to become double-positive. In addition, $CD4^+$sp T cells show higher upregulation of CD8α with proliferation, whereas the intensity of CD4 expression on $CD8\alpha^+$sp T cells stays more constant. All in all, *in vitro* developed double-positive T cells after stimulation are almost completely highly activated which is different to peripheral blood $CD4^+CD8\alpha^+$dp T cells, where only about 30% are constitutively $CD25^+$ (Bismarck et al., 2012). A higher proportion of peripheral blood $CD4^+CD8\alpha^+$dp T cells expresses CD25 compared to $CD4^+$sp and $CD8\alpha^+$sp T cells though (Bismarck et al., 2012). Presumably, canine $CD4^+CD8\alpha^+$dp T cells act similarly to their porcine and human counterpart and downregulate CD25 with termination of activation

and progression to memory state (Saalmüller et al., 2002; Gonzalez-Perez et al., 2013).

Having identified different progenitors of canine peripheral blood $CD4^+CD8\alpha^+$dp T cells, future work will have to unravel their function. Porcine $CD4^+CD8\alpha^+$dp cells are known to build the T helper memory pool (Zuckermann, 1999). For human double-positive T cells it was shown that the additional CD4 receptor expressed on $CD8^+$sp T cells can respond to IL-16 ligation and induce cellular chemotaxis (Kitchen et al., 2002). Furthermore, human $CD4^+$ T cell clones which express the additional CD8αα receptor after IL-4 stimulation have an additional cytolytic function (Paliard et al., 1988). Consequently, it is possible that on canine $CD4^+CD8\alpha^+$dp T cells the additional receptor is more than a sole activation marker and conveys an additional function to the previously single-positive T cells.

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2.3 unpublished work:

Canine peripheral blood $CD4^+CD8^+$ double-positive T cells: a heterogeneous population which occurs also in lymph nodes and has high capacity of IFN-γ production

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- practical experimental work
- data acquisition
- data analysis and interpretation
- manuscript writing

Gottfried Alber:

- support to experimental design and data interpretation
- manuscript revision

Heiner von Buttlar:

- experimental design
- assistance with practical experimental work, data analysis and interpretation
- support to manuscript writing

Canine peripheral blood $CD4^+CD8^+$ double-positive T cells: a heterogeneous population which occurs also in lymph nodes and has high capacity of IFN-γ production

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Abstract

The long believed mutually exclusive expression of CD4 or CD8 on peripheral blood T cells was challenged by the existence of mature $CD4^+CD8^+$ double-positive (dp) T cells in several species such as swine, monkey, human, and also the dog. Canine peripheral blood $CD4^+CD8^+$dp T cells comprise a small but stable αβ T cell subset, which displays an activated phenotype as shown by expression of CD25 and CD62L (BISMARCK et al. 2012). They can develop from either $CD4^+$ or $CD8^+$ single-positive (sp) T cells after activation (BISMARCK et al. 2014). Canine $CD4^+CD8α^+$dp T cells are a heterogeneous population, which can be further subdivided into three distinct subsets by their intensity of CD4 and CD8α expression: $CD4^{dim}CD8α^{bright}$, $CD4^{bright}CD8α^{bright}$, and $CD4^{bright}CD8α^{dim}$. This chapter focuses on three different subjects concerning canine $CD4^+CD8α^+$dp T cells: (i) A more detailed analysis of the frequency of double-positive T cell subsets among the total double-positive T cell population and their CD25 and CD62L expression, (ii) first insights into the occurrence of double-positive T cells in secondary lymphatic organs, and (iii) an initial characterization of their functional properties by determination of IFN-γ production. Detailed analysis of the above mentioned activation markers CD25 and CD62L for each distinct double-positive T cell subset revealed a lower frequency of $CD25^+$ cells among the $CD4^{dim}CD8α^{bright}$ subset compared with the other two $CD4^{bright}$ subsets. CD62L expression showed the lowest frequency among the $CD4^{bright}CD8α^{bright}$ subset, which consequently includes a higher rate of effector cells compared with the other two subsets. Besides peripheral blood, also in lymph nodes and spleen double-positive T cells were found. Determination of IFN-γ production after *in vitro* stimulation of canine PBMC identified canine $CD4^+CD8α^+$dp T cells as very potent IFN-γ producers. Among double-positive T cell subsets the $CD4^{dim}CD8α^{bright}$ subset has the highest percentage of IFN-$γ^+$ cells. In conclusion this study covers a diverse range of topics on canine double-positive T cells, which together provide a deeper understanding of the uniqueness and dynamics of double-positive T cells in the canine immune system.

Introduction

In previous studies we have identified canine $CD4^+CD8^+$ double-positive (dp) T cells in peripheral blood as a small but stable mature T cell subpopulation with an activated phenotype (BISMARCK et al. 2012). The number of double-positive T cells increases after *in vitro* stimulation with viral recall antigen (*Canine distemper virus*), *Staphylococcus aureus* enterotoxin B (SEB), or concanavalin A (ConA). We showed that $CD4^+CD8\alpha^+$dp T cells can develop from both, $CD4^+$ single-positive (sp) and $CD8\alpha^+$sp T cells (BISMARCK et al. 2014). Canine $CD4^+CD8\alpha^+$dp T cells can be further subdivided into three different subsets by their intensity of CD4 and CD8α expression: $CD4^{dim}CD8\alpha^{bright}$, $CD4^{bright}CD8\alpha^{bright}$, and $CD4^{bright}CD8\alpha^{dim}$. This division is useful for a more detailed analysis of CD8β expression of double-positive T cells. It was shown that similar to $CD8\alpha^+$sp T cells only the $CD4^{dim}CD8\alpha^{bright}$ subset mainly expresses the CD8αβ heterodimer (BISMARCK et al. 2012). This is in close association with the fact that $CD8\alpha^+$sp T cells can be the progenitors of the $CD4^{dim}CD8\alpha^{bright}$ subset (BISMARCK et al. 2014). Until now, we only determined the activation phenotype of the total population of peripheral blood double-positive T cells by analyzing their expression of CD25 (activation marker) and CD62L (L-selectin / T effector and T effector memory cell marker) (BEVERLEY 2008). Therefore, in the first part of this study, we wished to extend our previous analysis to the individual double-positive T cell subsets. This provides the basis for a more detailed characterization of canine $CD4^+CD8\alpha^+$dp T cells.

In the second part of this study we focus on the distribution of canine $CD4^+CD8\alpha^+$dp T cells in secondary lymphatic tissue. Information on the presence of double-positive T cells besides peripheral blood might provide a better understanding of their development and homeostatic dynamics in the dog. Porcine $CD4^+CD8^+$dp T cells, which are very prominent in peripheral blood, were also found in secondary lymphatic organs like spleen or mesenteric lymph node (JONJIC et al. 1987; SAALMÜLLER et al. 1987; JOLING et al. 1994; HAVERSON et al. 1999). The frequency of $CD4^+CD8^+$dp T cells in bronchial, mesenteric, mandibular, and retropharyngeal lymph node was similar to peripheral blood mononuclear cells (PBMC) (ZUCKERMANN and GASKINS 1996). There was also no significant difference in the proportion of $CD4^+$sp or $CD8^+$sp T cells between different lymph nodes (ZUCKERMANN and GASKINS 1996). In peripheral blood of several monkey species $CD4^+CD8\alpha^+$dp T cells were also found (AKARI et al. 1997; MURAYAMA et al. 1997; REIMANN et al. 1994). In contrary to swine, in cynomolgus monkey the frequency of $CD4^+CD8^+$dp T cells in spleen, inguinal and mesenteric lymph node was smaller compared with peripheral blood (AKARI et al. 1997). It is noticeable that the frequency of $CD4^+CD8^+$dp T cells in secondary lymphoid organs differs between species similar as the frequency in PBMC does (e.g. swine and monkey). However,

the number of $CD4^+CD8^+$dp T cells can also differ among individual animals of one species and between PBMC and secondary lymphatic organs (i.e. cynomolgus monkey).

The last and third part of this study will focus on the on functional properties of canine double-positive T cells, which are up to now, unclear. Knowledge about possible distinct functions of double-positive T cells might help to confine them from single-positive T cells and to confirm their uniqueness and importance during an immune response. Based on the fact that canine $CD4^+CD8\alpha^+$dp T cells have an activated phenotype it is reasonable and of great importance to analyze their cytokine profile, since cytokines are produced by activated cells to shape an immune response. IFN-γ plays an essential role in modulating an inflammatory response that is essential for clearance of intracellular pathogens by enhancing antigen-presenting capabilities of dendritic cells as well as antimicrobial activity of macrophages (SCHRODER et al. 2004; SCHREIBER and FARRAR 1993). It is well known that in the adaptive immune system $CD8^+$ cytotoxic T cells (GUIDOTTI and CHISARI 1999; SEN 2001) and $CD4^+$ Th1 cells (ROMAGNANI et al. 1998) are potent IFN-γ producers. In contrary to dog, IFN-γ production of $CD4^+CD8^+$dp T cells in some other species has been analyzed. Porcine $CD4^+CD8^+$dp T cells isolated from pseudorabies-vaccinated pigs produced high levels of IFN-γ after viral restimulation (ZUCKERMANN 1999). The IFN-γ immune response to porcine reproductive and respiratory syndrome virus (PRRSV) infection or vaccination is mainly dependent on $CD4^+CD8^+$dp T cells (MEIER et al. 2003). Furthermore, phenotypic characterization of porcine IFN-γ-producing lymphocytes after activation revealed that the $CD4^+CD8^+$dp T cell population includes the highest frequency of IFN-γ^+ T cells, with the highest per cell IFN-γ production compared with the other T cell subpopulations (RODRIGUEZ-CARRENO et al. 2002). In cynomolgus monkey it was shown that freshly isolated peripheral blood $CD4^+CD8^+$dp T cells express levels of IFN-γ which are comparable with $CD4^+$sp or $CD8^+$sp T cells. In addition, the ability of these $CD4^+CD8^+$dp T cells to produce IFN-γ upon activation was determined by stimulation of sorted T cell subsets with anti-CD3 antibody. It was shown that $CD4^+CD8^+$dp and $CD8^+$sp T cells are the main producers of IFN-γ (NAM et al. 2000). In humans a higher percentage of $CD4^+CD8^+$dp than $CD4^+$sp or $CD8^+$sp T cells produced IFN-γ under physiological conditions, in response to virus-infected cell lysates, HLA-A2-restricted peptides, or tetanus toxoid protein (NASCIMBENI et al. 2004).

This study focuses on three major points which will lead to better classification of double-positive T cells: (i) The percentage of each of the three distinct $CD4^+CD8\alpha^+$dp T cell subsets among the total double-positive T cell population and their CD25 and CD62L expression will be elucidated. (ii) Preliminary data on the frequency of canine $CD4^+CD8\alpha^+$dp T cells and their subsets in secondary lymphatic tissue compared with peripheral blood will be presented. (iii) The potential of canine $CD4^+CD8^+$dp T cells to produce IFN-γ will be determined in comparison to single-positive T cells.

Materials and methods

Animals

Blood was sampled by venipuncture into heparinized collection tubes (BD Vacutainer®, 10 ml, lithium heparin 17 IU/ml, Beckton Dickinson, Heidelberg, Germany). For analysis of the percentage of double-positive T cell subsets among the total double-positive T cell population and their CD25 and CD62L expression blood was sampled repeatedly over a period of 2 - 3 years from 12 healthy beagle dogs (6 female and 6 male; age range: 2.5 – 8.5 years). The dogs belong to the College of Veterinary Medicine, University of Leipzig, Leipzig, Germany. For IFN-γ studies blood was obtained from 15 healthy dogs. 8 dogs were of the above mentioned group of beagles (3 female and 5 male; age: 4 - 7 years). The other dogs belong to private owners (5 male and 2 female, 3 Labrador Retriever and 4 mixed breed; age: 2 - 9 years). All dogs were vaccinated against canine distemper, rabies, canine infectious hepatitis, parvovirus infection, parainfluenza and leptospirosis. The protocol for this study was approved by the Animal Care and Usage Committee of the Landesdirektion (state office) Sachsen in Leipzig, Germany (permit number: A 10/14).
Blood and tissue samples for analysis of anatomical distribution of $CD4^+CD8\alpha^+$dp T cells were taken from 5 otherwise healthy experimental dogs euthanized at Boehringer Ingelheim Pharma GmbH & Co. KG Development, Biberach, Germany. 4 dogs were of beagle breed (2 female and 2 male; age: 14 - 16 months). 1 dog was a Labrador Retriever mix (male; 2 years).

Isolation of PBMC

PBMC were isolated from heparinized canine whole blood by density gradient centrifugation. Blood was diluted 1:2 with phosphate buffered saline (PBS) and layered on a separation medium with a density of 1077 g/l (LSM 1077; PAA Laboratories GmbH, Coelbe, Germany / Bicoll Separating Solution; Biochrom AG, Berlin, Germany). Blood gradient was centrifuged without brakes at 500 xg for 30 min at room temperature in a centrifuge with swing bucket. Afterwards, the PBMC band was picked and cells were resuspended in PBS and centrifuged at 500 xg for 20 min at room temperature. Supernatant was discarded and cells were treated with erythrocyte lysis buffer (150 mM NH4Cl, 8 mM KHCO3, 2 mM EDTA; pH 7) for 10 min at room temperature. Lysis reaction was stopped with PBS and cells were centrifuged at 500 xg for 10 min at room temperature. Again, cells were washed once with PBS and twice with complete culture medium (RPMI 1640, PAA Laboratories GmbH, Coelbe, Germany) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Darmstadt, Germany), 100 units/ml penicillin and 100 µg/ml streptomycin (both PAA Laboratories

Germany GmbH, Coelbe, Germany). Between single washing steps, cells were centrifuged at 500 xg for 10 min at room temperature. For immediate flow cytometric staining cells were resuspended in PBS or for further *in vitro* culture in complete culture medium. Cell numbers were determined with a microscope using a hemocytometer (Laboroptik, Lancing, UK) and trypan blue (Sigma-Aldrich, Taufkirchen, Germany).

Generation of single cell suspensions of lymph node, spleen ,or thymus

A 2 x 2 cm piece of spleen, thymic tissue (size variable), or the whole lymph node was minced, afterwards passed through a 100 µm nylon cell strainer (BD Biosciences, Heidelberg, Germany) and resuspended in PBS. Cell suspensions were centrifuged at 500 xg for 10 min at 4°C. Splenic cells were treated with erythrocyte lysis buffer for 10 min at room temperature and lysis reaction was stopped with PBS. Again, the splenic cell suspension was centrifuged at 500 xg for 10 min at 4 °C. Splenic, thymic, and lymph node cells were used for subsequent flow cytometric cell staining.

In vitro stimulation of PBMC

Cells were stimulated and cultured in 96-well polystyrene flat-bottom plates (Becton Dickinson, Heidelberg, Germany) at a concentration of $1x10^6$ cells per 200 µl in complete culture medium for 2 days (or for 1, 2, 3, 5 or 6 days for previous time course studies) at 37°C in humidified atmosphere containing 5% CO_2. Cells were stimulated either with ConA (Biochrom AG, Berlin, Germany) or SEB (Sigma-Aldrich, Taufkirchen, Germany) at a concentration of 5 µg/ml. A combination of phorbol 12-myristate 13-acetate (PMA) and ionomycin at a concentration of 0.22 µg/ml each (Enzo Life Sciences, Lörrach, Germay) was added for the last 6 hours of 2 days of medium incubation. Medium incubation, ConA and SEB stimulations were treated with BrefeldinA (Sigma-Aldrich, Taufkirchen, Germany) at a concentration of 0.22 µg/ml for the last 6 h and PMA/ionomycin stimulation for the last 4 h.

Flow cytometry

Freshly isolated or stimulated cells were stained with the fixable viability dye eFluor780® (ebioscience, Frankfurt, Germany) according to manufacturer's description. Afterwards, cells were incubated for 15 min in a 3% dilution of a mixture of heat-inactivated rat/mouse/dog/goat normal serum to block Fc receptors. Staining for flow cytometry was

performed by use of the following antibodies: FITC-anti-canine CD4 (clone YKIX302.9) (ebioscience, Frankfurt, Germany), APC-anti-canine CD8α (clone YCATE55.9) (ebioscience, Frankfurt, Germany), PE-anti-canine CD5 (clone YKIX322.3) (ebioscience, Frankfurt, Germany), PE-anti-canine CD25 (clone P4A10) (ebioscience, Frankfurt, Germany) and cross-reactive PE-anti-human CD62L (clone FMC46) (Serotec, Morphosys AbD GmbH, Düsseldorf, Germany). Anti-dog CD8β (clone CA 15.4G2) (Serotec, Morphosys AbD GmbH, Düsseldorf, Germany) and anti-canine CD1a (clone CA13.9H11) (Peter F. Moore, Davis, CA, USA) were detected using PerCP/Cy5.5-goat anti-mouse IgG (clone poly 4053) (BioLegend, San Diego, CA, USA). As corresponding isotype controls FITC- or PE-ratIgG2a κ (clone eBR2a) (both: ebioscience, Frankfurt, Germany), APC-rat IgG1 κ (clone BRG1) (ebioscience, Frankfurt, Germany), PE-mouse IgG1 κ (clone P3.6.2.8.1) (ebioscience, Frankfurt, Germany), PE-mouse IgG2b κ (clone eBMG2b) (ebioscience, Frankfurt, Germany) and mouse IgG1 (BD biosciences Pharmingen™, San Diego, CA) were used. Antibody dilutions in fluorescence-activated cell sorting (FACS)-buffer (PBS with 10% FBS and 3% NaN_3 (Merck, Darmstadt, Germany)) were incubated for 30 min at 4°C. Following, cells were washed twice with FACS-buffer, centrifuging in between at 400 xg at 4°C for 3 min. Then, cells were washed once with PBS before fixation with 2% paraformaldehyde (PFA) for 20 min at 4°C.

For intracellular IFN-γ staining with cross-reactive (PEDERSEN et al. 2002) PE-anti-bovine IFN-γ (clone CC203) (Serotec, Morphosys AbD GmbH, Düsseldorf, Germany) after surface staining fixed cells were treated with Foxp3 / Transcription Factor Staining Buffer Set (ebioscience, Frankfurt, Germany). First, cells were incubated in Fixation/Permeabilization working solution for 60 min at room temperature and afterwards washed twice with Permeabilization Buffer working solution, centrifuging in between at 400 xg for 3 min at room temperature. After cells were incubated in a 3% dilution of a mixture of heat-inactivated rat/mouse/dog/goat normal serum in Permeabilization Buffer working solution for 15 min at room temperature, IFN-γ antibody diluted in Permeabilization Buffer working solution was added for additional 60 min. Then, cells were washed once with Permeabilization Buffer working solution, once with FACS-buffer, and once with PBS, centrifuging in between at 400 xg for 3 min at room temperature. Again, cells were fixed with 2% PFA for 20 min at 4°C.

Flow cytometric analysis was performed with a BD LSR Fortessa^{TM} flow cytometer (Beckton Dickinson, Heidelberg, Germany) and analyzed with BD FACS Diva^{TM} 6.1.3 (Beckton Dickinson, Heidelberg, Germany). Only living lymphocytes or lymphoblasts in case of stimulation after doublet-exclusion (FSC-A vs FSC-H) were included into subsequent analysis. For analysis of double-positive T cell subsets, CD25 or CD62L expression and for analysis of IFN-γ production the CD4 vs CD8α expression of the lymphocyte gate was analyzed. For analysis of anatomical distribution and CD8β expression of $CD4^+CD8\alpha^+$dp T cells the CD4 vs CD8α expression of $CD5^+$ lymphocytes was analyzed.

Statistical analysis

Statistical analysis was done using Graph Pad Prism 5.01 software (Graph-Pad Software, San Diego, CA; USA). D'Agostino-Pearson test was used to test for normality. For analysis of parametric data as global test the one-way analysis of variance was used to compare three or more groups. For subsequent comparison of single groups the Bonferroni's multiple comparison test was performed. Data are presented with the mean. For nonparametric data or comparisons including groups with nonparametric data the Kruskal-Wallis-H-test as global test for comparison of three or more groups with subsequent pairwise comparison of single groups by Dunn's post test was performed. Data are presented with the median. The level of confidence for significance is depicted in figure legends.

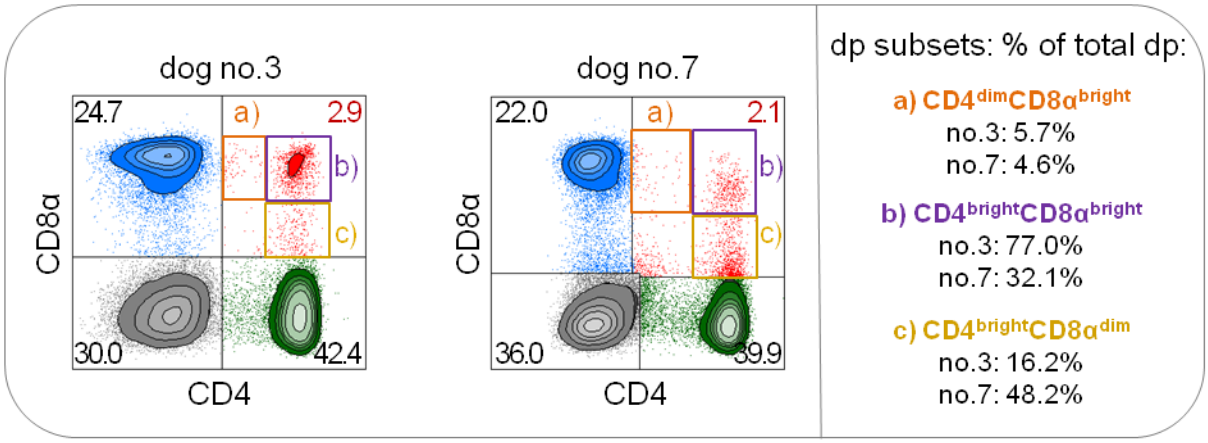
Results

I) Further differentiation of CD4^{+}CD8α^{+} double-positive T cells by their intensity of CD4 and CD8α expression

The CD4^{dim}CD8α^{bright} subset is the smallest and each of both CD4^{bright} subsets can be the largest double-positive T cell subset

Canine CD4^{+}CD8α^{+}dp T cells can be subdivided into three subsets by their intensity of CD4 and CD8α expression: CD4^{dim}CD8α^{bright}, CD4^{bright}CD8α^{bright}, and CD4^{bright}CD8α^{dim}. In a strict sense CD4^{bright}CD8α^{bright} cells show a lower intensity of CD8α expression than CD8α^{+}sp T cells, but it is still higher compared with the CD4^{bright}CD8α^{dim} subset (Fig. 1A). Analysis over a time period of 2 - 3 years from 12 dogs (7 - 14 blood samplings per dog) showed that in general the CD4^{dim}CD8α^{bright} subset is the smallest among peripheral blood double-positive T cells. Each of both CD4^{bright} subsets can comprise the largest double-positive T cell subset. This depends on individual dogs and can differ between repeated blood collections. Some dogs have a very prominent CD4^{bright}CD8α^{bright} subset at any time point of blood sampling (Fig. 1A; left plot, Fig. 1B; dog no. 3 and no. 9).

A



B

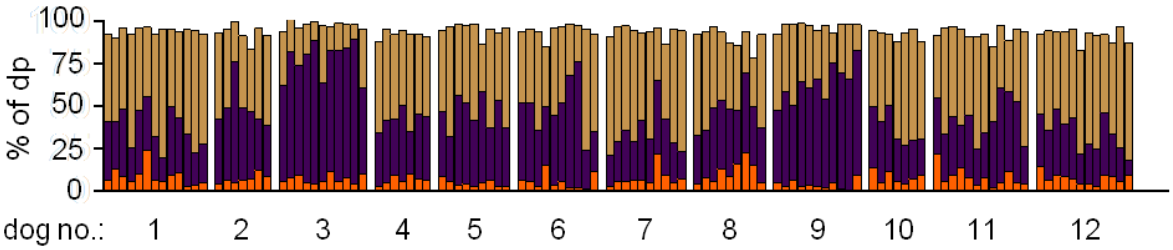


Fig. 1 The $CD4^{dim}CD8\alpha^{bright}$ subset is the smallest and each of both $CD4^{bright}$ subsets can be the largest subset of double-positive T cells

(A) The flow cytometry plots show living lymphocytes after doublet-exclusion for their CD4 vs CD8α expression. By their intensity of CD4 and CD8α expression three double-positive T cell subsets can be defined: $CD4^{dim}CD8\alpha^{bright}$, $CD4^{bright}CD8\alpha^{bright}$, and $CD4^{bright}CD8\alpha^{dim}$. Depending on individual dogs, either the $CD4^{bright}CD8\alpha^{bright}$ (left plot) or the $CD4^{bright}CD8\alpha^{dim}$ subset (right plot) is the largest of the total $CD4^{+}CD8\alpha^{+}$dp T cell population. (B) Shown is the proportion of double-positive T cell subsets ($CD4^{dim}CD8\alpha^{bright}$ = orange, $CD4^{bright}CD8\alpha^{bright}$ = purple, $CD4^{bright}CD8\alpha^{dim}$ = yellow) of the total $CD4^{+}CD8\alpha^{+}$dp T cell population (= 100%) for 12 dogs from different time points of blood sampling. Dogs are depicted with decreasing age from left to right.

The $CD4^{dim}CD8\alpha^{bright}$ subset is the least activated and the $CD4^{bright}CD8\alpha^{bright}$ subset is most likely to be of T effector / T effector memory phenotype

Detailed analysis of the activation-related surface markers CD25 and CD62L on double-positive T cell subsets, which we previously have performed for the total double-positive T cell population, revealed further differences. The $CD4^{dim}CD8\alpha^{bright}$ subset of all three subsets has the lowest frequency of $CD25^{+}$ cells, nevertheless, a higher frequency than the single-positive counterparts. Both $CD4^{bright}$ subsets do not differ significantly in their frequency

of CD25^{+} cells. The CD4^{bright}CD8α^{bright} subpopulation has the lowest frequency of CD62L^{+} cells, which identifies it most likely as T effector or T effector memory cells.

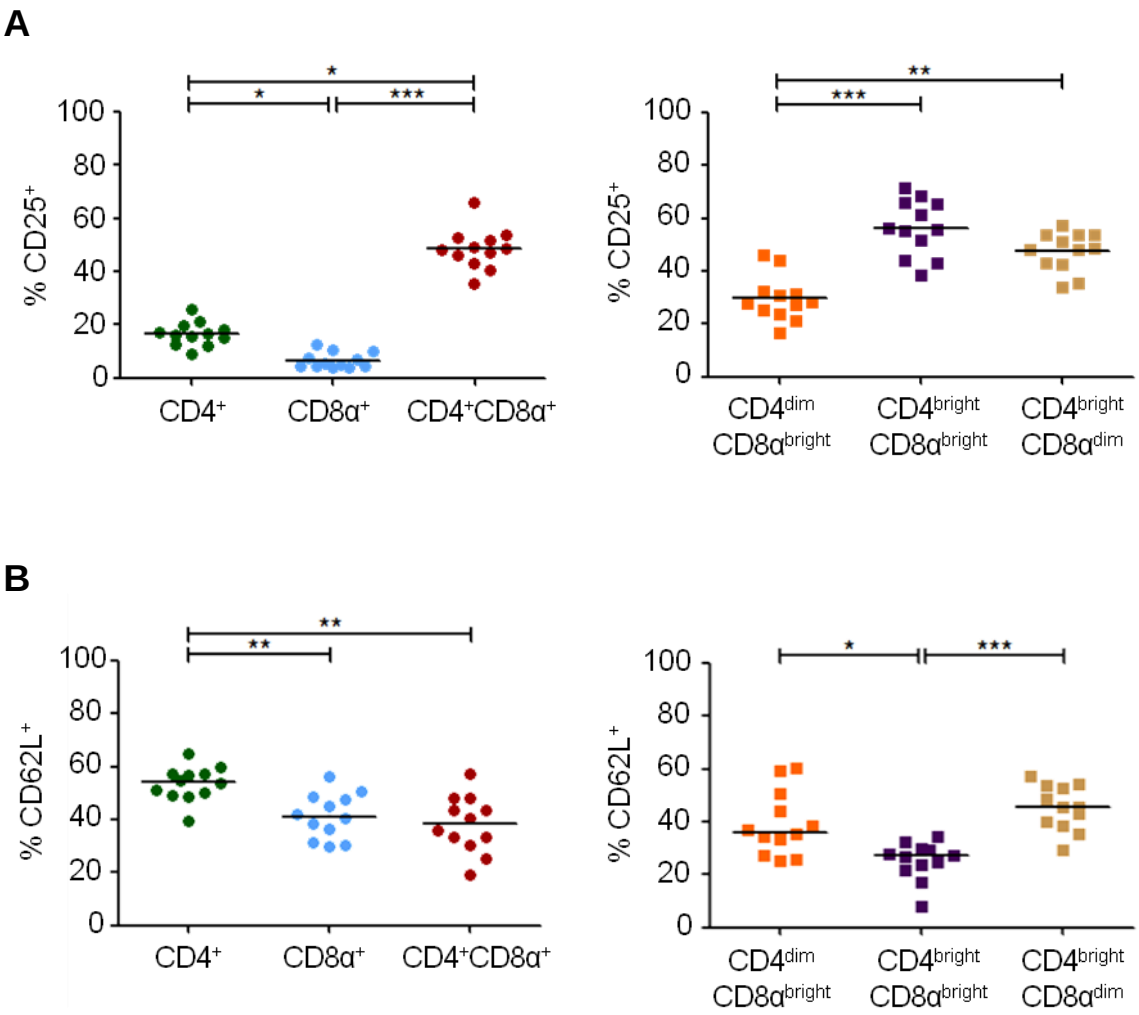


Fig. 2 Double-positive T cell subsets differ in their CD25 and CD62L expression

Each dot represents the median of one dog for CD25 or CD62L expression from several blood samplings (CD25: 4 - 10; CD62L: 8 - 14 per dog) over a time period of 2 – 3 years. (A) A higher frequency of CD4^{+}CD8α^{+}dp T cells expresses CD25 compared with CD4^{+}sp or CD8α^{+}sp T cells. Among double-positive T cell subsets the CD4^{dim}CD8α^{bright} subset has the lowest frequency of CD25^{+} cells. (B) CD4^{+}sp T cells have the highest CD62L^{+} proportion. CD4^{+}CD8α^{+}dp T cells show nearly the same CD62L expression as CD8α^{+}sp T cells. Comparison of the CD62L expression of double-positive T cell subsets shows that the CD4^{bright}CD8α^{bright} subset has the lowest frequency of CD62L^{+} cells. (A)+(B) Statistical analysis was performed with the Kruskal-Wallis-H-test for global comparison and subsequent multiple comparison between groups with Dunn's post test of either T cell subpopulations or double-positive T cell subsets. Statistical significant differences are indicated with asterisks (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$).

II) Anatomical distribution of CD4⁺CD8α⁺ double-positive T cells

CD4⁺CD8α⁺ double-positive T cells also exist in secondary lymphatic organs

In addition to canine peripheral blood we analyzed some secondary lymphatic tissues for the frequency of CD4⁺CD8α⁺dp T cells. Of interest were bronchial (lymphonodus (LN) tracheobronchialis), mesenteric (LN mesenterialis), mandibular (LN mandibularis), and the prescapular (LN cervicalis superficialis) lymph nodes as well as the spleen.

In all analyzed organs CD4⁺CD8α⁺dp T cells were found. Similar to PBMC they comprise the smallest T cell subpopulation as compared with both single-positive T cell subpopulations (mean: 3.7 ± SD 1.59; Fig. 3). With preliminary data available, comparison of the frequency of double-positive T cells between peripheral blood, lymph nodes, and spleen reveals no significant difference. However, it appears that the CD4⁺CD8α⁺dp T cell subpopulation among PBMC is smaller than in the secondary lymphatic organs. The proportion of either CD4⁺sp or CD8α⁺sp T cells does not significantly vary between the analyzed organs.

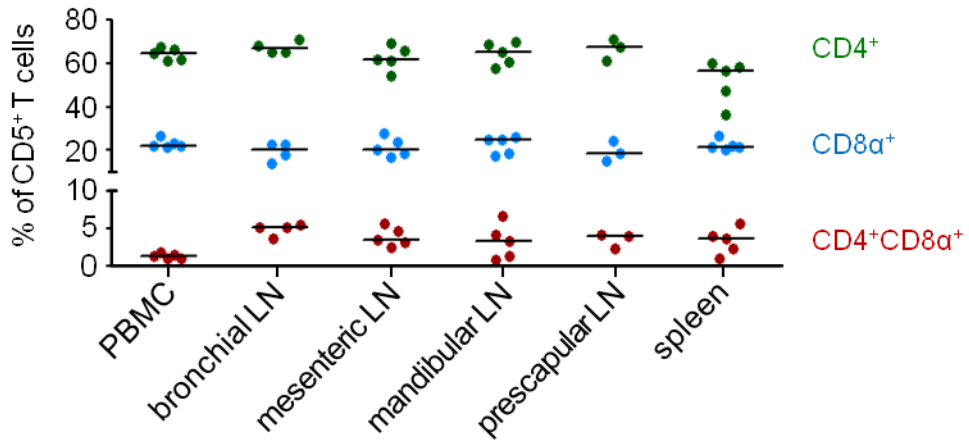


Fig. 3 CD4⁺CD8α⁺dp T cells also exist in lymph nodes and spleen

Depicted is the percentage of CD4⁺sp, CD8α⁺sp, and CD4⁺CD8α⁺dp T cells of PBMC; lymph nodes (LN), and spleen. Preliminary data indicate that the frequency of either single-positive or double-positive T cells does not differ significantly between peripheral blood, lymph nodes, and spleen. A trend can be seen that peripheral blood double-positive T cells and splenic CD4⁺sp T cells have a lower frequency compared with the other tissues. Statistical analysis of preliminary data was performed with Kruskal-Wallis-H-test and revealed no significance.

$CD4^+CD8\alpha^+$ double-positive T cell subsets vary between organs

$CD4^+CD8\alpha^+$dp T cells in lymph nodes and spleen can be divided into three subsets similar to peripheral blood (Fig. 5). Comparing the abundance of these double-positive T cell subsets between PBMC, spleen, and lymph nodes reveals some similarities but also differences (Fig. 4). The distribution of the three double-positive T cell subsets in PBMC is as described above and similar to spleen. A different composition of double-positive T cell subsets can be found in all analyzed lymph nodes. In contrast to PBMC and spleen in lymph nodes the proportion of the $CD4^{bright}CD8\alpha^{bright}$ subset is comparably small as the $CD4^{dim}CD8\alpha^{bright}$ subset. In lymph node the $CD4^{bright}CD8\alpha^{dim}$ subset is the predominant one. In addition, we analyzed the CD8β expression of double-positive T cells. The $CD4^{dim}CD8\alpha^{bright}$ subset expresses similar to $CD8\alpha^+$sp T cells in majority the CD8αβ heterodimer. In spleen the CD8β expression is generally reduced (data not shown).

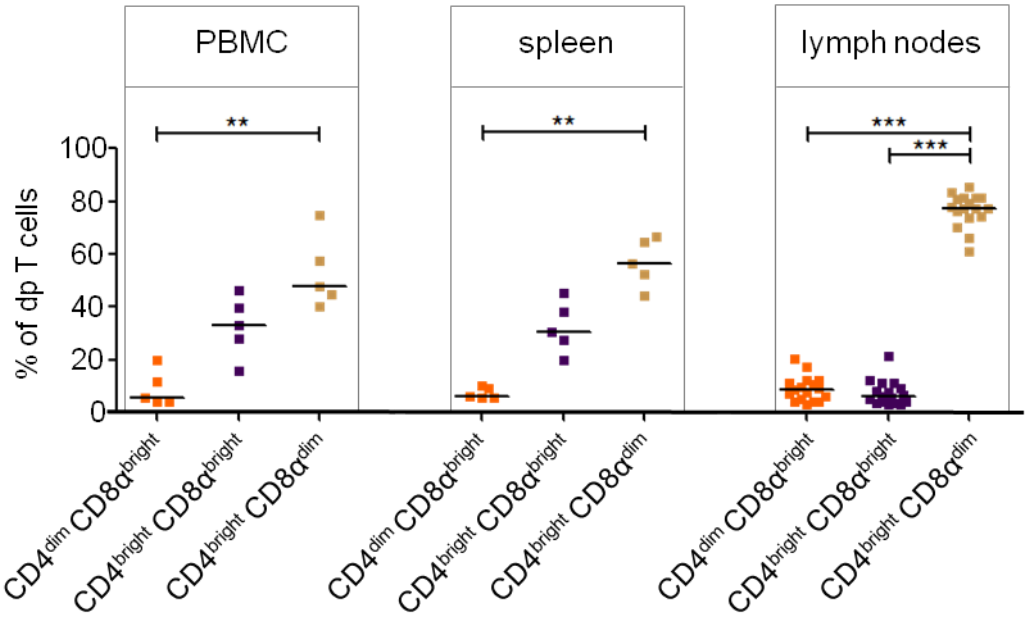


Fig. 4 Lymph nodes display a different composition of $CD4^+CD8\alpha^+$dp T cell subsets than peripheral blood

Shown is the percentage of double-positive T cell subsets of PBMC, spleen, and pooled lymph nodes (bronchial, mesenteric, mandibular, and prescapular). In all analyzed samples the $CD4^{bright}CD8\alpha^{dim}$ subset is the largest. The $CD4^{bright}CD8\alpha^{bright}$ subset in lymph nodes is similarly small as the $CD4^{dim}CD8\alpha^{bright}$ subset. In PBMC and spleen this population is intermediate. Kruskal-Wallis-H-test with Dunn's post test was performed to compare the frequency of $CD4^+CD8\alpha^+$dp T cell subsets of either PBMC, spleen or lymph nodes. Statistical significant differences are indicated with asterisks (** = $p < 0.01$; *** = $p < 0.001$).

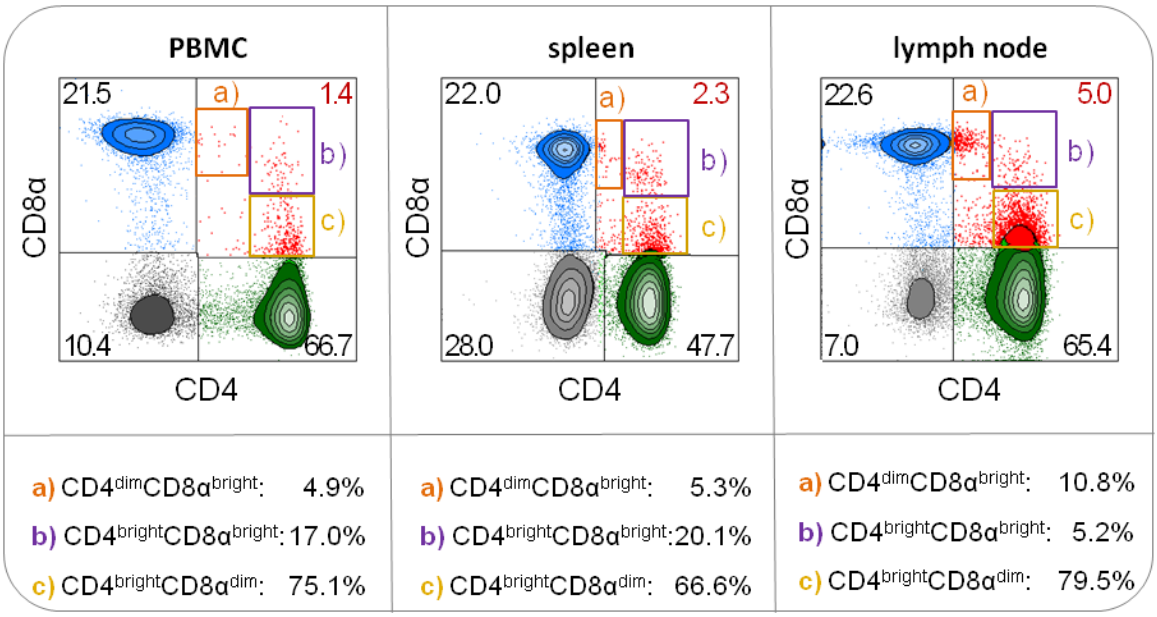


Fig. 5 Double-positive T cell subsets in peripheral blood, spleen, and lymph node

Shown are representative flow cytometry plots of PBMC (left), spleen (middle), and lymph node (right). Only living $CD5^+$ T cells after doublet-exclusion were included in further analysis. Depicted is their CD4 vs CD8α expression. In PBMC, spleen, and lymph node the three double-positive T cell subsets can be distinguished.

Canine lymph node $CD4^+CD8\alpha^+$ double-positive T cells are of mature phenotype

Peripheral blood $CD4^+CD8\alpha^+$dp T cells were already shown to be $CD1a^-$ mature T cells. Similarly, on canine lymph node $CD4^+CD8\alpha^+$dp T cells lack of CD1a was determined (Fig. 6A; right plot). As control, thymus was analyzed. In thymus $CD1a^+$ immature $CD4^+CD8\alpha^+$dp T cells (Fig. 6B; right plot) comprise the major T cell subpopulation (Fig. 6B; left plot).

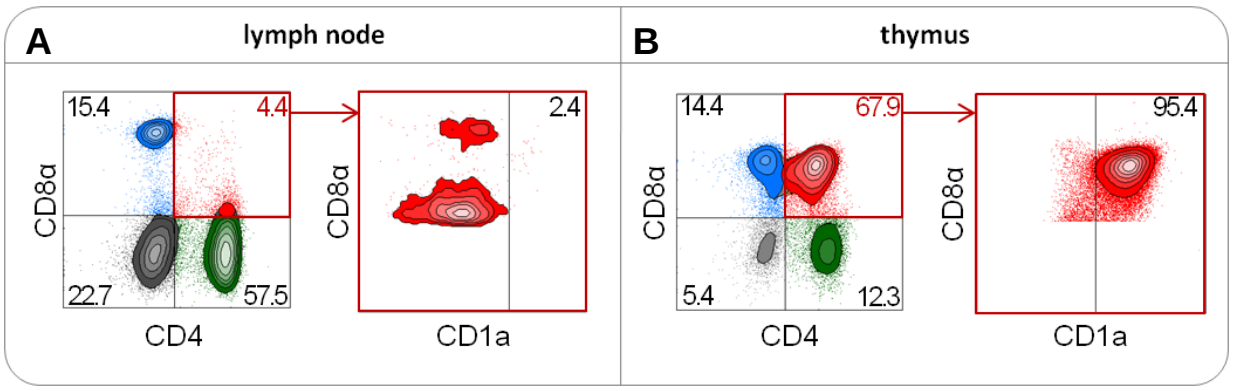


Fig. 6 Mature lymph node $CD4^+CD8\alpha^+$dp T cells lack CD1a expression in contrast to immature thymic $CD4^+CD8\alpha^+$dp T cells

Shown are representative flow cytometry plots of (A) lymph node and (B) thymus for their CD4 vs CD8α expression (left plot each). Only living $CD5^+$ T cells after doublet-exclusion were included into analysis. CD1a as a marker for immature T cells is not expressed on lymph node double-positive T cells (A; right plot) but on thymic double-positive T cells (B; right plot).

III) Functional analysis of CD4$^+$CD8α$^+$ double-positive T cells: IFN-γ

ConA, SEB, and PMA/ionomycin are appropriate stimuli to induce IFN-γ production in canine PBMC

For establishment of functional analysis of CD4$^+$CD8α$^+$dp T cells canine PBMC were stimulated with ConA or SEB for two days or with PMA/ionomycin for the last six hours of two days of medium incubation. Medium incubation served as negative control. To inhibit cytokine secretion cells were additionally treated with BrefeldinA, which prevents intracellular cytokine transport by disruption of the Golgi apparatus. Afterwards, cells were analyzed for IFN-γ production by intracellular cytokine staining with a cross-reactive anti-bovine IFN-γ antibody (clone CC203) (PEDERSEN et al. 2002). This antibody was also used successfully in other studies (FELLMAN et al. 2011; PAPADOGIANNAKIS et al. 2009; HORIUCHI et al. 2007) for characterization of canine IFN-γ production.

In several time course studies two days of stimulation appeared to be the best for a strong IFN-γ response after ConA or SEB stimulation (data not shown). After medium incubation, only a few lymphocytes produce IFN-γ (mean of 0.79% ± standard deviation (SD) 0.72%; n = 12) (representative flow cytometry plot in Fig. 9A; left). Stimulation leads to a significant increase of IFN-γ$^+$ lymphocytes compared with medium incubation ($p < 0.001$). SEB (representative flow cytometry plots in Fig. 9B; left) and ConA stimulation result in nearly the same frequency of IFN-γ producing lymphocytes (SEB: mean of 12.06% ± SD 4.45%; ConA: mean of 12.93% ± SD 5.81%; n = 12), whereas after PMA/ionomycin stimulation significantly more lymphocytes produce IFN-γ ($p < 0.001$; mean of 24.95% ± SD 9.50%; n = 11) (representative flow cytometry plots in Fig. 9C; left). It has to be mentioned that PMA/ionomycin stimulation leads to downregulation of surface CD4 and CD8α receptors (Fig. 9C). On the contrary, ConA and SEB stimulation induce lymphoblast development, which leads to a higher background autofluorescence of cells (Fig. 9B; left). Nevertheless, in both cases it is still possible to differentiate between the three double-positive T cell subsets.

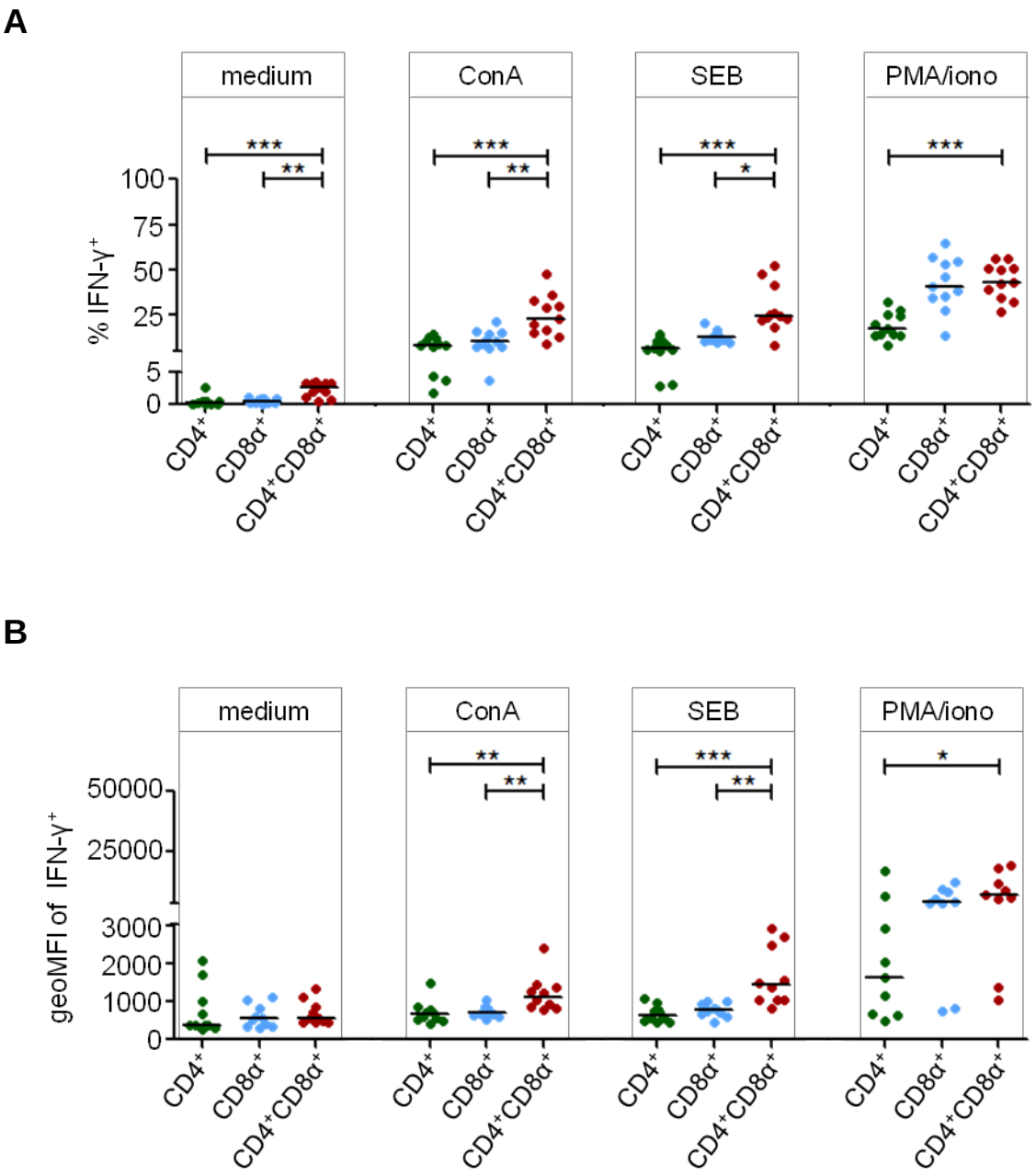


Fig. 7 $CD4^+CD8\alpha^+$dp T cells are very potent IFN-γ producers

PBMC were stimulated as indicated. (A) Plotted is the percentage of IFN-γ^+ cells of $CD4^+$sp, $CD8\alpha^+$sp, and $CD4^+CD8\alpha^+$dp T cells for medium incubation and each stimulus. An increase of IFN-γ^+ cells of each T cell subpopulation after stimulation as well as the higher frequency of IFN-γ^+ cells among $CD4^+CD8\alpha^+$dp T cells compared with $CD4^+$ or $CD8^+$sp T cells can be seen. (B) The geoMFI of IFN-γ^+ cells of $CD4^+CD8\alpha^+$dp T cells is higher than of IFN-γ^+ $CD4^+$sp or $CD8\alpha^+$sp T cells. (A)+(B) Data are shown with median ($n \geq 9$). Kruskal-Wallis-H-test with Dunn's post test was performed to compare either the (A) frequency or (B) geoMFI of IFN-γ^+ cells of $CD4^+CD8\alpha^+$dp T cells with $CD4^+$sp or $CD8\alpha^+$sp T cells. Statistical significant differences are indicated with asterisks (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$).

$CD4^+CD8\alpha^+$ double-positive T cells comprise a small population of IFN-γ^+ cells, but they are very potent IFN-γ producers

Analyzing the distribution of T cell subpopulations only among IFN-γ^+ lymphocytes shows that $CD4^+CD8\alpha^+$dp T cells comprise the smallest subpopulation compared with $CD4^+$ or $CD8\alpha^+$sp T cells (data not shown). This is independent of stimulation or medium incubation. However, looking at the proportion of cells which produces IFN-γ separately for each T cell subpopulation identifies a high proportion of $CD4^+CD8\alpha^+$dp T cells as source of IFN-γ (Fig. 7) as it is mentioned for effector cell populations (SEDER and AHMED 2003). In general, compared with medium incubation the percentage of IFN-γ^+ $CD4^+$sp, $CD8\alpha^+$sp, and $CD4^+CD8\alpha^+$dp T cells increases significantly after stimulation ($p < 0.01$; Fig. 7A). In detail, after medium incubation and after ConA stimulation a higher proportion of double-positive T cells than of $CD4^+$ or $CD8\alpha^+$sp T cells produces IFN-γ (Fig. 7A). After SEB or PMA/ionomycin stimulation no significant differences between $CD8\alpha^+$sp and $CD4^+CD8\alpha^+$dp T cells can be detected, but after SEB stimulation the trend of a higher frequency of IFN-γ^+ cells among double-positive T cells compared with $CD8\alpha^+$sp T cells can be seen (Fig. 7A). Importantly, after stimulation $CD4^+CD8\alpha^+$dp T cells produce more IFN-γ per cell than $CD4^+$ or $CD8\alpha^+$sp T cells. This can be seen by the higher geometric mean fluorescence intensity (geoMFI) of $CD4^+CD8\alpha^+$ IFN-γ^+ cells compared with the geoMFI of $CD4^+$ or $CD8\alpha^+$ IFN-γ^+ T cells (Fig. 7B).

IFN-γ production varies between double-positive T cell subsets

Having seen the high IFN-γ production capacity of the total $CD4^+CD8\alpha^+$dp T cell population we further analyzed whether the IFN-γ production differs between double-positive T cell subsets. Similar as for the total double-positive T cell population, the frequency of IFN-γ producing cells significantly increases in all three double-positive T cell subsets after stimulation compared with medium incubation ($p < 0.01$; Fig. 8). After medium incubation and after SEB stimulation a significant higher proportion of $CD4^{dim}CD8\alpha^{bright}$ cells produces IFN-γ compared with the other two double-positive T cell subsets (Fig. 8). The proportion of IFN-γ producing cells induced by ConA stimulation does not significantly differ between the three double-positive T cell subsets, but a trend can be seen that the $CD4^{bright}CD8\alpha^{dim}$ subset has the lowest frequency of IFN-γ producers (Fig. 8). PMA/ionomycin stimulation induces IFN-γ production in a smaller proportion of the $CD4^{bright}CD8\alpha^{dim}$ subset compared with the $CD4^{dim}CD8\alpha^{bright}$ and the $CD4^{bright}CD8\alpha^{bright}$ subsets (Fig. 8). There is no significant difference in the quantity of IFN-γ produced per cell between double-positive T cell subsets after stimulation, since no significant difference of the geoMFI of IFN-γ^+ cells of double-positive

T cell subsets could be detected (data not shown). For IFN-γ$^+$ cells of double-positive T cell subsets after medium incubation the geoMFI was not considered, because in this case the number of events was too low.

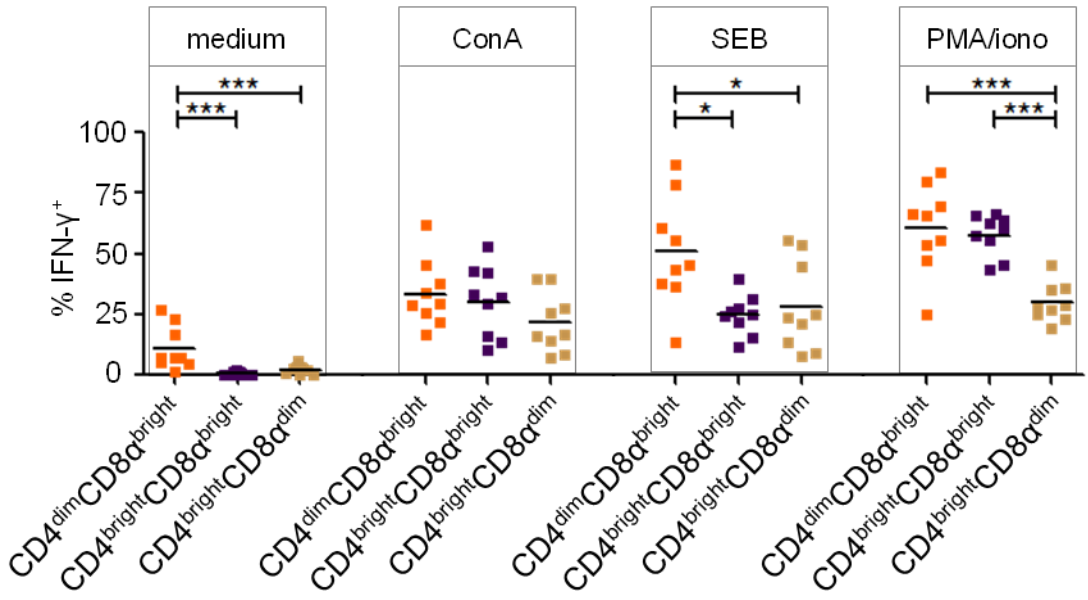
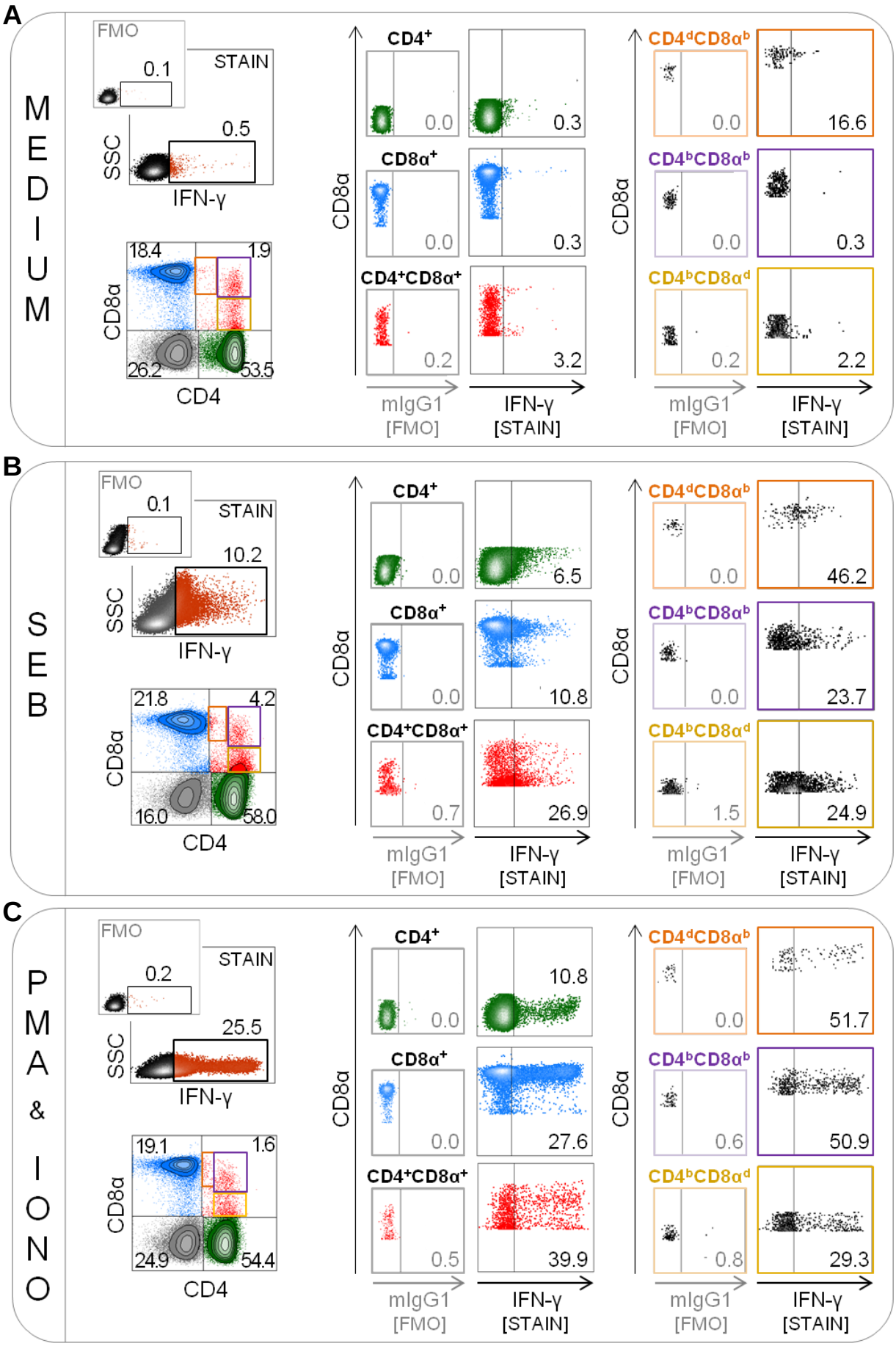


Fig. 8 Overall, the CD4^{dim}CD8α^{bright} subset shows highest percentage of IFN-γ producing cells

PBMC were stimulated as indicated. The percentage of IFN-γ$^+$ cells of double-positive T cell subsets is depicted. After medium incubation as well as SEB stimulation a higher percentage of the CD4^{dim}CD8α^{bright} subset produces IFN-γ compared with the other two subsets. After PMA/ionomycin and ConA stimulation the same trend can be seen. Proportions of IFN-γ$^+$ cells among the other two CD4^{bright} subsets differ depending on the stimulus. One-way analysis of variance followed by Bonferroni's multiple comparison test was performed to compare the frequency of IFN-γ$^+$ cells between all three double-positive T cell subsets of each stimulus. Data are shown with mean (n = 9). Statistical significant differences are indicated with asterisks (* = $p < 0.05$; *** = $p < 0.001$)

Fig. 9 (page 48) IFN-γ production after stimulation increases in all single-positive and double-positive T cell subpopulations as well as in double-positive T cell subsets

Cells were analyzed for their IFN-γ production after *in vitro* (A) medium, (B) SEB, and (C) PMA/ionomycin stimulation. For subsequent analysis cells were gated on living lymphocytes / lymphoblasts after doublet-exclusion. The left column of each panel shows the IFN-γ staining of lymphocytes with respective fluorescence minus one (FMO) controls (upper row) and the CD4 vs CD8α expression of lymphocytes including the differentiation of the three double-positive T cell subsets (lower row). The middle column of each panel shows IFN-γ staining with respective FMO controls of CD4$^+$sp, CD8α$^+$sp, and CD4$^+$CD8α$^+$dp T cells. The right column of each panel shows IFN-γ staining with respective FMO controls of double-positive T cell subsets: CD4^{dim}CD8α^{bright}, CD4^{bright}CD8α^{bright} and CD4^{bright}CD8α^{dim}. The increase of IFN-γ expression among all T cell subpopulations and double-positive T cell subsets after stimulation can be seen. Results from one representative experiment are shown (n ≥ 9).


A
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FMO
STAIN
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0.5
SSC
IFN-γ
18.4
1.9
26.2
53.5
CD8α
CD4
CD4⁺
CD8α⁺
CD4⁺CD8α⁺
0.0
0.3
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mIgG1
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B
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21.8
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CD8α
CD4
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CD4⁺CD8α⁺
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CD4ᵇCD8αᵈ
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23.7
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CD8α
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IFN-γ
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CD4ᵇCD8αᵈ
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51.7
0.6
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0.8
29.3

Discussion

Below, the newly gained more detailed view on canine $CD4^+CD8\alpha^+$dp T cells, first preliminary data of anatomical distribution and identified functional properties by means of IFN-γ production of double-positive T cells will be discussed.

I) Differentiation of $CD4^+CD8\alpha^+$ double-positive T cell subsets

Determination of $CD4^+CD8\alpha^+$ double-positive T cell subsets in peripheral blood depicts the $CD4^{dim}CD8\alpha^{bright}$ subset in all dogs as the smallest of all three. Each of both $CD4^{bright}$ subsets can comprise the largest double-positive T cell subset. This depends on the individual dog and on the individual blood sampling. Some dogs always have a very prominent $CD4^{bright}CD8\alpha^{bright}$ subset, in other dogs the major subset sometimes consists of $CD4^{bright}CD8\alpha^{dim}$ and sometimes of $CD4^{bright}CD8\alpha^{bright}$ cells. This might indicate that there is a dynamic change between these two $CD4^{bright}$ subsets in peripheral blood depending maybe on daily immunological challenges. This also could be in accordance with the possible different progenitors of canine $CD4^+CD8\alpha^+$dp T cells. The small $CD4^{dim}CD8\alpha^{bright}$ subset was shown to most likely originate from $CD8\alpha^+$sp T cells, which less efficiently upregulate the additional coreceptor. In contrast, both of the more numerous $CD4^{bright}$ subsets are likely to develop from $CD4^+$sp T cells (BISMARCK et al. 2014), which more frequently upregulate the additional coreceptor. Probably, before becoming $CD4^{bright}CD8\alpha^{bright}$, cells need to pass the $CD4^{bright}CD8\alpha^{dim}$ stage.

A more detailed analysis of $CD4^+CD8\alpha^+$dp T cell subsets emphasizes the heterogeneity of canine $CD4^+CD8\alpha^+$dp T cells. In addition to the already known distinct CD8β expression of double-positive T cell subsets (BISMARCK et al. 2012), they also show a different expression of CD25 and CD62L. Comparing the frequency of activated $CD25^+$ cells reveals that the $CD4^{dim}CD8\alpha^{bright}$ subset among double-positive T cells has the lowest proportion of $CD25^+$ cells, which is still higher than the frequency of $CD25^+$ $CD4^+$ or $CD8^+$sp T cells. The different frequencies of $CD25^+$ cells among double-positive T cell subsets could be linked to their progeny from $CD4^+$ or $CD8\alpha^+$sp T cells (BISMARCK et al. 2014). $CD8\alpha^+$sp T cells have a lower frequency of $CD25^+$ cells than $CD4^+$sp T cells. Thus, the $CD4^{dim}CD8\alpha^{bright}$ subset has a lower frequency of $CD25^+$ cells than both $CD4^{bright}$ subsets, because it descends from $CD8\alpha^+$sp T cells. Likewise, both $CD4^{bright}$ subsets as possible descendants of $CD4^+$sp T cells show a higher frequency of $CD25^+$ cells. This is in great contrast to human and monkey $CD4^+CD8\alpha^+$dp T cells which were described to be of resting $CD25^-$ T memory phenotype (AKARI et al. 1997; COLOMBATTI et al. 1998; SALA et al. 1993; TONUTTI et al. 1994). For porcine $CD4^+CD8^+$dp T cells an enriched low CD25 expression among double-positive T cells compared with

$CD4^+$sp T cells was described (SAALMÜLLER et al. 2002). Under pathological conditions for human double-positive T cells, e.g. in pleural effusions of breast cancer, which are of $CD4^{dim}CD8^{bright}$ phenotype, or in atopic dermatitis, a higher frequency of $CD25^+$ double-positive T cells compared with single-positive T cells was described (DESFRANCOIS et al. 2009; BANG et al. 2001; SALA et al. 1993; TONUTTI et al. 1994).

In contrast, we analyzed the constitutive higher frequency of $CD25^+$ double-positive T cells in healthy dogs. This CD25 expression could have two reasons: On the one hand double-positive T cells could permanently encounter antigen and thus become activated. On the other hand a permanent CD25 expression could favor them also as regulatory T (Treg) cells. Treg cells are known to constitutively express CD25 (SAKAGUCHI et al. 1995). Upon stimulation they express CD25 more persistently and at higher levels than activated naïve T cells. Furthermore, activated naïve T cells downregulate CD25 after activation is ceased, whereas Treg cells constantly keep their CD25 expression level (KUNIYASU et al. 2000). Among porcine $CD4^+CD8\alpha^+$dp T cells also $FoxP3^+CD25^+$ Treg cells could be detected (KÄSER et al. 2008). Percentages of the $CD4^+CD8\alpha^+$dp T cells among $FoxP3^+CD25^+$ cells, seem to differ between studies (KÄSER et al. 2008; SILVA-CAMPA et al. 2012). Hence, a portion of canine $CD4^+CD8\alpha^+$dp T cells could also be of Treg cell type.

CD62L is a secondary lymphoid tissue homing marker (ARBONES et al. 1994; GALLATIN et al. 1983) which is downregulated after activation and thus low CD62L expression has been linked to an effector stage (HAMANN et al. 1988; JUNG et al. 1988; BEVERLEY 2008). The low frequency of CD62L expression identifies most cells of the $CD4^{bright}CD8\alpha^{bright}$ subset as either T effector or T effector memory cells. Human $CD4^+CD8^+$dp T cells as well as canine double-positive T cells show a lower frequency of $CD62L^+$ cells than $CD4^+$sp T cells but almost the same as $CD8^+$sp T cells (NASCIMBENI et al. 2004). For porcine $CD4^+CD8\alpha^+$dp T cells using qPCR also a lower CD62L mRNA expression compared with $CD4^+$ T helper cells was found (REUTNER et al. 2013).

II) Anatomical distribution of $CD4^+CD8\alpha^+$ double-positive T cells

Preliminary data predict that the anatomical distribution of canine $CD4^+CD8\alpha^+$dp T cells does not differ significantly between peripheral blood and secondary lymphoid tissue. It appears that the frequency of double-positive T cells in peripheral blood is lower compared with lymph nodes or spleen. This is in great contrast to cynomolgus monkey. In this species secondary lymphatic organs show a drastically lower frequency of double-positive T cells compared with peripheral blood (AKARI et al. 1997). In six – seven-months-old swine, double-positive T cells do not seem to differ in their distribution between peripheral blood and lymph

nodes (ZUCKERMANN and GASKINS 1996). Of note is that the general frequency of $CD4^+CD8^+$dp T cells in porcine peripheral blood is clearly higher compared with dog.

In contrary to some other species, where double-positive T cells consist of a more homogeneous population, in dog further differentiation of double-positive T cells is useful. Consequently, frequencies of double-positive T cell subsets in secondary lymphatic organs were analyzed. Whereas in peripheral blood and spleen $CD4^{bright}CD8\alpha^{dim}$ and $CD4^{bright}CD8\alpha^{bright}$ subsets predominate, in lymph nodes the $CD4^{bright}CD8\alpha^{dim}$ subset represents the main population. T cells circle constantly throughout lymph nodes to encounter foreign antigen with consequent activation (SPRENT and TOUGH 1994; ZINKERNAGEL et al. 1997). From our progenitor studies we recognized that $CD4^{bright}CD8\alpha^{bright}$dp T cells mainly develop from $CD4^+$sp T cells after activation (BISMARCK et al. 2014). This activation most likely occurs in secondary lymphatic organs. When naïve $CD4^+$sp T cells encounter antigen in lymph node some of them could begin with the upregulation of the additional CD8α receptor and leave the lymph node as activated cells.

Recirculation of T cells is amongst others connected with the secondary lymphatic tissue homing factor CD62L (ARBONES et al. 1994; WARNOCK et al. 1998)**.** Since the $CD4^{bright}CD8\alpha^{bright}$ subset is the one with the lowest proportion of $CD62L^+$ cells, especially for this double-positive T cell subset a T effector or T effector memory function is likely. Thus, $CD4^{bright}CD8\alpha^{bright}$ T cells preferentially not recirculate between peripheral blood and lymph node. This would explain their relatively smaller frequency there. Another or additional explanation could be that the CD8α expression on $CD4^+$ T cells is a dynamic process and cells reach their maximum CD8α expression mainly after leaving the lymph node. This cannot be elucidated yet.

Since the spleen was not perfused, we cannot exclude that a picture comparable to lymph node is seen in the white pulp that is masked by the cells of the red pulp. In future, if possible, perfusion of the spleen could be performed or immunohistology might provide more insights in differences of the white and red pulp. Similarly, in a study on the memory T cell development of $CD8^+$sp T cells in mice it was found that $CD62L^-$ T effector and T effector memory cells were reduced in lymph nodes and predominantly found in spleen (OEHEN and BRDUSCHA-RIEM 1998).

For peripheral blood $CD4^+CD8\alpha^+$dp T cells we already have shown that they are mature T cells, since they lack CD1a expression (BISMARCK et al. 2012). Canine CD1a has been characterized as an antigen-presenting molecule, but was also described to be present on thymocytes (LOORINGH VAN BEECK et al. 2008). In contrast to thymic immature double-positive T cells, which express CD1a, double-positive T cells in lymph node are mature T cells lacking CD1a expression. As described by others we found thymic areas amongst large areas of involution in dogs of 14 – 24 months of age (GOFF et al. 1987). Canine thymus showed the

presence of a large double-positive T cell population, and a smaller population of $CD4^+$sp, $CD8^+$sp, and $CD4^-CD8^-$ double-negative (dn) T cells.

III) Functional analysis of $CD4^+CD8\alpha^+$ double-positive T cells: IFN-γ

This study shows that canine $CD4^+CD8\alpha^+$dp T cells can be very potent IFN-γ producers after stimulation with different agents. Resting T cells usually do not produce IFN-γ or only produce very low levels of IFN-γ. Therefore, the use of PMA/ionomycin is a widely used method to induce broad cytokine production and was already shown to work for canine cells (PAPADOGIANNAKIS et al. 2009; HORIUCHI et al. 2007; PEDERSEN et al. 2002). The combination of PMA/ionomycin leads to downregulation of CD4 and also CD8α surface receptors, which was already described for canine cells (PAPADOGIANNAKIS et al. 2009) and human T cell clones (BLUE et al. 1987; PELCHEN-MATTHEWS et al. 1993). Thus, in addition, we stimulated cells with ConA or SEB. This also works well and does not lead to downregulation of surface receptors of interest. In contrary, already two days of stimulation can lead to a moderate increase of $CD4^+CD8\alpha^+$dp T cells. This was beneficial for our purpose of characterization of their IFN-γ production. In summary, ConA and SEB are also useful stimuli for further studies on cytokine expression of activated canine $CD4^+CD8\alpha^+$dp T cells.

Here, we succeeded in measuring IFN-γ production of double-positive T cells. This cytokine is produced on the one hand by $CD8^+$ cytotoxic T cells and on the other hand by $CD4^+$ Th1 cells (SCHRODER et al. 2004). So we were able to analyze a hallmark feature of cytotoxic T cells and Th1 cells in canine $CD4^+CD8\alpha^+$dp T cells. We also would have liked to determine a Th2 phenotype for canine $CD4^+CD8\alpha^+$dp T cells by analyzing IL-4 production of cells. However, a commercially available anti-bovine IL-4 antibody described to be cross-reactive (clone CC303) (PEDERSEN et al. 2002), which also was used in other studies (HORIUCHI et al. 2007; PAPADOGIANNAKIS et al. 2009; FELLMAN et al. 2011), did not stain properly in our hands. This was also observed by another group, which in detail analyzed the staining properties of different IL-4 antibodies that were described to be cross-reactive with the canine species (LAUBER et al. 2010).

In a previous study we have shown that $CD4^+CD8\alpha^+$dp T cells can develop from $CD4^+$sp or $CD8\alpha^+$sp T cells. Data indicated that the $CD4^{dim}CD8\alpha^{bright}$ subset develops from $CD8\alpha^+$sp T cells, the $CD4^{bright}CD8\alpha^{dim}$ subset and the $CD4^{bright}CD8\alpha^{bright}$ subset mainly from $CD4^+$sp T cells (BISMARCK et al. 2014). As previously published (PAPADOGIANNAKIS et al. 2009) in our study we also see that a higher percentage of $CD8\alpha^+$sp T cells than of $CD4^+$sp T cells produces IFN-γ. Likely, a higher frequency of the $CD4^{dim}CD8\alpha^{bright}$ subset as descendant of $CD8^+$sp T cells produces IFN-γ than the other two double-positive subsets

which descend from $CD4^+$sp T cells. Generally, in most of the studies on $CD4^+CD8\alpha^+$dp T cells of other species and IFN-γ production a further distinction of double-positive T cell subsets was not done. For swine and also for the cynomolgus monkey this might not be necessary. Porcine $CD4^+CD8^+$dp T cells only have $CD4^+$sp T cells as their progenitor resulting in a homogeneous population of $CD4^+CD8\alpha\alpha^+$dp T cells (SAALMÜLLER et al. 2002). The progeny of cynomolgus monkey $CD4^+CD8^+$dp T cells is not clarified, but a further differentiation of double-positive T cells might also not be necessary since they mainly seem to consist of $CD4^{bright}CD8\alpha\alpha^{dim}$dp T cells (NAM et al. 2000; AKARI et al. 1997; REIMANN et al. 1994). Human $CD4^+CD8^+$dp T cells can derive from $CD4^+$ or $CD8^+$sp T cells (BLUE et al. 1986). In the study mentioned above on effects of stimulation with virus-infected cell lysates, HLA-A2-restricted peptides, or tetanus toxoid protein human double-positive T cells were divided in two different subsets (NASCIMBENI et al. 2004). Although, no differences in the cytokine profile (including IFN-γ) for these two distinct double-positive T cell subsets were observed. In conclusion, different progeny of double-positive T cells could explain different capabilities of IFN-γ production. This again emphasizes the heterogeneity of canine double-positive T cells.

Interestingly, on single cell level, $CD4^+CD8\alpha^+$dp T cells are high producers of IFN-γ. In the murine system this high production capability was linked to polyfunctionality of T cells (DARRAH et al. 2007; FORBES et al. 2008). Whether this holds true for canine T cells and whether polyfunctionality can be connected to the $CD4^+CD8\alpha^+$dp phenotype needs to be elucidated in future experiments.

Concluding remarks

In summary, the further distinction of double-positive T cells into their subsets is reasonable, since they exhibit different CD25 and CD62L expression. Preliminary data imply that $CD4^+CD8\alpha^+$dp T cells also appear in secondary lymphatic organs, albeit with another distribution of their subsets. This suggests that the $CD4^{bright}CD8\alpha^{bright}$ subset mainly is not part of the recirculating T cell pool. These experiments build the first attempts for functional characterization of canine $CD4^+CD8\alpha^+$dp T cells by identifying them as very potent IFN-γ producers, again with diversity among double-positive T cell subsets. This all could be in accordance with different origin of either $CD4^+$sp or $CD8\alpha^+$sp T cells. Definitely, more data on secondary lymphatic tissue $CD4^+CD8\alpha^+$dp T cells and functional properties are needed to understand their dynamic and function in the canine immune system.

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3 Discussion

The properties and functions of $CD4^+$ single-positive (sp) and $CD8^+$sp T cells during an immune response are very well known. In this work canine $CD4^+CD8^+$ double-positive (dp) T cells were identified to be a distinct but heterogeneous T cell subpopulation with an activated phenotype. In the following, the unique position of $CD4^+CD8^+$dp T cells in the canine system will be discussed. This discussion section is intended to complement but not reiterate the discussion parts of the preceding two publications and the chapter describing unpublished and preliminary results.

3.1 Canine $CD4^+CD8^+$ double-positive T cells are mature αβ T cells and no artifact

The discovery of $CD4^+CD8^+$dp T cells in peripheral blood was against the long believed paradigm of a mutually exclusive $CD4^+$sp or $CD8^+$sp T cell lineage commitment. Thus, also the possibility was discussed that double-positive T cells could be recent thymic emigrants (RTE) which did not completely mature in thymus. The existence of $CD4^+CD8^+$dp immature RTE was shown in lymph nodes of eight-week-old rats (HOSSEINZADEH and GOLDSCHNEIDER 1993) and three-day-old mice, but not in lymph nodes of adult mice (BONOMO et al. 1994). Therefore, this rather seems to be a phenomenon of younger animals. The occurrence of double-positive immature RTE in peripheral blood was not analyzed in these studies, although in mouse spleen, which was not mentioned to have been perfused and thus contained peripheral blood, no double-positive immature RTE occurred (BONOMO et al. 1994).

In contrary, the maturity of $CD4^+CD8^+$dp T cells found in peripheral blood of other species, such as swine (PESCOVITZ et al. 1990; SAALMÜLLER et al. 1989), cynomolgus monkey (AKARI et al. 1997), and human (ORTOLANI et al. 1993) was confirmed by the absence of the thymic marker CD1, CD1b, or CD1a, respectively. In rats, maturity of $CD4^+CD8^+$dp T cells was reasoned by a high expression level of αβ T cell receptor (TCR), which is expressed at low levels on immature thymocytes (KENNY et al. 2000)**.** Interestingly, for rat reports on the effect of thymectomy are diverse: in cyclosporine-treated, allografted rats (GODDEN et al. 1985) thymectomy did not but in healthy rats (KENNY et al. 2000) thymectomy did lead to a transient decrease of $CD4^+CD8^+$dp T cells. In human umbilical cord blood also $CD1a^-$ mature double-positive T cells were found (RES et al. 1997). This implied that also already mature $CD4^+CD8^+$dp T cells might emigrate from thymus. In contrast, the origin of double-positive T cells in cynomolgus monkey was implied to be extrathymic, since with the age of 10 years,

when thymic involution is thought to be completed, the percentage of double-positive T cells increases (NAM et al. 1998).

In our study canine $CD4^+CD8^+$dp T cells in peripheral blood and lymph nodes were also identified to be mature T cells which do not express the thymic marker CD1a. TCR staining of canine $CD4^+CD8^+$dp T cells revealed the utilization of αβ TCR (BISMARCK et al. 2012) similarly to known double-positive T cells in other species (COLOMBATTI et al. 1998; KENNY et al. 2000; SALA et al. 1993; TONUTTI et al. 1994).

To exclude the possibility of a flow cytometric artifact due to cellular doublets or dead cells (KELLY et al. 1988), for analysis of canine $CD4^+CD8^+$dp T cells only living cells with subsequent doublet-exclusion were used. In addition, CD4 and CD8α mRNA was detected in purified canine $CD4^+CD8^+$dp T cells. The cell sort for this analysis revealed a high purity (at least 99.7%) and the double-positive phenotype was maintained during flow cytometric re-analysis.

3.2 Activated phenotype of $CD4^+CD8^+$ double-positive T cells

To analyze the activation-related phenotype of $CD4^+CD8^+$dp T cells several activation markers such as CD25 (ORTEGA et al. 1984; UCHIYAMA et al. 1981) and CD62L were determined. CD25 is the alpha chain of the interleukin-2 (IL-2) receptor. By its additional expression on the cell surface, the low affinity IL-2 receptor switches to high affinity and cells are more responsive to IL-2, which is released in their local milieu and induces proliferation (GREENE and LEONARD 1986; SMITH 1989). CD62L is a homing marker for secondary lymphoid tissue (ARBONES et al. 1994; GALLATIN et al. 1983) and regulates circulating and homing properties of T cells. Upon activation, CD62L is shed from the cell surface (CHAO et al. 1997). Its downregulation can be linked to an effector stage (HAMANN et al. 1988; JUNG et al. 1988) and is used to differentiate naïve T cells from T effector cells and likewise T central memory cells (T_{CM}) from T effector memory cells (T_{EM}) (BEVERLEY 2008).

Canine $CD4^+CD8^+$dp T cells in peripheral blood were shown to constantly express CD25 in a higher frequency compared with $CD4^+$ or $CD8^+$sp T cells (BISMARCK et al. 2012). CD62L is expressed at a lower frequency on double-positive T cells than on $CD4^+$sp T cells but quite similar as on $CD8^+$sp T cells (BISMARCK et al. 2012 and unpublished). With regard to the activation phenotype, analogies between canine and porcine species can be seen. Porcine $CD4^+CD8^+$dp T cells also show a higher frequency of $CD25^+$ cells among $CD4^+CD8^+$dp T cells compared with single-positive T cells (see table 1) (SAALMÜLLER et al. 2002). The permanent CD25 expression of a higher frequency of double-positive T cells might suggest that at least

some of them are regulatory T (Treg) cells. Treg cells are known to constitutively express CD25 (SAKAGUCHI et al. 1995) and are characterized by their transcription factor FoxP3 (forkhead box P3) (FONTENOT et al. 2003; HORI et al. 2003). Among porcine $CD4^+CD8^+$dp T cells also $FoxP3^+CD25^+$ Treg cells could be detected (KÄSER et al. 2008). Presently, as reported by others (personal communication TOBIAS KÄSER, Edinburgh, Scotland, European Veterinary Immunology Workshop, 2nd - 4th September 2012), a reliable protocol for flow cytometric staining of canine transcription factor FoxP3 could not be established. The constitutive CD25 expression among canine and porcine $CD4^+CD8^+$dp T cells is in great contrast to human, monkey, and chicken double-positive T cells which in peripheral blood do not express CD25 (AKARI et al. 1996; COLOMBATTI et al. 1998; MURAYAMA et al. 1997; SALA et al. 1993; TONUTTI et al. 1994). The CD62L expression profile of canine double-positive T cells is similar as for other species where L-selectin expression was analyzed (see table 1)(NASCIMBENI et al. 2004; REUTNER et al. 2013).

It was shown that *in vitro* upon polyclonal concanavalin A (ConA) or oligoclonal *Staphylococcus aureus* enterotoxin B (SEB) stimulation as well as viral restimulation with *Canine distemper virus* (CDV) the portion of $CD4^+CD8^+$dp T cells increases (BISMARCK et al. 2012). Newly developed double-positive T cells exhibit the highest frequency of $CD25^+$ cells, strongest downregulation of CD62L, and a higher proportion of proliferated cells compared with $CD4^+$ or $CD8^+$sp T cells. This emphasizes the link between the double-positive state and activation.

Our progenitor studies revealed that upon *in vitro* activation $CD4^+$ and to a lesser extent also $CD8^+$sp T cells can be progenitors of $CD4^+CD8^+$dp T cells (BISMARCK et al. 2014). It is noteworthy, that not all activated $CD4^+$ or $CD8^+$sp T cells upregulate an additional coreceptor. Therefore, activation does not necessarily lead to double-positive development in canine single-positive T cells. Distinct signals or requirements of progenitor cells for double-positive T cell development remain to be elucidated.

3.3 Different single-positive T cell progenitors point to distinct coreceptor as well as activation marker expression and functional properties of canine $CD4^+CD8^+$ double-positive T cell subsets

On $CD4^+CD8^+$dp T cells two different isoforms of the CD8 receptor were detected: the CD8αα homodimer and the CD8αβ heterodimer. Thus, all canine double-positive T cells express a CD4 receptor and the CD8α chain but can differ in CD8β expression. Interestingly, the intensity of CD4 and CD8α expression differs among double-positive T cells, which allows further division into three subsets: $CD4^{dim}CD8\alpha^{bright}$, $CD4^{bright}CD8\alpha^{bright}$ and $CD4^{bright}CD8\alpha^{dim}$

(BISMARCK et al. 2012). The progenitor studies identified either $CD4^+$ or $CD8\alpha^+$sp T cells as double-positive T cell origin. Upon *in vitro* activation, $CD4^+$sp T cells give rise to both $CD4^{bright}$ subsets and $CD8\alpha^+$sp T cells mainly to the $CD4^{dim}CD8\alpha^{bright}$ subset. This *in vitro* observation led to the assumption that also *in vivo* double-positive T cells have two distinct progenitors. This can be further confirmed by an overview on all available data:

1) **The different intensity of CD4 and CD8α expression**: The first point is obvious. Double-positive T cell subsets which exhibit a $CD4^{bright}$ but $CD8\alpha^{dim}$ expression are likely to be descendants of $CD4^+$sp T cells. Subsets which exhibit a $CD8\alpha^{bright}$ but $CD4^{dim}$ expression should be descendants of $CD8^+$sp T cells. For the subset with a $CD4^{bright}$ and $CD8\alpha^{bright}$ expression a conclusion cannot be drawn.

2) **The proportion of the subsets of the total $CD4^+CD8\alpha^+$dp T cell population:** The size of double-positive T cell subsets correlates with the efficiency of single-positive T cells to upregulate the additional coreceptor. $CD8\alpha^+$sp T cells, with a low efficiency of double-positive T cell development, lead to a small $CD4^{dim}CD8\alpha^{bright}$ subset. $CD4^+$sp T cells, with a higher efficiency of double-positive T cell development, lead to the larger $CD4^{bright}CD8\alpha^{dim}$ and $CD4^{bright}CD8\alpha^{bright}$ subsets.

3) **The CD8 receptor composition:** Similar to $CD8\alpha^+$sp T cells the $CD4^{dim}CD8\alpha^{bright}$ subset mainly expresses the CD8αβ heterodimer.

4) **The frequency of $CD25^+$ cells (activation marker):** The $CD4^{dim}CD8\alpha^{bright}$dp T cell subset has a lower frequency of $CD25^+$ cells than both $CD4^{bright}$ subsets. This is similar to the lower frequency of $CD25^+$ cells among $CD8\alpha^+$sp T cells compared with $CD4^+$sp T cells.

5) **The CD62L (L-selectin) expression:** The $CD4^{dim}CD8\alpha^{bright}$ subset shows a tendency of a lower frequency of $CD62L^+$ cells compared with the $CD4^{bright}CD8\alpha^{dim}$ subset, which is similar to a lower frequency of $CD62L^+$ cells among $CD8\alpha^+$sp T cells compared with $CD4^+$sp T cells. The $CD4^{bright}CD8\alpha^{bright}$ subset is clearly an exception with its low frequency of $CD62L^+$ cells.

6) **The IFN-γ production:** The $CD4^{dim}CD8\alpha^{bright}$ subset overall exhibits the highest frequency of IFN-γ producing cells compared with both $CD4^{bright}$ subsets, which is similar to $CD8\alpha^+$sp T cells compared with $CD4^+$sp T cells.

In conclusion, these various lines of experimental evidence strongly argue for $CD8\alpha^+$sp T cells as the progenitors of the $CD4^{dim}CD8\alpha^{bright}$ subset and $CD4^+$sp T cells as the progenitors of both $CD4^{bright}$ subsets *in vivo*. Since very few $CD4^{bright}CD8\alpha^{bright}$ cells also express CD8β and *in vitro* to a smaller extent $CD8\alpha^+$sp T cells could highly upregulate CD4, it is conceivable that also *in vivo* some $CD8^+$sp T cells are progenitors of the $CD4^{bright}CD8\alpha^{bright}$ subset. However, *in vivo*

additional possibilities of double-positive T cell development could occur. Presently, it cannot be excluded that already mature $CD4^+CD8\alpha^+$dp T cells emigrate from thymus in peripheral blood as it was implied in some studies for human and rat, as mentioned above (KENNY et al. 2000; RES et al. 1997). Based on this double-positive T cells also could be self-renewing. To further investigate this, studies on *in vivo* longevity of canine double-positive T cells might be helpful.

As can be seen in table 1 the progeny of double-positive T cells described in other species differs from dog. Porcine as well as monkey double-positive T cells represent a homogeneous $CD4^+CD8\alpha\alpha^+$dp T cell population (AKARI et al. 1996; MURAYAMA et al. 1997; SAALMÜLLER et al. 2002). Thus, further division into subsets by CD4 and CD8αβ expression in these two species is not necessary or possible. Similar to canine, human $CD4^+CD8^+$dp T cells can be of both $CD4^+$ or $CD8^+$ origin (BLUE et al. 1985). In accordance, human double-positive T cells can also be divided in subsets: $CD4^{dim}CD8\alpha\beta^{bright}$, $CD4^{bright}CD8\alpha\beta^{bright}$ and $CD4^{bright}CD8\alpha\alpha^{dim}$ (SALA et al. 1993; TONUTTI et al. 1994). The $CD4^{bright}CD8\alpha^{bright}$ subset in human is mostly observed in cases of immune diseases such as multiple sclerosis (MUNSCHAUER et al. 1993), Kawasaki disease (HIRAO and SUGITA 1998) and atopic dermatitis (BANG et al. 2001).

3.4 Lymph node as a possible site of $CD4^+CD8^+$ double-positive T cell induction

Naïve T cells circulate constantly between blood and lymph node. In lymph node naïve T cells are activated, if they get in contact with an antigen-presenting cell (APC) which expresses cognate antigen by MHC and provides costimulatory signals. As shown by us, *in vitro* activation leads in a portion of single-positive T cells to upregulation of the additional CD4 or CD8α coreceptor (BISMARCK et al. 2014). Canine naïve single-positive T cells could be activated in lymph node after antigen recognition and become double-positive. Analysis of double-positive T cell subsets in lymph nodes revealed a different composition of subsets compared with peripheral blood (BISMARCK et al. unpublished). The $CD4^{bright}CD8\alpha^{bright}$ subset in lymph node is clearly smaller than in peripheral blood and the $CD4^{bright}CD8\alpha^{dim}$ subset in lymph node is larger than in peripheral blood.

This points to the following possible mechanism: Naïve $CD4^+$sp T cells become activated in lymph node. Some of these activated $CD4^+$sp T cells begin to upregulate the additional coreceptor and become $CD4^{bright}CD8\alpha^{dim}$. These activated $CD4^{bright}CD8\alpha^{dim}$ effector cells migrate into peripheral blood, where some of them eventually could become $CD4^{bright}CD8\alpha^{bright}$. The fate of these $CD4^{bright}CD8\alpha^{bright}$ effector cells could be divergent. Either they die after

accomplishing their task or they develop into memory cells (SPRENT and TOUGH 2001). Also, both options can proceed in parallel. Memory development would include downregulation of CD25 after the period of activation is passed. Moreover, the $CD4^{bright}CD8\alpha^{bright}$ subset includes the lowest frequency of $CD62L^{+}$ cells among double-positive subsets, which diminishes their homing to lymph nodes. Consequently, the possibility that the progression of $CD4^{bright}CD8\alpha^{dim}$ cells to $CD4^{bright}CD8\alpha^{bright}$ cells occurs after emigration from lymph node in peripheral blood combined with the low ability of the $CD4^{bright}CD8\alpha^{bright}$ subset to home to lymph nodes could explain its small abundance there. Whether diminished lymph node homing ability of these effector cells redirects their migration to inflamed tissue is an interesting topic for future analysis to reveal their position in the effector and memory system of T cells.

Similar developmental pathway could be presumed for double-positive T cell development from $CD8\alpha^{+}$sp T cells. Since $CD8\alpha^{+}$sp T cells show a lower efficiency of additional coreceptor upregulation, a low frequency of the $CD4^{dim}CD8\alpha^{high}$ subset in lymph node and also in peripheral blood is not surprising.

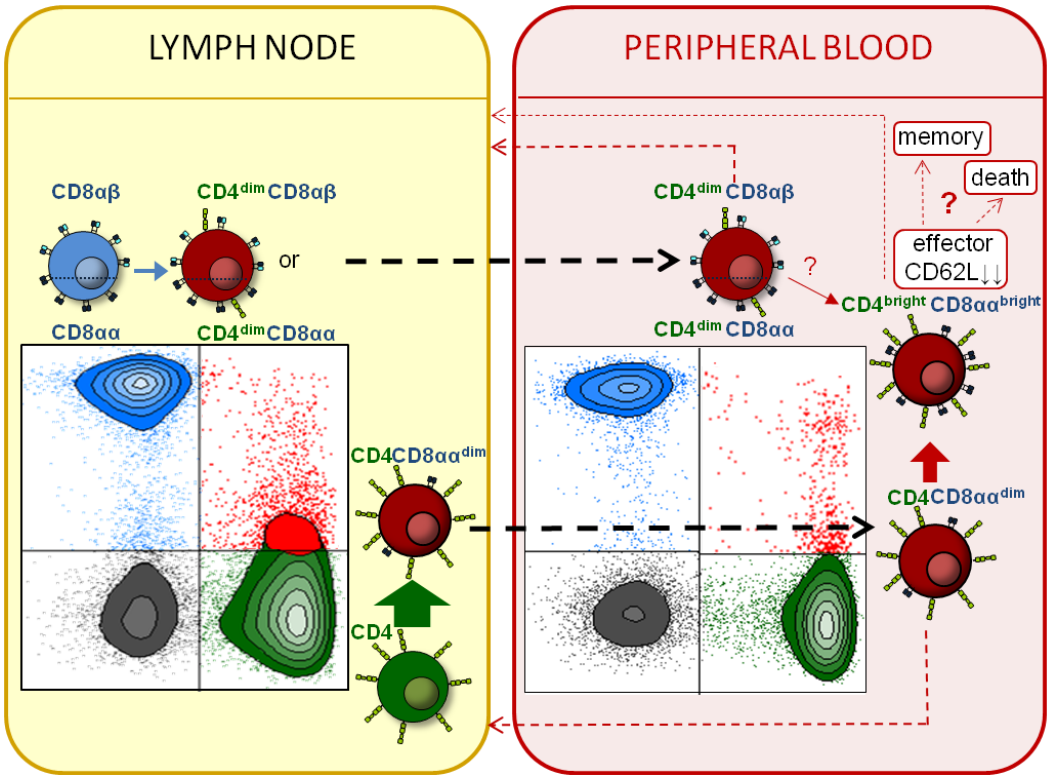

Fig. 1 Possible development of canine peripheral blood $CD4^{+}CD8\alpha^{+}$dp T cells

Double-positive T cells might develop in lymph node from either $CD4^{+}$sp or $CD8^{+}$sp T cells. After migration of double-positive cells with a dim coreceptor expression into peripheral blood further progression might occur there. The fate of effector double-positive T cells is still unclear. Dashed lines indicate migration of cells (thick dashed lines = high frequency of cells migrates; thin dashed lines = low frequency of cells migrates).

3.5 Potential role of memory among $CD4^+CD8^+$ double-positive T cells

In some other species $CD4^+CD8^+$dp T cells are associated with a memory phenotype (see table 1). Induction of memory is still not fully understood. For a long time it was not even clear whether T memory cells develop from proliferating effector cells or from separate precursors (AHMED and GRAY 1996). Today, it is believed that for memory T cell development at least some effector functions need to be expressed (OPFERMAN et al. 1999; SAPAROV et al. 1999).

For the dog, presently the lack of reagents does not allow for further distinction between naïve and memory T cells. In other species, naïve T cells, T_{EM} and T_{CM} cells can be better subdivided, for example by expression of CCR7 and CD45RA (MICHIE et al. 1992): naïve T cells = $CD45RA^+$ $CCR7^+$, T_{EM} = $CD45RA^-$ $CCR7^+$ and T_{CM} = $CD45RA^+$ $CCR7^-$ (SALLUSTO et al. 1999). Unfortunately, both antibodies do not cross-react with canine cells. As mentioned in the previous chapter, it is possible that some canine $CD4^+CD8\alpha^+$dp T cells are of memory type. Findings from the canine *in vitro* studies could be in favor of this assumption (BISMARCK et al. 2012). It is known that memory T cells show a stronger rate of cell division (turnover) compared with naïve T cells *in vivo* (TOUGH and SPRENT 1994). Consistent with this, a higher proliferative capacity of canine double-positive T cells in comparison with single-positive T cells after seven days of cell-culture medium incubation was observed (BISMARCK et al. 2012).

More recently it was described that an additional transient expression of CD8αα homodimers on $CD8\alpha\beta^+$ T cells upon antigenic stimulation promotes survival and differentiation into memory cells (MADAKAMUTIL et al. 2004). Nevertheless, memory formation can also appear without this transient CD8αα expression (CHANDELE and KAECH 2005; ZHONG and REINHERZ 2005). In human peripheral blood also $CD8\alpha\alpha^+$ T cells were found for which a memory phenotype was implied (KONNO et al. 2002). It is assumed that CD8αα is upregulated upon activation at the beginning of an immune response. In this phase the antigen dose is high and thus CD8αα preserves effector T cells for memory formation that emerge under strong activation conditions. These are preferentially T_{EM} (CHEROUTRE and LAMBOLEZ 2008). Whether this CD8αα expression during memory formation is also at least transiently present on $CD4^+$T cells might be an interesting topic.

Hence, it is reasonable that some canine double-positive T cells could be of a memory phenotype, since they (i) show a stronger turnover *in vitro* and (ii) express CD8αα which recently was associated with memory formation. This applies especially for the $CD4^{bright}CD8\alpha^{bright}$ subset: with its high CD8αα expression it preferably could evolve to $T_{EM.}$ Indeed, this subset includes a higher frequency of $CD62L^-$ effector cells (BISMARCK et al. unpuplished). Whether the expression of CD8αα on canine double-positive T cells is permanent or only transient and cells return to a single-positive phenotype or whether double-

positive T cells are mainly activated effector cells which die later remains to be investigated. Further studies on this topic in dogs are necessary when appropriate tools for analysis are available.

3.6 Possible therapeutic applications of canine $CD4^+CD8^+$ double-positive T cells in cancer treatment

In human $CD4^+CD8^+$dp T cells, especially the $CD4^{bright}CD8^{bright}$ subset is associated with neoplasms and other diseases (BAGOT et al. 1998; BERNARD et al. 1981; DESFRANCOIS et al. 2009; ORTOLANI et al. 1993)**.** Besides surgery, radiation and chemotherapy, activated lymphocyte therapy is used as cancer treatment and leads to clinically significant anti-tumor responses in patients (DUDLEY et al. 2008; ROSENBERG and DUDLEY 2009; ROSENBERG et al. 2008). This method has been also transferred to small animal medicine (HOSHINO et al. 2008).

Autologous *ex vivo* proliferating and activated lymphocytes were sequentially re-injected into healthy dogs. Among *ex vivo* activated T cells the percentage of $CD4^+CD8^+$dp T cells increased from culture cycle to culture cycle. Activated lymphocytes are supposed to express direct cytotoxic functions against tumor cells. In addition, they might be able to indirectly induce cell-mediated immunity with T lymphocyte and NK cell activation by cytokine secretion (KWAK et al. 2000). The therapy had no severe adverse effects on healthy dogs. Amongst others, after an initial phase of 14 days the serum IFN-γ level transiently increased in treated dogs. It has not been evaluated whether this effect was mediated by $CD4^+$sp, $CD8^+$sp, or $CD4^+CD8^+$dp T cells. The pro-inflammatory cytokine IFN-γ plays an important role in the anti-tumor immune response. It can inhibit cell proliferation and tumor angiogenesis, leads to surface MHC-I expression, and can facilitate enhanced trafficking of cytotoxic T cells to the tumor (BOEHM et al. 1997; NALDINI and CARRARO 2005). In addition, in several human anti-tumor treatment studies it was observed that IFN-γ production was associated with an improved clinical prognosis (KUSUDA et al. 2005; MARTH et al. 2004; ZHANG et al. 2003).

To further enhance the therapeutic efficacy an additional enrichment of double-positive T cells after the *ex vivo* culture for example by magnetic or fluorescence-activated cell sorting could be performed. Consequently, highly activated and very potent IFN-γ producing double-positive T cells could lead to an additional or prolonged IFN-γ increase in serum and thus possibly improve anti-tumor treatment. Another possibility could be local treatment of a solid tumor by direct injection of an enriched double-positive T cell suspension. Dog and human show similarities in spontaneous tumor development and morphology (KHANNA et al. 2006), thus improvement of cancer therapy could be beneficial for both species.

3.7 Interspecies differences reveal a unique canine $CD4^+CD8\alpha^+$ double-positive T cell population

	Origin	Subsets & CD8 receptor	Phenotype related to activation	Function
DOG	*in vitro*: $CD4^+$ $CD8^+$ [11]	$CD4^{bright}$ $CD8\alpha\alpha^{dim}$ $CD4^{bright}$ $CD8\alpha\alpha^{bright}$ $CD4^{dim}$ $CD8\alpha\beta^{bright}$ [11; 12]	*in vitro* activation: coreceptor↑ = activation / $CD25^{bright}$↑↑ [12] peripheral blood: CD25↑ [12] CD62L↓ [12]	• IFN-γ production ???
SWINE	*in vitro*: $CD4^+$ [108]	$CD4^{bright}$ $CD8\alpha\alpha^{dim}$ [108]	*in vitro* activation: coreceptor↑ = activation / CD25↑↑ [108] peripheral blood: CD25↑ [108] CD62L↓ (mRNA) [100]	• T memory cells [108] • IFN-γ production [102] • Treg/regulatory function [48] • no cytolytic function [92; 129]
MONKEY	? extra-thymic origin [81]	$CD4^{bright}$ $CD8\alpha\alpha^{dim}$ [4]	peripheral blood: $CD25^-$ [4]	• resting memory phenotype [4] • cytotoxic activity [80] • IFN-γ production [80]
HUMAN	*in vitro:* $CD4^+$ $CD8^+$ [13]	$CD4^{bright}$ $CD8\alpha\alpha^{dim}$ $CD4^{bright}$ $CD8\alpha\beta^{bright}$ * $CD4^{dim}$ $CD8\alpha\beta^{bright}$ [111; 122]	*in vitro* activation: coreceptor↑ = activation [13; 119] peripheral blood: $CD25^-$ [21; 111; 122] CD62L↓ [82]	• T effector memory with antiviral function [82] • IFN-γ production [82] • CD4 receptor on $CD8^+$: chemotactic to IL-16 [58] • CD8 receptor on $CD4^+$: cytolytic function [89]
			during infection: CD25↑ [26]	*associated with disease* [132]
RAT	*in vivo: mature/* immature RTE * [44; 52]	$CD4^+CD8\alpha\alpha^+$ $CD4^+CD8\alpha\beta^+$ [44; 52]	*in vitro* activation: coreceptor↑ = activation [53; 96] peripheral blood: CD25↑ [44; 52]	• IFN-γ production [53]
CHICKEN	?	$CD4^+CD8\alpha\alpha^+$ [66]	peripheral blood: $CD25^-$ [66]	• comparable to $CD4^+$ T cells [66]

Table 1 Interspecies differences of $CD4^+CD8^+$dp peripheral blood T cells

Table 1 Interspecies differences of $CD4^+CD8^+$dp peripheral blood T cells

This table depicts roughly major facts regarding origin, subset & CD8 receptor composition, activation phenotype and functional properties of $CD4^+CD8^+$dp T cells in different species. Arrows indicate either a higher (↑) or a lower (↓) percentage of receptor expression. Asterisk (*) indicates that description differs between reports. Numbers in brackets refer to the reference list at the end of this thesis.

3.8 Concluding remarks

Overall, this work provides a mandatory basic characterization of canine $CD4^+CD8\alpha^+$dp T cells: Canine double-positive T cells are a unique T cell subpopulation with a high activation potential. They display a heterogeneous group, which is in accordance to their different origin from either $CD4^+$ or $CD8\alpha^+$sp T cells. In comparison with their single-positive T cell progenitors they stand out with a more explicit activation profile and IFN-γ producing capacity. A memory phenotype at least for some canine double-positive T cells can be assumed.

3.9 Outlook

Generally, the limited availability of veterinary and particularly anti-canine antibodies for use in flow cytometry limits characterization of double-positive T cells using this method. The great advantage of flow cytometry is to specifically analyze distinct subpopulations, which was shown to be useful for the heterogeneous canine double-positive T cells. Other immunological methods as enzyme-linked immunosorbent assay (ELISA) or real-time reverse transcription quantitative polymerase chain reaction (RT-qPCR) only provide information for the whole cell culture.

Nevertheless, a solution for this problem could be the combination of flow cytometry and fluorescence-activated cell sorting with subsequent real-time RT-qPCR, as already performed for clarification of mechanisms involved in double-positive T cell development (BISMARCK et al. 2014).

Open questions which were raised by basic characterization of double-positive T cells remain to be elucidated:

(i) Of interest, as indicated above, is the clarification of the possible memory phenotype of double-positive T cells.

(ii) Functional analysis: With identification of $CD4^+CD8\alpha^+$dp T cells as potent IFN-γ producers first steps for functional classification were performed. It would be helpful to provide further insights on production of typical Th1 and Th2 cytokines or cytotoxic T lymphocyte (CTL) effector molecules. This could be combined with determination of

subset specific transcription factors. Thus, the important question whether the $CD4^+CD8\alpha^+$dp phenotype correlates with a specialized function could be addressed.

In addition, confirmation and extension of anatomical distribution of $CD4^+CD8\alpha^+$dp T cells, especially in inflamed tissues and niches of memory cell survival, could provide more insights on their dynamics and role in the canine immune system.

4 Summary

Doris Bismarck

Canine $CD4^+CD8^+$ double-positive T cells: unique cells with an activated phenotype, remarkable heterogeneity, different origins, and a high capacity of interferon-gamma production

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73 pages, 20 figures, 1 table, 189 references

Keywords: dog, $CD4^+CD8^+$ double-positive T cells, activated phenotype, progenitors, Interferon-gamma, anatomical distribution

Introduction: T cells play an important role in the adaptive immune response of dogs and any other vertebrate species. It is generally accepted that, after passing an immature $CD4^+CD8^+$ double-positive (dp) state during thymic development, T cells, before emigrating into peripheral blood, differentiate into either $CD4^+$ single-positive (sp) T-helper or $CD8^+$sp cytotoxic T cells. However, in dogs, and also in some other species, a distinct peripheral blood T cell population exists which expresses both coreceptors simultaneously: $CD4^+CD8^+$dp T cells.

Aims of study: Objective of this study was a basic phenotypical characterization of canine peripheral blood $CD4^+CD8^+$dp T cells and identification of their origin. In addition, screening of secondary lymphatic organs and initial functional analysis by determination of interferon-gamma (IFN-γ) production intended to further elucidate the role of $CD4^+CD8^+$dp T cells in the canine immune system.

Material and methods: Freshly isolated (sample size: n = 19 dogs) or stimulated canine peripheral blood mononuclear cells (PBMC) and also lymph node and splenic cells (n = 5 dogs) of dogs were analyzed using flow cytometry. For phenotypic characterization of peripheral blood $CD4^+CD8^+$dp T cells their activation state was determined by cell surface expression of CD25 and CD62L (L-selectin) and as functional property IFN-γ production upon stimulation was analyzed (n ≥ 12 dogs) (BISMARCK et al. 2012 and unpublished). To identify the origin of double-positive T cells (n = 12 dogs), fluorescence-activated cell sorting was performed to purify $CD4^+$sp or $CD8^+$sp T cells as possible progenitors. By cell tracing experiments origin and conditions of $CD4^+CD8^+$dp T cell development were investigated. Real-

time reverse transcription quantitative polymerase chain reaction was performed for certain experimental setups to substantiate *de novo* synthesis of selected surface receptors (n = 3 dogs) (BISMARCK et al. 2014). Statistical analysis was done using one-way analysis of variance with subsequent post-hoc tests.

Results: This work identified $CD4^+CD8^+$dp T cells in canine peripheral blood as mature αβ T cells. They comprise a small T cell subpopulation (average of 2.4% of T cells) which exhibits a more activated phenotype than $CD4^+$sp or $CD8^+$sp T cells.

Upon oligoclonal, polyclonal, or viral recall stimulation of PBMC the proportion of $CD4^+CD8^+$dp T cells increases. These stimulation-induced double-positive T cells, again, possess a more activated phenotype than $CD4^+$sp or $CD8^+$sp T cells. The higher activation of double-positive T cells in comparison with $CD4^+$sp or $CD8^+$sp T cells after oligoclonal and polyclonal stimulation is also reflected by their higher IFN-γ production on the single cell level.

It was demonstrated that $CD4^+$sp and to a lesser extent $CD8^+$sp T cells can be progenitors of $CD4^+CD8^+$sp T cells. The CD8 receptor is composed of two chains which either assemble as a CD8αβ heterodimer or a CD8αα homodimer. Double-positive T cells which develop from $CD4^+$sp T cells express the CD8αα homodimer. In contrast, on the majority of double-positive T cells which develop from $CD8^+$sp T cells the CD8αβ heterodimer (about 80%) was detected. Consequently, the total population of canine double-positive T cells has in common that it possesses a CD4 receptor and a CD8α chain. On the basis of the different intensity of CD4 and CD8α expression three distinct double-positive T cells subsets can be defined: $CD4^{dim}CD8\alpha^{bright}$, $CD4^{bright}CD8\alpha^{bright}$, and $CD4^{bright}CD8\alpha^{dim}$ (^{bright} = high expression; ^{dim} = low expression). These subsets contribute to the total double-positive T cell population with the $CD4^{dim}CD8\alpha^{bright}$ subset always being the smallest.

The $CD4^{bright}CD8\alpha^{bright}$ subset has a special position among the three double-positive T cell subsets. It mainly includes effector cells which are characterized by an existing CD25 and lack of CD62L expression. CD62L mediates extravasation of T cells into secondary lymphatic organs. This study for the first time describes the occurrence of $CD4^+CD8^+$dp T cells (average 3.7% of T cells) in secondary lymphatic organs. It revealed that the portion of the $CD4^{bright}CD8\alpha^{bright}$ subset in lymph nodes is smaller than in peripheral blood which is in accordance with their effector phenotype.

Conclusions: This work identifies canine peripheral blood $CD4^+CD8^+$dp T cells of different origin as a unique but heterogeneous T cell subpopulation. Double-positive T cells display an activated phenotype and can be potent IFN-γ producers. Thus, $CD4^+CD8\alpha^+$dp T cells might be crucial effector cells during cell-mediated immunity. This could be exploited therapeutically for example in novel strategies for anti-tumor treatment.

5 Zusammenfassung

Doris Bismarck

Canine $CD4^+CD8^+$ doppelt-positive T-Zellen: eine einzigartige, aktivierte und heterogene T-Zellpopulation unterschiedlichen Ursprungs mit ausgeprägter Fähigkeit zur Interferon-gamma Produktion

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Schlüsselwörter: Hund, $CD4^+CD8^+$ doppelt-positive T-Zellen, aktivierter Phänotyp, Vorläuferzellen, Interferon-gamma, anatomische Verteilung

Einleitung: T-Zellen spielen eine zentrale Rolle in der adaptiven Immunantwort von Hunden und anderen Vertebraten. Es galt lange die allgemeine Auffassung, dass T-Zellen während ihrer Reifung im Thymus zwar ein $CD4^+CD8^+$ doppelt-positives (dp) Stadium durchlaufen, aber vor dem Übergang ins Blut zu $CD4^+$ einfach-positiven T-Helferzellen oder $CD8^+$ einfach-positiven zytotoxischen T-Zellen ausreifen. Sowohl bei Hunden als auch in einigen anderen Spezies wurden allerdings inzwischen T-Zellen im peripheren Blut beschrieben, die beide Oberflächenrezeptoren gleichzeitig exprimieren: $CD4^+CD8^+$dp T-Zellen.

Ziele der Untersuchung: In dieser Arbeit sollte eine grundlegende Charakterisierung von $CD4^+CD8^+$dp T-Zellen im peripheren Blut des Hundes in Bezug auf ihren Phänotyp und ihre Herkunft erfolgen. Weiterhin sollten Untersuchungen in sekundären lymphatischen Organen sowie erste funktionelle Analysen der Interferon-gamma (IFN-γ) Produktion zum Verständnis der Rolle von $CD4^+CD8^+$dp T-Zellen im Immunsystem des Hundes beitragen.

Materialien und Methoden: Der Nachweis $CD4^+CD8^+$dp T-Zellen (Stichprobenumfang: n = 19 Hunde) erfolgte mittels Durchflusszytometrie in frisch isolierten beziehungsweise in *in vitro* stimulierten peripheren mononukleären Blutzellen (PBMC) sowie in Lymphknoten- und Milzzellen (n = 5 Hunde). Des Weiteren wurde der Aktivierungszustand der $CD4^+CD8^+$dp T-Zellen des peripheren Blutes durchflusszytometrisch bestimmt, indem die Expression der Oberflächenmarker CD25 und CD62L (L-Selektin) sowie ihre IFN-γ Produktion nach Stimulation (n ≥ 12 Hunde) analysiert wurde (BISMARCK et al. 2012 und unveröffentlicht). Die Untersuchungen zur Herkunft und Bedingungen der Entstehung von $CD4^+CD8^+$dp T-Zellen (n = 12 Hunde) basierten auf der fluoreszenz-aktivierten Zellsortierung der möglichen $CD4^+$ und $CD8^+$ einfach-positiven Vorläuferzellen und anschließenden Zellmarkierungs-Experimenten. In einigen experimentellen Konditionen wurde mittels quantitativer Echtzeit-reverser Transkriptase-Polymerase-Kettenreaktion die *de novo* Synthese ausgewählter Oberflächenrezeptoren untersucht (n = 3 Hunde) (BISMARCK et al. 2014). Die statistische

Auswertung der Daten erfolgte mittels einfacher Varianzanalyse mit anschließenden Post-Hoc-Mehrfachvergleichen.

Ergebnisse: In der vorliegenden Arbeit konnten im peripheren Blut des Hundes $CD4^+CD8^+$dp T-Zellen als reife αβ T-Zellen identifiziert werden. Sie stellen eine kleine T-Zellpopulation (ca. 2,4% der T-Zellen) dar, die über einen stärker aktivierten Phänotyp als $CD4^+$ oder $CD8^+$ einfach-positive T-Zellen verfügt. Nach oligoklonaler oder polyklonaler Stimulation sowie nach viraler Restimulation von PBMC nimmt der Anteil an $CD4^+CD8^+$dp T-Zellen zu. Diese weisen interessanterweise wiederum einen höheren Aktivierungsgrad als die $CD4^+$ und $CD8^+$ einfach-positiven T-Zellen auf. Die stärkere Aktivierung doppelt-positiver T-Zellen im Vergleich zu einfach-positiven T-Zellen nach oligoklonaler und polyklonaler Stimulation spiegelt sich auf Einzelzellebene zudem in der erhöhten Produktion von IFN-γ wider.

Es konnte gezeigt werden, dass $CD4^+$ und in einem geringeren Maße auch $CD8^+$ T-Zellen Vorläufer von $CD4^+CD8^+$dp T-Zellen sind. Der CD8 Rezeptor besteht aus zwei Ketten, die entweder als CD8αβ Heterodimer oder als CD8αα Homodimer vorliegen. Doppelt-positive T-Zellen, die nach *in vitro* Stimulation aus $CD4^+$ T-Zellen hervorgehen, verfügen über einen CD8αα Rezeptor. Im Gegensatz dazu ist auf doppelt-positiven T-Zellen, die aus $CD8^+$ T-Zellen entstehen, überwiegend der CD8αβ Rezeptor (ca. 80%) nachweisbar. Eine Gemeinsamkeit aller doppelt-positiver T-Zellen des Hundes besteht also darin, dass sie einen CD4 Rezeptor und eine CD8α Kette besitzen. Aufgrund der unterschiedlichen Intensität der CD4 und CD8α Expression kann eine weitere Unterteilung in drei Subpopulationen erfolgen: $CD4^{dim}CD8\alpha^{bright}$, $CD4^{bright}CD8\alpha^{bright}$ und $CD4^{bright}CD8\alpha^{dim}$ (^{bright} = starke Expression; ^{dim} = schwache Expression). Diese Subpopulationen tragen prozentual unterschiedlich zur Gesamtpopulation der doppelt-positiven T-Zellen bei, wobei die $CD4^{dim}CD8\alpha^{bright}$ Subpopulation den geringsten Anteil hat.

Eine besondere Stellung innerhalb der doppelt-positiven T-Zellen nimmt die $CD4^{bright}CD8\alpha^{bright}$ Population ein. Sie beinhaltet hauptsächlich Effektor-Zellen, die durch eine vorhandene CD25 und fehlende CD62L Expression gekennzeichnet sind. CD62L vermittelt den Eintritt von T-Zellen in sekundäre lymphatische Organe. In diesen konnte im Rahmen der vorliegenden Arbeit erstmals ebenfalls eine $CD4^+CD8^+$dp T-Zellpopulation (ca. 3,7% der T-Zellen) nachgewiesen werden. Dabei stellte sich heraus, dass der Anteil der $CD4^{bright}CD8\alpha^{bright}$ Subpopulation in Lymphknoten im Einklang mit dem Effektor-Phänotyp geringer ist als im Blut.

Schlussfolgerungen: Zusammenfassend werden in dieser Arbeit $CD4^+CD8^+$dp T-Zellen des Hundes als eine heterogene T-Zellpopulation charakterisiert, die aus $CD4^+$ und in geringerem Maße aus $CD8^+$ einfach-positiven Vorläuferzellen entstehen kann. Doppelt-positive T-Zellen sind durch einen aktivierten Phänotyp und ein hohes Potential zur IFN-γ-Produktion gekennzeichnet. Das weist auf eine wichtige Rolle dieser einzigartigen Zellpopulation in der zellulären Immunität hin. Demzufolge könnten $CD4^+CD8^+$dp T-Zellen therapeutisch, beispielsweise in der Tumortherapie, von großem Nutzen sein.

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