Investigation of immunomodulatory properties of neurovirulent viruses – *in vitro* and *in vivo* effects of canine distemper virus

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Investigation of immunomodulatory properties of neurovirulent viruses – in vitro and in vivo effects of canine distemper virus

Thesis

Submitted in partial fulfillment of the requirements for the degree

Doctor of Philosophy (PhD)

awarded by the University of Veterinary Medicine Hannover

by

Visar Qeska

(Republic of Kosovo)

Hannover 2013
Visar Qeska was supported by Young cell scheme VI, European Council and Kosovo government, Department of Pathology, Veterinary University Hannover, Germany, and Center for Systems Neuroscience (ZSN), Hannover. This study was in part supported by the German Research Foundation (FOR 1103, BA 815/10-2 and BE 4200/1-2).
To Lejla
“Truth is not a democracy”

(Neil deGrasse Tyson)
Parts of the thesis have been published / submitted in peer-reviewed journals previously:


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<td>APC</td>
<td>antigen presenting cell</td>
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<td>bmDCs</td>
<td>bone marrow derived dendritic cells</td>
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<td>CCL22</td>
<td>C-C motif chemokine 22</td>
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<td>CCR4</td>
<td>C-C chemokine receptor type 4</td>
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<td>CDV</td>
<td>canine distemper virus</td>
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<td>CNS</td>
<td>central nervous system</td>
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<td>DCs</td>
<td>dendritic cells</td>
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<td>DL</td>
<td>demyelinating leukoencephalitis</td>
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<td>EAE</td>
<td>experimental autoimmune encephalitis</td>
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<td>Flt3L</td>
<td>Fms-related tyrosine kinase 3 ligand</td>
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<td>Foxp3</td>
<td>forkhead box P3</td>
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<td>FV</td>
<td>Friend retrovirus</td>
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<td>GM-CSF</td>
<td>granulocyte macrophage-colony stimulating factor</td>
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<td>HIV</td>
<td>human immunodeficiency virus</td>
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<td>IHC</td>
<td>Immunohistochemistry</td>
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<td>IFN</td>
<td>interferon</td>
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<td>IL</td>
<td>interleukin</td>
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<td>LC</td>
<td>Langerhans cells</td>
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<td>MHC II</td>
<td>major histocompatibility complex class II</td>
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<td>moDC</td>
<td>monocyte-derived dendritic cells</td>
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<td>MS</td>
<td>multiple sclerosis</td>
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<td>MV</td>
<td>measles virus</td>
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<td>PBMC</td>
<td>peripheral blood mononuclear cells</td>
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<td>PMS</td>
<td>periodical microstructure</td>
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<td>rc</td>
<td>recombinant canine</td>
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<td>rh</td>
<td>recombinant human</td>
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<td>SLAM</td>
<td>signalling lymphocyte activation molecule</td>
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<td>TME</td>
<td>Theiler's murine encephalomyelitis</td>
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<td>TMEV</td>
<td>Theiler's murine encephalomyelitis virus</td>
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Chapter 1: Aims and hypothesis of the present study

Canine distemper virus (CDV) infection causes a long lasting immunosuppression in dogs. Due to its profound lymphotropism and preferential infection of CD150-expressing immune cells, generalized lymphoid depletion and severe lymphopenia can be found during the acute distemper phase, which is similar to human measles virus (MV) infection (von Messling et al. 2006; Beineke et al. 2009; Sellin et al. 2009). Moreover, due to demyelination that occurs with disease progression, canine distemper represents a spontaneous model for human myelin disorders such as multiple sclerosis (MS) (Vandevelde and Zurbriggen, 2005; Sips et al. 2007). Several publications demonstrated the important role of regulatory T cells (Treg) and dendritic cells (DC) for the development of chronic central nervous system (CNS) disorders (Wu and Laufer, 2007; Zozulya and Wiendl, 2008). Interestingly, although a therapeutic effect of these immunomodulatory cells can be observed in autoimmune disorders, both beneficial and detrimental effects have been described in infectious disorders (Belkaid and Rouse, 2005; Schneider-Schaulies and Dittmer, 2006; Zozulya and Wiendl, 2008). Krakowka et al. (1982) discussed the existence of a mononuclear suppressor cell population that causes inhibition of immune responses in CDV-infected dogs. However, this cell population, which potentially includes Treg and/or DCs, has not been identified yet. During the chronic phase of CDV-infection, viral antigen can be found in cells with a DC-like morphology in splenic germinal centers (Wünschmann et al. 2000). Thus, similar to the proposed effects of MV upon human DCs, it is hypothesized that CDV also inhibits the differentiation of canine DCs, which might subsequently lead to persistent depressive effects upon the immune system (Beineke et al. 2009; Céspedes et al. 2010; Reuter et al. 2010). Moreover, Treg have been demonstrated to cause long lasting immunosuppression in human measles (Sellin et al. 2009; Griffin 2010). Since so far none of the above mentioned cell populations have been investigated in canine distemper infection, the aims of the project were to determine disease phase-dependant phenotypical changes and the associated cytokine expression in lymphoid organs of CDV-infected dogs and to testify the hypothesis that a peripheral depletion of Treg
causes a lack of CNS-infiltrating immunomodulatory cells in the predemyelinating phase of CDV-infection. The latter might represent a potential prerequisite for immune mediated demyelination. Moreover, the effect of CDV upon canine DCs was investigated \textit{in vitro} to depict further potential parallels between canine distemper and human measles.
Chapter 2: General introduction

2.1 Canine distemper virus (CDV)

Morbilliviruses belong to the Paramyxoviridae family and include a number of highly pathogenic viruses, such as measles virus (MV), rinderpest virus, canine distemper virus (CDV), and peste-des-petits-ruminants virus, which cause devastating diseases in humans and animals (Beineke et al. 2009; Langedijk et al. 2011). In the last decades, morbilliviruses additionally emerged as causative agents of several mass-mortalities in bottlenose dolphins, Siberian seals, harbor seals, and striped dolphins (Osterhaus et al. 1990; Lipscomb et al. 1994; Saliki et al. 2002; Beineke et al. 2010; Stimmer et al. 2010).

Canine distemper is a fatal disease of carnivores with a worldwide distribution which affects mainly dogs. CDV-infection is also found in other animals including felidae, mustelidae, procyonidae, phocidae, tayassuidae, and non-human primates (Macaca fuscata; Pringle 1999; Sips et al. 2007; Beineke et al. 2009). Efforts to prevent the CDV infection by vaccination are largely successful (Patel et al. 2012). However, even with a broad vaccination regiment, distemper outbreaks have been reported in France, Germany, USA, Japan and Finland (Mori et al. 1994; Johnson et al. 1995; Beineke et al. 2009). Moreover, canine distemper has been observed in vaccinated animals following infection with genetically different CDV strains (Simon-Martínez et al. 2008). CDV-infection represents a systemic disease which affects the respiratory and gastrointestinal tract, skin, lymphoid tissues, and CNS (Krakowka et al. 1980; Baumgärtner et al. 1989). Moreover, among other morbilliviruses, CDV-infection shows a high incidence of CNS complications (Rudd et al. 2006). Pathological changes that occur during CDV-induced demyelinating leukoencephalitis (DL) show remarkable similarities with human multiple sclerosis (MS), making DL a naturally occurring translational model for human demyelinating disorders (Baumgärtner and Alldinger, 2005). Additionally, the disease course and pathogenesis in canine distemper resemble those of human MV infection including, fever, rash, respiratory signs, lymphopenia, and
profound immunosuppression with generalized depletion of lymphoid organs during the acute disease phase (von Messling et al. 2006; Beineke et al. 2009; Sellin et al. 2009). Thus, CDV-infection of dogs is further appreciated as a model to investigate morbillivirus induced alterations of the immune system.

2.1.1 Viral properties of canine distemper virus

CDV is an enveloped, negative-sense, single-stranded RNA virus. Similar to other paramyxoviruses CDV contains six structural proteins: the nucleocapsid (N), phospho (P), large (L), matrix (M), hemagglutinin (H) and fusion (F) protein, and two accessory non-structural proteins (C and V) found as extratranscriptional units within the P gene (Örvell, 1980; Dhiman et al. 2004; Röthlisberger et al. 2010) (Fig. 1). The lipid envelope surrounding the virion contains two surface proteins (F and H), which mediate virus entry into the cell. During morbillivirus infection, the initial interaction with the host cell is mediated by the envelope-anchored attachment protein, the H protein, an essential viral component, which, assisted by the viral fusion protein, initiates virus cell entry (Stern et al. 1995; von Messling et al. 2001; Langedijk et al. 2011). The N, P and L proteins are responsible for virus replication, while the M protein connects the surface glycoproteins and N protein during viral maturation (von Messling et al. 2001; Röthlisberger et al. 2010). Co-expression of both H and F glycoprotein are sufficient and necessary to induce cell fusion. Moreover, the H protein represents the major factor determining CDV cell tropism (Stern et al. 1995; Plattet et al. 2005). The cell fusion in paramyxovirus infection seems to be a complex process. The current model proposes that the H protein undergoes conformational changes after binding with the host cell, which also affects the structure of the F protein resulting in insertion of hydrophobic fusion peptide into the cell membrane with the result of final binding to the host cell (Lamb 1993; Plattet et al. 2005). On the other side, von Messling et al. (2001) reported that the fusogenicity is solely determined by properties of the H protein. Moreover, the F protein
might have a key role in viral persistence (Plattet et al. 2005; Plattet et al. 2007). Additionally, M and N proteins which are important for viral budding might represent co-factors for the ability of CDV to induce persistent infection (Stettler et al. 1997; Plattet et al. 2007). Additionally, the role of V proteins accounts for rapid viral multiplication in lymphocytes and the inhibition of interferon signaling pathways (von Messling et al. 2006; Röthlisberger et al. 2010).

![Figure 1](image.png)

Figure 1. Schematic diagram of the morbillivirus genome and cell receptors for canine distemper virus (CDV). A) The morbillivirus virion contains the RNA genome and six structural proteins: the nucleocapsid (N), phospho (P), matrix (M), fusion (F), hemagglutinin (H) and large (L) protein. The H and F proteins are associated with the envelope. The V and C proteins are non-structural proteins. Additionally, CDV uses the signalling lymphocyte activation molecule (SLAM) as a receptor for cell entry. Additional receptors which are supposed to support infection are CD9 and CD46. B) The non-segmented RNA genome contains six genes. The P gene encodes for the P, C and V proteins. The P and C proteins are translated from overlapping reading frames on a functionally bicistronic mRNA and the V protein is translated from V mRNA, which is formed after insertion of a single nucleotide by RNA editing. Modified from Yanagi et al. (2006).

After virus attachment, the virus replicates in the cytoplasm of the host cell, and cytoplasmic as well as intranuclear inclusions can be found in many cell types (Baumgärtner et al. 1989). Until now, only one serotype and several co-circulating CDV
genotypes with differences in virulence and cell tropism have been described (Haas et al. 1999).

2.1.2 Canine distemper virus infection, receptors and cell tropism

For MV several proteins that serve as receptors for virus entry have been described. The signaling lymphocyte activation molecule (SLAM), also known as CD150, has been identified as an ultimate and universal morbillivirus receptor (Fig. 1) (Hahm et al. 2004; von Messling et al. 2006; Sato et al. 2012). SLAM is a glycosylated transmembrane protein that is constitutively expressed on immature thymocytes, DCs, CD45RO\textsuperscript{high} memory T cells and a proportion of B cells, and is rapidly induced on T and B cells after activation (Cocks et al. 1995; Tatsuo et al. 2000; Beineke et al. 2009). The expression of SLAM on leukocytes in MV and CDV-infection accounts for the preferential infection of lymphoid organs (lymphotropism) with subsequent lymphoid depletion and immunosuppression (Wünschmann et al. 2000; Minagawa et al. 2001; Wenzlow et al. 2007; Langedijk et al. 2011). For instance, during early CDV-infection, SLAM is upregulated on lymphoid cells, which is supposed to enhance virus amplification in the host (Wenzlow et al. 2007). Beside the SLAM receptor, certain strains of MV are able to infect cells by interaction with the CD46 receptor (Naniche et al. 1993; Erlenhöfer et al. 2002). CD46 serves as a receptor predominantly for attenuated viruses and only for few wild type strains \textit{in vitro} (Erlenhöfer et al., 2002). Whether wild type MV \textit{in vivo} interacts with CD46 or not, and whether all MV-strains have the ability to use SLAM as a receptor, is still not known (Dörig et al. 1993; Erlenhöfer et al. 2002; Yanagi et al. 2006). The role of CD46 in CDV-infection has not yet been confirmed, although the lack of SLAM in certain CDV-target cells supports the assumption of SLAM independent infection pathways (Wenzlow et al. 2007, Beineke et al. 2009). So far, CD46 molecules have been identified only in neoplastic lymphoid cells of dogs (Suter et al., 2005). Additional receptors, such as CD9, are discussed as possible factors for CDV-infection.
of Vero cells. CD9, a tetraspan transmembrane protein, was shown to induce cell-to-cell fusion, but not virus-to-cell fusion (Löffler et al. 1997; Schmid et al. 2000). Since no direct binding of the virus with CD9 can be demonstrated, this receptor is supposed to represent a co-factor for viral infection as part of the receptor complex or by effecting the expression of other receptor molecule (Löffler et al. 1997). Recently, nectin-4 was identified as a new receptor for MV (Mühlebach et al. 2011). Its role in canine distemper remains to be determined.

CDV is a pantropic virus that shows a broad cell tropism. Accordingly, CDV can be found in cells of the respiratory, gastrointestinal and urinary tract, as well as in lymphoid tissues, endocrine organs and the central nervous system (CNS; Baumgärtner et al. 1989; Gröne et al. 2004; Beineke et al. 2009). Moreover, infection of various cell types can be found in canine distemper (Vandevelde and Zurbriggen, 2005; Seehusen et al. 2007). In the CNS, astrocytes, microglia, and oligodendrocytes, can get infected regardless of the CDV strain, while the infection of neurons is strain dependent (Pearce-Kelling et al. 1991; Orlando et al. 2008). During the acute disease stage, astrocytes represent the main cell population infected by CDV (Alldinger et al. 2000; Seehusen et al. 2007). Interestingly, even though CNS lesions are characterized by white matter vacuolization and demyelination, only a limited number of oligodendrocytes can be found to be infected (Zurbriggen et al. 1998; Vandevelde and Zurbriggen, 2005).

2.1.3 Pathogenesis and clinical manifestation of canine distemper

The disease course including the duration and severity of clinical signs depends mainly on the virulence of the strain as well as on the age and immune status of the animal. Transmission of the virus is facilitated by sneezing, coughing and close contact. Accordingly, animals are infected primarily by inhalation of viruses and infective droplets, respectively (Krakowka et al. 1980). Initially, virus replicates in lymphoid tissue of the upper respiratory tract. Here, monocytes and macrophages are the first cells that
get infected and propagate the virus (Appel et al. 1970). The incubation period varies from one to four weeks (Krakowka et al. 1980; Beineke et al. 2009). Animals display a broad spectrum of clinical signs including lethargy, anorexia, dehydration, weight loss, pneumonia, and neurological signs. Furthermore, development of a biphasic fever represents a characteristic clinical finding (Wright et al. 1974). During the first viremic phase (three to six days post-infection), generalized infection of all lymphoid tissues with lymphopenia, profound immunosuppression and transient fever is observed. The second viremia takes place several days later, and is associated with high fever and infection of parenchymal tissues such as the respiratory tract, gastrointestinal tract, skin, and CNS (Appel et al. 1969, Krakowka et al. 1980, Beineke et al. 2009). During this disease stage, various signs such as conjunctivitis, nasal discharge, anorexia, neurological disturbances, gastrointestinal signs and respiratory signs can be observed (Krakowka et al. 1980; Beineke et al. 2009). Respiratory signs are a consequence of virus-induced rhinitis and interstitial pneumonia, which can exceed to suppurative bronchopneumonia due to secondary bacterial infection. Vomiting, diarrhea and dehydration follow infection of the gastrointestinal tract (Greene and Apple, 1998; Decaro et al. 2004;). Neurologic signs depend on viral distribution in the CNS and include hyperesthesia, cervical rigidity, seizures, cerebellar and vestibular signs, as well as paraparesis or tetraparesis with sensory ataxia (Deem et al. 2000; von Rüden et al. 2012). Neurological manifestations include encephalopathy, acute encephalitis, subacute to chronic demyelinating encephalitis, and polioencephalitis (Nessler et al. 1999; Rudd et al. 2010; Wyss-Fluehmann et al. 2010). Recovery depends on the immune state of the animal. Particularly, a strong and effective cellular immune response can eliminate the virus prior to the infection of parenchymal tissues, while weak and delayed cellular and humoral immune responses lead to virus spread and persistence, respectively
2.1.4 Pathology of lymphoid organs and mechanism of immunosuppression

Systemic CDV-infection causes depletion of multiple lymphoid tissues such as spleen, lymph nodes, thymus and mucosa associated lymphatic tissues (MALT) of dogs. Microscopic changes in lymphoid organs include loss of B and T cell areas, formation of giant cells, intracytoplasmic inclusion bodies in lymphoid cells, follicular necrosis, and thymic atrophy (Fig. 2) (Iwatsuki et al. 1995; Wünschmann et al. 2000; Beineke et al. 2009). Moreover, syncytia formation and cell death of immune cells lead to complete loss of secondary follicles (Iwatsuki et al. 1995). During the acute disease stage, CDV antigen is found mostly in lymphocytes and macrophages, located predominantly in T cell areas (Apple et al., 1969; Iwatsuki et al., 1995, Wünschmann et al., 2000; Schobesberger et al., 2005). At this, CD4\(^+\) T cells are the primarily affected cell type and the first population that gets depleted (Iwatsuki et al., 1995; Wünschmann et al., 2000). Lymphopenia is associated with a rapid loss of CD4\(^+\) T helper cells, CD8\(^+\) cytotoxic T cells, CD21\(^+\) B cells, and macrophages (Wünschmann et al. 2000). Changes in lymphoid organs correlate with the amount of CDV antigen (Wünschmann et al. 2000). During the chronic disease phase, the virus is cleared from lymphoid organs and reconstitution of lymphoid cells takes place (Krakowka et al. 1980). However, despite regeneration and lymphoid repopulation, long lasting phenotypical alterations are observable in lymphoid organs in chronically infected dogs (Wünschmann et al. 2000). During chronic infection, viral antigen can be found in germinal centers within leukocytes with DC-like morphology (Wünschmann et al. 2000). Thus, similar to the proposed effects of MV upon human DCs, it is hypothesized that CDV also modulates the function of canine DCs, which leads to persistent immune depressive effects (Wünschmann et al. 2000; Beineke et al., 2009). For instance, CDV-infection of thymic DCs may result in impaired maturation of T cells with release of CD5-negative T cells. The CD5-negative T cell population potentially contains autoreactive lymphocytes, which might be responsible for the induction of myelin specific immunity in canine distemper (Wünschmann et al. 2000). However, so far, functional properties of DCs in
canine distemper have not been investigated. Mechanisms of CDV-induced immunosuppression remain largely undetermined (Beineke et al. 2009). In human measles, apoptosis of immune cells is considered as one cause for severe leukopenia (Okada et al. 2000). Similarly, apoptosis of infected and non-infected immune cells in lymphoid organs contribute to lymphoid depletion and impaired immune responses in CDV-infected dogs (Okada et al. 2000; Schobesberger et al. 2005). This indicates that directly virus-mediated as well as virus-independent mechanisms might be involved in leukocyte apoptosis. Proposed mechanisms for apoptotic cell death include an over-activation of immune responses and activation of Fas-pathways, respectively (Schobesberger et al. 2005; Beineke et al. 2009).

Figure 2. Effects of canine distemper virus (CDV) upon immune cells. CDV-infection of leukocytes is supposed to cause an inhibition of plasma cell differentiation, reduced proliferation of lymphocytes, increased lymphocyte apoptosis induction and diminished function of antigen presenting cells (APCs), such as dendritic cells.

After viral elimination from the peripheral blood, decreased antigen presentation and lymphocyte maturation is supposed to contribute to persistent immunosuppression
CDV causes modulation of antigen presenting abilities of monocytes by the inhibition of IL-1 (Krakowka et al., 1987). Furthermore, CDV-N protein potentially modulates antigen presentation by inhibiting the production of IL-12 in DCs, as described for MV-infected DCs (Schneider-Schaulies and Dittmer, 2006), while morbillivirus V proteins act as interferon antagonists and cytokine inhibitors (von Messling, 2006). Krakowka et al., (1982) discussed the existence of a mononuclear suppressor cell population that causes long lasting immunosuppression. Interestingly, regulatory T cells (Treg) have been demonstrated to cause long lasting immunosuppression in infectious diseases, such as human measles (Piccirillo and Shevach 2001; Sellin et al. 2009; Reuter et al. 2012). However, until now the function of Treg in the pathogenesis of canine distemper has not been investigated yet. Similarly, the role of DCs in CDV-induced immunopathology remains enigmatic.

Further studies have to focus on CDV-dependent and independent mechanisms of immunosuppression as well as upon the role of DCs and Treg for immune alteration and neuroinflammation in canine distemper.

2.1.5 Neuropathology of canine distemper

Infection of the CNS represents the a serious complication of canine distemper, often with poor prognosis (Carvalho et al. 2012). Dependent upon the host immune response and virus strain, polioencephalitis and demyelinating leukoencephalitis (DL) can be discriminated (Pearce-Kelling et al. 1990; Orlando et al. 2008). Polioencephalitis is a rare manifestation of CDV-infection and can be subclassified as old dog encephalitis, inclusion body encephalitis and post vaccinal encephalitis (Bestetti et al. 1978; Vandevelde et al. 1980; Nessel et al. 1999). Grey matter lesions are predominantly located in cortical areas and brain stem nuclei with neurons and astrocytes representing the most affected cell populations (Nessel et al. 1999). Histologically neuronal degeneration and necrosis with gliosis, inclusion bodies and infiltration of macrophages
and lymphocytes can be found (Nesseler et al. 1997; Beineke et al. 2009). Leukoencephalitis is the more common manifestation and shows a progressive disease course. White matter lesions are subclassified as acute lesions, subacute lesions without inflammation, subacute lesions with inflammation and chronic lesions with inflammation (Alldinger et al. 1993; Wünschmann et al. 1999; Wünschmann et al. 2000; Beineke et al. 2009). Demyelinating foci are located predominately in proximity to the ventricles, cerebellar velum, cerebellar peduncles and optic tract (Summers and Appel, 1994). Acute DL is characterized by focal to multifocal or diffuse vacuolization of the white matter which develops during the period of severe immunosuppression (Wünschmann et al. 1999; Seehusen et al. 2007). Lesions consist of mild gliosis with reactive astrocytes and few gemistocytes (Baumgärtner et al. 1989; Alldinger et al. 1993). Here, astrocytes represent the main target cells for CDV-infection (Alldinger et al. 2006; Seehusen et al. 2007; Carvalho et al. 2012). Spread of the virus among astrocytes does not require infectious particles (Wyss-Fluehmann et al. 2010; Carvalho et al. 2012). The usage of gap junctions of the astrocytic synapse-like network represents a possible mechanism for CDV spread as described for herpes simplex virus (Wyss-Fluehmann et al. 2010). Acute and subacute lesions without inflammation are characterized by a lack of perivascular cuffing (Tipold et al. 1999; Wünschmann et al. 1999). The initial vacuolization might be caused by restricted infection of oligodendrocytes. Experiments in vitro and in vivo revealed a down-regulation of myelin-specific genes (Zurbrigggen et al. 1998; Vandevelde and Zurbrigggen, 2005). During the subacute disease course astrocytic hypertrophy and hyperplasia (astrogliosis and astrocytosis) with formation of gemistocytes and multinucleated astrocytes as well as gitter cells can be observed, although mononuclear perivascular infiltrates are initially still absent (Tipold et al. 1999; Seehusen et al. 2007; Seehusen and Baumgärtner, 2010). Subsequent inflammatory stages of DL coincide with recovery of the immune system. CNS lesions are characterized by the presence of perivascular infiltrations of lymphocytes, plasma cells and macrophages (Vandevelde et al. 1982). Chronic CNS lesions are associated with prominent perivascular infiltrations (more than three layers
of monocytic inflammatory cells) and profound myelin loss (Vendevelde et al. 1982; Beineke et al. 2009; Vandevelde and Zurbriggen, 2005). A correlation between microglial activation and loss of myelin has been described (Stein et al. 2004). Virus-activated microglia release myelinotoxic substances which leads to bystander demyelination (Fig. 3; Alldinger et al. 1996; Vandevelde and Zurbriggen, 2005; Beineke et al. 2009). Recent studies revealed the existence of oligodendrocytes in demyelinating lesions, indicating that primary demyelination precedes the loss of myelin-forming cells in DL (Schobesberger et al. 2002). Moreover, an increased apoptotic rate particularly in the granular layer of the cerebellar grey matter in DL indicates the possibility of demyelination as a secondary process following Wallerian degeneration or loss of astrocytic support, respectively (Moro et al. 2003; Beineke et al. 2009; Del Puerto et al. 2010; Seehusen and Baumgärtner, 2010).

**Figure 3. Possible mechanisms of demyelination in canine distemper.** A) During early infection demyelination is a consequence of direct and indirect effects (e.g. loss of trophic support) upon oligodendrocyte which causes myelin alteration and loss. B) During advanced stages immune mediated processes including the release of myelinotoxic substances by activated microglia (bystander demyelination) contribute to progressive myelin damage. Modified from Carvalho et al. (2012).
2.1.6 Immune responses in canine distemper demyelinating leukoencephalitis

The host defense during CDV-infection initially relies on innate immune responses, although for complete virus elimination the activation of humoral and cellular immune responses are required (Carvalho et al. 2012). Protective humoral immunity in canine distemper is achieved by the production of antibodies against viral nucleoproteins, followed by the development of specific immunoglobulins against viral envelope proteins (Miele and Krakowka, 1983; Rima et al. 1991).

Lesion development of DL represents a biphasic event with initial tissue damage directly induced by the virus and subsequent immune mediated inflammation as a consequence of viral persistence and delayed type hypersensitivity (Baumgärtner et al. 1989; Alldinger et al. 1996). The infiltration of CD8^+ T cells correlates with virus replication and the appearance of early immune responses against the N-protein (Tipold et al. 1999). These T cells contribute to viral clearance but also to initial tissue damage by antibody independent cytotoxicity (Wünschmann et al. 1999). In subacute lesions prominent numbers of CD4^+ T cells and B cells can be found. While CD8^+ T cells might function as cytotoxic effectors, CD4^+ T cells are supposed to contribute to delayed type hypersensitivity reactions in advanced lesions (Wünschmann et al. 1999). Additionally, major histocompatibility complex class II (MHC II) is upregulated within areas with low or absent viral antigen in chronic foci, which indicates virus-independent, immune mediated mechanisms of demyelination (Alldinger et al. 1996). Thus, while during the acute phase myelin damage is ascribed as directly virus mediated, demyelination in chronic lesions is a result of collateral damage, e.g. due to an over-activation of microglia/macrophages (bystander demyelination; Beineke et al. 2009) (Fig 3). Repopulation of peripheral lymphoid organs is supposed to be a prerequisite for CNS-infiltration of immune cells during inflammatory stages (Wünschmann et al. 2000). For instance, with recovery of the immune system an infiltration of CD4^+ T cells, B cells and IgG-producing plasma cells in the brain as well as CDV-specific humoral immune responses in the cerebrospinal fluid can be observed (Vandevelde et al. 1982; Beineke
et al. 2009). The increased antibody production by plasma cells might enhance demyelination by an antibody dependent T cell mediated cytotoxicity (Alldinger et al. 1996; Wünschmann et al. 1999).

2.1.7 Cytokine expression in canine distemper

Cytokines are important signalling molecules involved in cell communication and orchestration of immune responses in infectious and immune mediated disorders (Rothwell 1997). In DL, infiltration of immune cells and accompanied demyelination is followed by a tremendous up-regulation of several cytokines (Spitzbarth et al. 2012). Cytokine expression can be directly induced by the virus or by autocrine and paracrine regulatory loops during CDV-infection (Gröne et al. 2002; Markus et al. 2002; Beineke et al. 2009).

During early DL lesion development pro-inflammatory cytokines, such as IL-6, IL-12 and TNF-α, are up-regulated, while the anti-inflammatory cytokines, IL-10 and TGF-β, remain unchanged (Markus et al. 2002; Beineke et al. 2008). The pro-inflammatory cytokine environment in the brain during acute CDV-infection is indicative of insufficient counter regulatory mechanisms, potentially causing early immune over-activation and initial tissue damage in the brain. Similarly, expression of neuroprotective and Treg inhibitory cytokines such as IL-10 and TGF-β is insufficient in canine spinal cord injury, potentially leading to an activation of CNS resident immune cells (Spitzbarth et al. 2011). Initial infiltration of CD8+ T cells in the brain is associated with the expression of chemoattractant cytokines such as IL-8 (Gröne et al. 1998; Tipold et al. 1999). In advanced stages of DL, production of IL-12 within the CDV-infected CNS might trigger Th1-biased immune responses (Gröne et al. 2000; Wünschmann et al. 2000; Beineke et al. 2009; Spitzbarth et al. 2012). In addition, IL-12 is known to play a role in demyelinating diseases such as MS and experimental autoimmune encephalomyelitis (EAE). IL-12 also accounts for the maturation of monocyte-derived dendritic cells (moDCs) and probably the activation of microglia (Fox and Rostami, 2000; Sugiura et
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al. 2010; Spitzbarth et al. 2012). In the cerebrospinal fluid of CDV-infected dogs, independent of the disease course, pro-inflammatory cytokines (TNF and IL-6) and anti-inflammatory cytokines (IL-10 and TGF-β) are often observed simultaneously (Frisk et al. 1999; Beineke et al. 2009).

Cytokine analysis of whole blood samples of dogs with DL revealed an expression of IL-2, IL-6, TNF and TGF-β. IL-6 can be detected in the blood of dogs with early CNS lesions, while TGF-β is found predominately during late stages of the disease, indicative of a delayed onset of peripheral immunomodulatory processes in advanced disease stages (Gröne et al. 1998). IL-1, IL-6 and TNF affect the permeability of the blood brain barrier and represent a prerequisite for infiltration of leukocytes and enhancement of neuroinflammation (Gröne et al. 1998; Beineke et al. 2008; Beineke et al. 2009). The lack of IFN-γ expression in peripheral blood leukocytes of CDV-infected dogs might account for an inadequate antiviral immunity in affected dogs (Gröne et al. 1998). Similarly, in experimental CDV-infection of ferrets, early infection (3 days post infection) is characterized by a lack of significant cytokine responses, probably as a consequence of virus-mediated immunosuppression (Svitek and von Messling, 2007). Interestingly, in the same animal model, as reported in MV-infected children, with disease progression a switch from Th1 to Th2 cytokine responses was observed (Svitek and von Messling, 2007). Referring to this, prolonged IL-10 expression of peripheral leukocytes is supposed to cause long lasting immune alterations in measles patients (Svitek and von Messling, 2007). However, so far, cytokine responses in lymphoid organs of CDV-infected dogs have not been investigated.

2.2 Dendritic cells

The term DCs referred to a heterogeneous group of multifunctional leukocytes (Bodey et al. 1997). They represent the most potent antigen-presenting cell (APC) population. So far, no other functions of DCs then antigen presentation and regulation of immune responses are known (Steinman, 2007). They serve as sentinels of the immune system
and initiate immune responses (Banchereau et al. 2000). DCs exist in at least two states, the immature and mature stage (Banchereau et al. 2000). They are found in various tissues, such as skin, lymphoid organs, airways and intestinal tract. The cells are able to capture and process antigens, migrate to T cell areas of lymphoid organs and present antigens on their cell surface via MHC molecules. The antigen uptake by DCs is conducted by receptor-mediated endocytosis and phagocytosis using different receptors such as Fc-receptors, lectin receptors, macrophage mannose receptors, ICAM-3, and toll-like receptors (Bhardwaj, 2003). Following antigen uptake, DCs undergo a process of maturation and migrate to the draining lymph node. After recognition of the antigen by T cells, Th1 or Th2 immune responses are initiated. The polarization of T cells depends on co-stimulatory molecules and cytokine expression of the DC (Macatonia et al. 1995, Bhardwaj, 2003). DCs have a unique ability to present antigens from non-replicating viruses to CD8+ T cells (Smed-Sörensen et al. 2012). In comparison of other APCs (e.g. macrophages or B cells), DCs have a 1000 fold higher efficiency to activate resting T cells, which demonstrates the pivotal role of DCs for the initiation of adaptive immune responses against infectious agents (Klagge and Schneider-Schaulies, 1999; Bhardwaj, 2003). In addition to antigen recognition by T cell receptors, interaction between CD28 on the T cell surface with co-stimulatory molecules on DCs is required for optimal T cell activation (Björck et al. 1997; Weis and Wardrop, 2010). Although all DCs share a common ability to process and induce immune responses indirectly by activating T cells, they differ by the expression of surface markers, localization and cytokine production (Wu and Liu, 2007). A deregulation of DC function is involved in immune-mediated tissue damage and immunosuppression in human and veterinary medicine such as histocytic tumours, leishmaniasis, atopic dermatitis, inflammatory bowel disease (Vanloubbeeck et al. 2003; Moore, 2008; Cerquetella et al. 2010; Ricklin et al. 2010; Silva et al. 2012).
2.2.1 Dendritic cells in dogs

Similar to other species, canine DCs generated \textit{in vitro} are non-adherent cells with characteristic cytoplasmic projections (dendrites). They form clusters in culture and have the ability to induce mixed leukocyte reactions (Goodel et al., 1985). So far, two subsets of DCs, myeloid and lymphoid lineage DCs (Fig.4), have been recognized in dogs. Myeloid DCs express MHC II, CD34 and CD14 and derive from monocyte and bone marrow cells, while lymphoid DCs are MHC II$^+$, CD34$^+$ and CD14$^-$ (Tizard, 2009).

Canine DCs can be generated \textit{in vitro} from CD14$^+$ peripheral blood mononuclear cells (PBMC) and the bone marrow by separation of CD34$^+$ progenitor cells. As in humans and mice, differentiation of precursor cells into DCs is induced by stimulation with different cytokines, such as recombinant GM-CSF and IL-4 (Hägglund et al. 2000; Ibisch et al. 2005; Bonnefont-Rebeix et al. 2006; Wijewardana et al. 2006; Wang et al. 2007a,b; Bund et al. 2010; Sugiura et al. 2010; Mielcarek et al. 2011; Fitting et al. 2011).

Canine DCs show an abundant formation of the Golgi apparatus and endoplasmic reticulum but lack large lysosomal organelles (Ibisch et al. 2005). A unique ultrastructural feature of canine DCs is the presence of periodical microstructures in the cytoplasm (Ibisch et al. 2005; Isotani et al. 2006). Additionally, in contrast to human and mouse Langerhans cells canine Langerhans cells lack classical Birbeck granules (Moore et al. 1996). Canine moDCs express CD14 which contrast with human and murine moDCs (moDCs; Ibisch et al. 2005; Wijewardana et al. 2006; Ricklin Gutzwiller et al. 2010). CD14 expression of bone marrow-derived DCs (bmDCs) is under debate (Hägglund et al. 2000; Weber et al. 2003; Ricklin Gutzwiller et al. 2010).

Compared to monocytes and macrophages canine moDCs and bmDCs show high expression levels of MHC II, CD1a, and CD40, as well as of the co-stimulatory molecules CD80 and CD86 (Ibisch et al. 2005; Bonnefont-Rebeix et al. 2006; Wang et al. 2007a; Ricklin Gutzwiller et al. 2010; Sugiura et al. 2010). Phenotypical analyses also enable the discrimination between canine bmDCs and moDCs (Ricklin Gutzwiller et al. 2010).
Figure 4. Dendritic cell (DC) lineages of dogs. Two DC lineages have been identified in dogs: the myeloid and lymphoid lineage. The myeloid lineage consists of three different subsets which differ in localization, function and phenotype (Langerhans cells, bone marrow derived DCs and monocyte derived DCs). Plasmacytoid DCs originate from the lymphoid lineage.

Canine Langerhans cells express CD1c, CD11c, CD80, MHC II and E-cadherin in situ, which parallels the surface molecule expression pattern of human cells. However, in contrast to humans, canine Langerhans cells lack S100, ATPase and ICAM-1 (Moore et al. 1996; Zaba et al. 2009; Baines et al. 2008; Ricklin-Gutzwiller et al. 2010). Different subsets of canine DCs exhibit differences in the ability to induce mixed leukocyte reactions and cytokine expression (Andrea et al. 1995; Syme et al. 2005; Wang et al. 2007b; Ricklin Gutzwiller et al. 2010).

Understanding species-specific properties of canine DCs is pivotal for future studies upon the role of this cell type in infectious disorders.
2.2.2 Dendritic cells in viral diseases

The immune response to viruses is a complex interplay between the pathogen and innate and adaptive immune responses which aims to eradicate the infectious agent with minimal damage to the host (Lambotin et al. 2010). The interaction between different viruses and DCs includes the alteration of DC functions, such as endocytosis, vesicle trafficking, immunological synapse formation, apoptosis induction and cytokine production (Harman et al. 2006; Cunningham et al. 2010). Virus infection of DCs can lead to a productive infection and subsequent release of infectious particles, as observed for human immunodeficiency virus (HIV), MV, Epstein-Barr virus, and human cytomegalovirus (Li et al. 2002; Beck et al. 2003; Donaghy et al. 2003; Schneider-Schaulies et al. 2003; Rinaldo and Piazza, 2004). Alternatively, virus particles can be transferred from DCs directly to other cell types, as observed for HIV infection (Rinaldo and Piazza, 2004). As demonstrated in murine models for Sendai virus-, Moloney leukemia virus-, herpes simplex virus-, and influenza virus-infection, DC-mediated priming of the immune response leads to viral elimination (Kast et al. 1998; Hengel et al. 1987; Nonacs et al. 1992; Klagge and Schneider-Schaulies, 1999). For instance, in influenza virus infection, virus antigen can be found on all APCs, but only DCs are able to induce effective immune responses (Hamilton-Easton and Eichelberger, 1995; Klagge and Schneider-Schaulies, 1999). In contrast to macrophages, influenza virus-infected DCs do not undergo rapid cell death. Interestingly, influenza virus-infected monocytes are unable to differentiate into DCs, which leads to the assumption that virus-mediated DC inhibition might account for an impairment of virus-specific immunity (Boliar and Chambers, 2010). In addition, several other viruses that cause persistent infection, such as human cytomegalovirus, murine cytomegalovirus and Epstein-Barr virus are able to manipulate DCs, which leads to inadequate protective immune responses (Rinaldo and Piazza, 2004).

Herpes simplex virus infection of DCs leads to productive infection with down-regulation of the co-stimulatory molecules CD80, CD86 and CD40, but not of MHC I and MHC II,
which suggests that herpes simplex virus proteins target signal transduction pathways that control the expression of co-stimulatory molecules (Mikloska et al. 2001). HIV has been demonstrated to interact with DCs and modulate their function, which represents a prototypical model of DC-virus interaction (Rinaldo and Piazza, 2004). DCs are supposed to be the early targets of HIV and by the ability to cluster T cells they can spread the virus within the host (Klagge and Schneider-Schaulies, 1999). In addition, in advanced stages of HIV infection, DCs become the virus reservoir and therefore contribute to virus persistence. Once the DCs incorporate the HIV, they transport the virus to the draining lymph node to induce an immune response (Klagge and Schneider-Schaulies, 1999). Subsequently, DCs undergo a killing process due to feedback mechanisms that remove APCs after stimulation of T cell responses. Interestingly, only immature DCs can get infected, while mature DCs do not support the replication of HIV (Knight et al. 1997; Klagge and Schneider-Schaulies, 1999).

Previously it was shown that during MV infection epithelial cells of the upper respiratory tract are the first cells to be infected (Esolen et al. 1993). Since epithelial cells express only CD46, which is a receptor only for attenuated MV-strains, and lymphocytes, which are one of the most affected cells in measles, are not present in large numbers in respiratory epithelium, it was concluded that other cells might account for early MV entry (Tatsuo et al. 2000; de Swart et al. 2007; de Witte et al. 2008). Moreover, undifferentiated monocytes, which express CD46, are relatively resistant to MV replication (Fugier-Vivier et al. 1997). CD150, which is necessary for virus entry, is expressed in T cells, B cells, macrophages, and DCs (de Swart et al. 2007). In vivo infection of DCs has been described in animal experiments using cotton rats, transgenic mice and macaques. (de Swart et al. 2007) Thus, infection of DCs of the respiratory tract and subsequent migration of these cells to draining lymph nodes is supposed to contribute to virus spread in the organism. Furthermore, dysfunction of DCs following MV infection is supposed to represent one of the factors for long lasting and profound immune suppression in measles patients. (Servet-Delprat et al. 2000) However, DC
infection in human patients has not been confirmed until now (Hahm et al. 2005; de Swart et al. 2007; Griffin, 2010). Different studies have demonstrated MV infection of different subtypes of myeloid DCs \textit{in vitro} (Fugier-Vivier et al. 1997; Murabayashi et al. 2002; Ohgimoto et al. 2007). Here, the increased susceptibility of mature DCs for MV infection is in part a consequence of higher CD150 expression levels (Klagge et al. 2004). The H protein of MV determines the tropism for moDCs. The induction of syncytia formation of infected DCs is a characteristic feature of MV wild type strains (Fugier-Vivier et al. 1997; Murabayashi et al. 2002; Griffin 2010), while vaccine strains can indeed infect and replicate in DCs, although only small amounts of infectious virus are produced due to an instability of the M protein (Ohgimoto et al. 2007; Griffin 2010). Interference of MV with APCs represents an important cause for immunosuppression. During MV infection of cultured immature DCs, infected and non-infected cell undergo a maturation process (Zilliox et al. 2006). This maturation is associated with an up-regulation of CD40, CD80, CD86 and MHC II, while CD1a and CD34 are down-regulated (Fig. 5) (Schnorr et al. 1997; Servet-Delprat et al., 2000; Zilliox et al. 2006).

The infection also results in rapid production of type I interferon (IFN) which also contributes to DC maturation, but without the ability to prevent viral spread (Schneider-Schaulies et al. 2002; Schneider-Schaulies and Meulen, 2002; Zilliox et al. 2006). Moreover, in murine models, MV infection impairs the differentiation of DCs \textit{in vivo}, characterized by a down-regulation of co-stimulatory molecules, MHC class I and MHC II (Oldstone et al. 1999; Hahm et al. 2005; Trifilo et al. 2006). In addition to the modulation of antigen presenting function of DCs, MV infection suppresses the production of IL-12 (Fugier-Vivier et al. 1997). Impairment of IL-12 expression in MV-infected DCs coincides with a high percentage of apoptotic DCs and inhibition of CD40 signalling (Fugier-Vivier et al. 1997; Servet-Delprat et al. 2000). Inhibition of IL-12 secretion can be observed with disease progression predominantly in the late stage of the disease, which might lead to insufficient Th1 immune responses in measles patients (Schneider-Schaulies et al. 2002)
Figure 5. Proposed mechanisms of interaction between measles virus (MV) and dendritic cells (DC). MV induces maturation of DCs, characterized by an up-regulation of co-stimulatory molecules (CD80/86), CD40 and MHC II, while impairing the production of IL-12 and T cell stimulatory ability. Modified from Kerdiles et al. (2006).

2.3 Regulatory T cells

Immune homeostasis is mainly regulated by Treg (Vignali et al. 2009). Treg are essential for maintaining immune tolerance and therefore prevent autoimmune diseases and limit chronic inflammatory processes (Vignali et al. 2009). Expression of the forkhead-winged helix transcription factor Foxp3 regulates the transcription of genes involved in immune modulation and represents a Treg-specific marker molecule (Brunkow et al. 2001; Hori et al. 2003; Biller et al. 2007; Feuerer et al. 2009). Additionally Treg are characterized by the expression of CD25, CTLA-4, and GITR (Zheng and Rudensky, 2007). Foxp3+ Treg use the αβ T cell antigen receptor (TCR) for antigen recognition and have a broad TCR repertoire (Feuerer et al. 2009; Relland et al.
2012). So far, two main sources of Tregs have been described: thymic Foxp3\(^+\) Treg generated in the thymus (natural Treg) and Treg which are induced in the periphery by different T cell derived factors (adaptive Treg; Mills 2004; Feuerer et al. 2009; Miyara and Sakaguchi, 2007). For example, adaptive Tregs can originate from CD4\(^+\) effector T cells due to IL-2 and TGF-β stimulation. These T cells, although unstable, show an expression of Foxp3 and immunosuppressive properties (Chen et al. 2003; Fantini et al. 2004; Floess et al. 2007; Feuerer et al. 2010). Immature and mature DCs have the ability to induce proliferation of Treg in vitro and in vivo (Yamazaki et al. 2007). In the CNS, activated microglia and DCs have the ability to attract thymic Treg via the production of CCL22, which interacts with CCR4 on Treg (Vulcano et al. 2001; Kipnis et al. 2004). Treg can also be induced under inflammatory conditions by astrocytes and neurons (Lowther and Hafler, 2012). A novel population of natural Treg has recently been identified in the peripheral blood of human beings. These cells express CD4 or CD8 but lack Foxp3-expression (Feger et al. 2007; Zozulya and Wiendl, 2008).

Several mechanisms are involved in Treg-mediated immunosuppression, which can be grouped as suppression by cytokines, suppression by cytolysis, suppression by metabolic disruption, and suppression by the modulation of DC function (Fig. 6) (Vignali et al. 2009; Miyara and Sakaguchi, 2007). Treg can inhibit the proliferation and function of Natural Killer T cells, CD4\(^+\) T cells, CD8\(^-\) T cells, and B cells, as well as the maturation and antigen presenting capacity of DCs (Piccirillo and Shevach, 2001; Azuma et al. 2003; Misra et al. 2004; Lim et al. 2005). A major function of Treg is to respond to signals associated with tissue destruction and to minimize collateral tissue damage (Belkaid and Rouse, 2005). Treg are involved in gastrointestinal immune homeostasis, as demonstrated in mouse colitis models (Belkaid and Rouse, 2005). Similar beneficial effects have been observed in mouse models of *Leishmania major* infection (Aseffa et al. 2002; Liu et al. 2003; Belkaid and Rouse, 2005; Rai et al. 2012). Here, the disease severity is enhanced in the absence of Treg, while application of CD4\(^+\)CD25\(^+\) Treg reverses pathological lesions (Liu et al. 2003; Rai et al. 2012).
Figure 6. Schematic diagram of mechanisms involved in regulatory T cell (Treg)-mediated immunosuppression. Treg-mediated mechanisms of immunosuppression can be grouped in the following categories: (i) suppression of immune responses by inhibitory cytokines (IL-10, IL-35 and TGF-β); (ii) cytolysis by the release of granzymes (Grz); (iii) metabolic disruption by cytokine deprivation via IL-2 receptor α (CD25) with subsequent lymphocyte apoptosis, cyclic AMP-mediated inhibition, or CD39/CD73 and adenosine receptor (A2A)-mediated immunosuppression; (iv) modulation of dendritic cells (DC) by down-regulation of MHC II and CD80/86 which leads to a reduced antigen presenting capacity as well as via cytotoxic T lymphocyte antigen-4 (CTLA4)–CD80/CD86-mediated induction of indoleamine 2,3-dioxygenase (IDO) which is a potent immunosuppressive molecule. Modified from Vignali et al. (2008) and Miyara and Sakaguchi, (2007).
2.3.1 Regulatory T cells in viral diseases

Immunologic self tolerance is critical for the prevention of autoimmunity and maintenance of immune homeostasis in the CNS (Valencia et al. 2006). However, the role of Treg during different infectious diseases remains dubious, since both beneficial (reduction of immune mediated tissue damage) and detrimental effects (reduction of protective immune responses) of Treg have been described in infectious disorders (Fig. 7; Lund et al. 2008; Göbel et al. 2012; Herder et al., 2012). In contrast to this, Treg exhibit beneficial effects by reducing bystander tissue damage in the CNS during the acute phase of coronavirus infection of mice (Cecere et al. 2012). For instance, depletion of Treg increases the mortality of mice infected with in neurotropic mouse hepatitis virus, while the adoptive transfer of Tregs increases the rate of survival in infected animals (Anghelina et al. 2009). The clinical outcome of coronavirus-induced encephalitis depends on the balance between pro-inflammatory modalities required for virus clearance and anti-inflammatory factors to prevent deleterious immune responses (Anghelina et al. 2009). Treg-mediated immunosuppression in coronavirus-infected mice is associated with the production of TGF-β or IL-35 (Anghelina et al. 2009; Vignali et al. 2009).

![Figure 7. Dualism of regulatory T cells (Treg) in virus infection.](image)

**Figure 7. Dualism of regulatory T cells (Treg) in virus infection.** With increasing immune responses during the disease course the number of Treg increases in the inflamed tissue in order to limit excessive inflammation and tissue damage. The suppressive function of Treg also reduces protective immune responses which favors virus persistence and probably enhances immunopathology in the chronic disease phase. Modified from Belkaid and Rouse, (2005).
In persistent viral infection (e.g. HIV), where an equilibrium between viral proliferation and the immune response is established, viral removal becomes difficult, which leads to life threatening diseases (Dittmer et al. 2004). Induction and/or expansion of Treg cells by viruses is a highly efficient strategy to prevent effector T cell activation (Mills, 2004; Schneider-Schaulies and Dittmer, 2006). The role of Treg in MV infection is under debate, since differing findings have observed in animal experiments and human patients (Yu et al. 2008; Li et al. 2008; Sellin et al. 2009). Probably the interplay between the immunoregulatory and effector response during MV infection could be critical for pathogenesis, and the adequate balance between these two arms of immunity may play an essential role for the disease outcome (Sellin et al. 2009).
3 Chapter 3: Regulatory T cells in canine distemper virus infection

Depletion of Foxp3+ regulatory T cells as a putative prerequisite for lesion initiation in canine distemper virus induced demyelinating leukoencephalitis

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Abstract

Canine distemper virus (CDV) infection causes demyelinating leukoencephalitis in dogs, sharing similarities with human myelin disorders and is therefore appreciated as a translational animal model for multiple sclerosis (MS). In viral neurological diseases, an ambiguous function of regulatory T cells (Treg), with both beneficial effects by reducing immunopathology and detrimental effects by inhibiting antiviral immunity, has been described. However, the role of Treg in the pathogenesis of canine distemper has not been investigated yet. The aim of the present study was to test the hypothesis that peripheral lymphoid depletion influences immunomodulatory mechanisms in the brain of CDV-infected dogs. Immunohistochemistry revealed a lack of Foxp3$^+$ Treg in predemyelinating and early demyelinating lesions which was associated with the accumulation of CD3$^+$ T cells, L1$^+$ macrophages/microglia and GFAP$^+$ astrocytes. Together with CD79α$^+$ B cells, a delayed infiltration of Foxp3$^+$ Treg was observed in chronic demyelinating lesions. Splenic depletion of Foxp3$^+$ Treg was associated with an increased mRNA-expression of tumor necrosis factor in the acute disease phase, indicative of a pro-inflammatory microenvironment and lack of immunological counter regulation in peripheral lymphoid organs. In conclusion, disturbed immune regulatory mechanisms represent a potential cause for excessive neuroinflammation and early lesion development in canine distemper leukoencephalitis, as discussed for immune mediated myelin disorders such as MS.

Introduction

Distemper in dogs is caused by the canine distemper virus (CDV), a morbillivirus which is closely related to the human measles virus [1–3]. The disease course and pathogenesis of canine distemper are similar to human measles, including fever, rash, respiratory signs, lymphopenia, and profound immunosuppression with generalized
depletion of lymphoid organs during the acute disease phase [4–6]. Central nervous system (CNS) infection and neurological complications can be observed more frequently in infected dogs compared to measles patients, usually affecting children [7–9]. Depending on CDV strain, host immune status, and age, naturally infected dogs develop demyelinating leukoencephalomyelitis, which shares similarities with human myelin disorders, such as multiple sclerosis (MS) as well as measles virus associated post-infectious encephalomyelitis and subacute sclerosing panencephalitis [2,10,11].

Regulatory T cells (Treg), characterized by expression of the transcription factor *forkhead* box P3 (Foxp3), play a key role in the maintenance of immunological tolerance and therefore prevent autoimmune CNS disease [12–17]. However, in infectious CNS diseases Treg exhibit both beneficial effects by reducing immune mediated tissue damage and detrimental effects due to their immunosuppressive properties, causing disease exacerbation or persistence, respectively [18,19]. For instance, Treg reduce antiviral immunity in experimental Theiler’s murine encephalomyelitis [20,21], a rodent model for demyelinating disorders as well as in Friend retrovirus mouse model [22] and experimental herpesvirus infection of mice [23,24]. However, the impact of Treg upon morbillivirus-induced immunological alterations during early infection and CNS manifestation remains enigmatic [25], since reports that Treg are increased in measles patients [26,27] have been contradicted by others [28]. Moreover, different rodent models for measles virus infection came to ambiguous conclusions regarding Treg-related effects upon immune responses, probably attributed to disease course-dependant effects or mouse strain-specific responses to virus infection [5,29–31]. Thus, in addition to rodent models, there is an increasing interest in spontaneous and experimental canine diseases as translational large animal models for human CNS disorders [32–34].

Demyelination in canine distemper represents a biphasic process with directly virus induced neurodegeneration, microglial activation and CD8-mediated cytotoxicity during the early phase [35–37]. In comparison, during the chronic phase, reconstitution of
Chapter 3: Regulatory T cells in CDV infection

Peripheral lymphoid organs facilitates immune mediated mechanisms with delayed type hypersensitivity and progressive myelin loss in the CNS of CDV-infected dogs [4,38,39]. A proinflammatory cytokine environment in the brain during acute CDV-infection is indicative of insufficient counter regulatory mechanisms, potentially causing early immune over-activation and initial tissue damage in the brain [40–43]. Similarly, expression of neuroprotective and Treg-specific cytokines such as IL-10 and TGF-β is insufficient in canine spinal cord injury, leading to an activation of CNS resident immune cells [44]. Similar to the mechanisms in demyelinating leukoencephalomyelitis in CDV-infection, an early stimulation of microglia and lack of immunoregulation is discussed as a requirement for myelin damage in MS patients [45–48]. However, so far, the role of immunomodulatory cells, especially Treg, in the pathogenesis of canine distemper has not been investigated.

Since the initiation of inflammation in myelin disorders is influenced by an immunological imbalance of the peripheral immune system [49–51], the aim of the present study was to determine disease phase-dependant phenotypical changes and cytokine expression in lymphoid organs in canine distemper. Special emphasis was given to testify the hypothesis that peripheral depletion of Treg causes a lack of CNS-infiltrating immunomodulatory cells in the predemyelinating phase of CDV infection, which has the ability to enhance early neuroinflammation and represents a potential prerequisite for immune mediated demyelination.

Materials and Methods

Animals and tissue selection
A total of 23 dogs of different breeds and age with spontaneous CDV-infection and five control animals were selected for this study (Table 1). Animals were clinically examined at the Small Animal Clinic of the University of Veterinary Medicine Hannover (Germany) and sacrificed by an overdose of pentobarbital (100 mg/kg intravenously) on the
owner’s request due to worsening of clinical signs related to CDV-infection, such as neurological (seizures), respiratory (coughing, sneezing) and intestinal signs (diarrhea), respectively, and poor prognosis. Infected dogs which died spontaneously as a consequence of systemic distemper, showed seizures prior to death and were directly submitted to necropsy by pet owners (animals 16, 17, 21, 27; Table 1). Main pathological findings in the brain and extra-neuronal tissues of affected animals are listed in table 1. All dogs were examined for research purposes at the Department of Pathology of the University of Veterinary Medicine Hannover (Germany) with the permission by the owners. Five non-infected healthy animals (beagles) without neurological signs were obtained from an animal experiment performed at the Institute for Parasitology of the University of Veterinary Medicine Hannover, which was approved and authorized by the local authorities (Niedersächsisches Landesamt für Verbraucherschutz- und Lebensmittelsicherheit (LAVES), Oldenburg, Germany, permission number 08A580) and used as controls (Table1).

Infected animals were grouped according to the most advanced and dominating brain lesion (SI-SIII, see below). For morphological and phenotypical characterization, paraffin embedded spleen tissue was available from all CDV-infected and control animals. Out of these, immunohistochemical analyses of paraffin embedded brain tissue were performed in 15 CDV-infected and 5 control dogs. For cytokine expression analyses by reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR), frozen spleen tissue was available from 11 CDV-infected and five control dogs (Table 1).

**Histology and phenotyping**

Paraffin embedded tissue slices (4μm thickness) from the spleen and brain were stained with hematoxylin and eosin (HE) for morphological examination. The brain tissue was additionally stained with luxol fast blue (LFB) for detecting myelin loss and myelinophagia (active demyelination). Antigen detection was performed by the avidin-
biotin-peroxidase complex method as previously described [35,52]. In brief, paraffin embedded tissues were deparaffinized in Roticlear (Carl Roth GmbH, Karlsruhe, Germany) and rehydrated through graded alcohols. Endogenous peroxidase activity was suppressed with 0.5% H$_2$O$_2$ in methanol, followed by incubation with primary antibody overnight at 4°C. Specificity controls included substitution of the respective monoclonal antibody with ascitic fluid from nonimmunized BALB/cJ mice or rabbit normal serum and in the case of the anti-Foxp3 antibody, a rat immunoglobulin isotype control, was used. Spleen tissue of a healthy dog was used as positive control for the detection of lymphoid cells. Except for the lectin BS-1, incubation with primary antibodies was followed by incubation with biotinylated secondary antibodies for 30 minutes at room temperature. Subsequently, the avidin-biotin-peroxidase complex (VECTASTAIN Elite ABC Kit; Vector Laboratories, PK 6100, Burlingame, CA) was added and incubated for 30 minutes at room temperature. Antigen-antibody reactions were visualized by incubation with 3,3'-diaminobenzidine-tetrahydrochloride-H$_2$O$_2$ in 0.1 mol/L imidazole, pH 7.1 for 5 minutes, followed by counterstaining with hematoxylin.

**Histological evaluation and phenotypical characterization of white matter lesions in the cerebellum**

HE- and LFB-staining of white matter lesions of dogs suffering from CDV-infection and of control dogs were evaluated morphologically by light microscopy. The cerebellar white matter of 15 naturally infected dogs and five control dogs were examined and lesions were classified as described by Wünschmann et al. (1999) with slight modifications [37]. Briefly, groups were classified as acute non-inflammatory encephalitis (SI), subacute non-inflammatory encephalitis (SII) and subacute to chronic inflammatory encephalitis (SIII). Acute white matter lesions (SI) were characterized by hypercellularity and vacuolization whereas SII and SIII lesions showed active demyelination as demonstrated by decreased intralesional LFB-staining and the presence of LFB$^+$-myelinophages (gitter cells).
### Chapter 3: Regulatory T cells in CDV infection

**Table 1. Characteristics of dogs used in the study.**

<table>
<thead>
<tr>
<th>Dog no.</th>
<th>Brain lesion (group)</th>
<th>Age [month]</th>
<th>Sex</th>
<th>Breed</th>
<th>Main pathological findings in extra-neuronal tissues</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>4.5</td>
<td>n.d.</td>
<td>Beagle</td>
<td>None</td>
</tr>
<tr>
<td>2</td>
<td>Control</td>
<td>4.5</td>
<td>n.d.</td>
<td>Beagle</td>
<td>None</td>
</tr>
<tr>
<td>3</td>
<td>Control</td>
<td>4.5</td>
<td>n.d.</td>
<td>Beagle</td>
<td>None</td>
</tr>
<tr>
<td>4</td>
<td>Control</td>
<td>4.5</td>
<td>n.d.</td>
<td>Beagle</td>
<td>None</td>
</tr>
<tr>
<td>5</td>
<td>Control</td>
<td>4.5</td>
<td>n.d.</td>
<td>Beagle</td>
<td>None</td>
</tr>
<tr>
<td>6</td>
<td>SI</td>
<td>3</td>
<td>♀</td>
<td>mongrel</td>
<td>Interstitial pneumonia, catarrhal enteritis</td>
</tr>
<tr>
<td>7</td>
<td>SI</td>
<td>72</td>
<td>♂</td>
<td>mongrel</td>
<td>Catarrhal rhinitis</td>
</tr>
<tr>
<td>8</td>
<td>SI</td>
<td>3</td>
<td>♂</td>
<td>mongrel</td>
<td>Interstitial pneumonia, catarrhal enteritis</td>
</tr>
<tr>
<td>9</td>
<td>SI</td>
<td>3.5</td>
<td>♀</td>
<td>Jack Russell Terrier</td>
<td>Interstitial pneumonia, catarrhal enteritis</td>
</tr>
<tr>
<td>10</td>
<td>SI</td>
<td>n.d.</td>
<td>♀</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>11</td>
<td>SI</td>
<td>2</td>
<td>♀</td>
<td>n.d.</td>
<td>Interstitial pneumonia, catarrhal enteritis</td>
</tr>
<tr>
<td>12</td>
<td>SI</td>
<td>10</td>
<td>♂</td>
<td>mongrel</td>
<td>Interstitial pneumonia, catarrhal enteritis, catarrhal rhinitis</td>
</tr>
<tr>
<td>13</td>
<td>SI</td>
<td>n.d.</td>
<td>♀</td>
<td>mongrel</td>
<td>n.d.</td>
</tr>
<tr>
<td>14</td>
<td>SII</td>
<td>7</td>
<td>♀</td>
<td>Husky</td>
<td>Interstitial pneumonia</td>
</tr>
<tr>
<td>15</td>
<td>SII</td>
<td>2</td>
<td>♀</td>
<td>Labrador Retriever</td>
<td>Interstitial pneumonia</td>
</tr>
<tr>
<td>16</td>
<td>SII</td>
<td>3</td>
<td>♂</td>
<td>Shih Tzu</td>
<td>Interstitial pneumonia</td>
</tr>
<tr>
<td>17</td>
<td>SII</td>
<td>5</td>
<td>♀</td>
<td>Chihuahua</td>
<td>Interstitial pneumonia</td>
</tr>
<tr>
<td>18</td>
<td>SII</td>
<td>12</td>
<td>♀</td>
<td>Bassett Hound</td>
<td>Catarrhal enteritis</td>
</tr>
<tr>
<td>19</td>
<td>SII</td>
<td>4</td>
<td>♂</td>
<td>mongrel</td>
<td>Interstitial pneumonia, catarrhal enteritis</td>
</tr>
<tr>
<td>20</td>
<td>SII</td>
<td>3</td>
<td>♀</td>
<td>Jack Russell Terrier</td>
<td>None</td>
</tr>
<tr>
<td>21</td>
<td>SII</td>
<td>6</td>
<td>♂</td>
<td>mongrel</td>
<td>Interstitial pneumonia</td>
</tr>
<tr>
<td>22</td>
<td>SIII</td>
<td>5</td>
<td>♀</td>
<td>mongrel</td>
<td>n.d.</td>
</tr>
<tr>
<td>23</td>
<td>SIII</td>
<td>12</td>
<td>♂</td>
<td>mongrel</td>
<td>Interstitial pneumonia</td>
</tr>
<tr>
<td>24</td>
<td>SIII</td>
<td>7</td>
<td>♂</td>
<td>mongrel</td>
<td>Interstitial pneumonia</td>
</tr>
<tr>
<td>25</td>
<td>SIII</td>
<td>8</td>
<td>♀</td>
<td>Retriever</td>
<td>None</td>
</tr>
<tr>
<td>26</td>
<td>SIII</td>
<td>5.5</td>
<td>♂</td>
<td>German Shepherd Dog</td>
<td>n.d.</td>
</tr>
<tr>
<td>27</td>
<td>SIII</td>
<td>24</td>
<td>♀</td>
<td>mongrel</td>
<td>None</td>
</tr>
<tr>
<td>28</td>
<td>SIII</td>
<td>4</td>
<td>♀</td>
<td>Dachshund</td>
<td>Interstitial pneumonia</td>
</tr>
</tbody>
</table>
n.d. = not determined; S1 = acute non-inflammatory encephalitis; SII = subacute non-inflammatory encephalitis; SIII = subacute to chronic inflammatory encephalitis; ♀ = female; ♂ = male; § paraffin embedded spleen tissue available; † used for immunohistochemical analyses of brain tissue; ‡ frozen spleen tissue available

While SII lesions were dominated by glial responses (microgliosis and astrogliosis) without perivascular cuffing, SIII lesions displayed marked lymphohistiocytic infiltration within the neuroparenchyma and perivascular spaces, indicative of an advanced disease process. The control group (C) showed no histopathological alterations. Immunohistochemistry and lectin histochemistry for quantifying inflammatory cell infiltrations and glial responses in the brain were evaluated by using a morphometric grid at a high power field resolution (number of cells/0.0625 mm²). The absolute number of labeled cells was counted in white matter lesions (S1-SIII) and controls (C). Small lesions were counted in total, otherwise a maximum of ten randomly distributed high power fields within large lesions were counted [53].

**Histological scoring of lymphoid depletion and characterization of phenotypical changes in the splenic white pulp**

The degree of lymphoid depletion in spleen sections of CDV-infected and control animals, expressed as the cellularity score, was evaluated semi-quantitatively by light microscopy as described by Wünschmann et al. (2000) [38]: 4 = normal architecture, 3 = mild, 2 = moderate, 1 = severe depletion. In a normal spleen (cellularity score 4; Fig. S1A), the white pulp areas showed a regular architecture, with numerous primary and secondary follicles. Mildly depleted spleens (cellularity score 3; Fig. S1B) were characterized by a reduced size of primary follicles and loss of secondary follicles, whereas marked reduction of the white pulp size and loss of secondary follicles were indicative of moderate depletion (cellularity score 2; Fig. S1C). In severely depleted spleens, the white pulp was no longer recognizable (cellularity score 1; Fig. S1D).
Table 2. List of markers used for immunohistochemistry and lectin histochemistry

<table>
<thead>
<tr>
<th>Antibody specificity</th>
<th>Target</th>
<th>Primary antibody</th>
<th>Pretreatment / dilution</th>
<th>Secondary antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDV 3991</td>
<td>CDV-NP</td>
<td>Monoclonal, mouse anti-CDV-NP (C. Örvell, Stockholm, Sweden)</td>
<td>None / 1:6000</td>
<td>GaM-b</td>
</tr>
<tr>
<td>CDV D110</td>
<td>CDV-NP</td>
<td>Monoclonal, mouse anti-CDV-NP (A. Zurbriggen, Bern, Switzerland)</td>
<td>Citrate buffer / 1:1000</td>
<td>GaM-b</td>
</tr>
<tr>
<td>CD3</td>
<td>T cells</td>
<td>Polyclonal, rabbit anti-human (A0452; Dako)</td>
<td>Citrate buffer / 1:1000</td>
<td>GaR-b</td>
</tr>
<tr>
<td>CD79α</td>
<td>B cells</td>
<td>Monoclonal, mouse anti-human (HM57, Dako)</td>
<td>Citrate buffer / 1:60</td>
<td>GaM-b</td>
</tr>
<tr>
<td>Foxp3</td>
<td>Regulatory T cells</td>
<td>Monoclonal, mouse anti-rat (FJK-16s; eBioscience)</td>
<td>Citrate buffer / 1:10</td>
<td>RaRt-b</td>
</tr>
<tr>
<td>L1</td>
<td>Macrophages</td>
<td>Monoclonal, mouse anti-human (MAC387; Dako)</td>
<td>Citrate buffer / 1:200</td>
<td>GaM-b</td>
</tr>
<tr>
<td>MHC II</td>
<td>APC</td>
<td>Monoclonal, mouse anti-human (TAL.1B5; Dako)</td>
<td>Citrate buffer / 1:80</td>
<td>GaM-b</td>
</tr>
<tr>
<td>GFAP</td>
<td>Astrocytes</td>
<td>Polyclonal, rabbit anti-bovine (Z0334; Dako)</td>
<td>None / 1:2000</td>
<td>GaR-b</td>
</tr>
<tr>
<td>BS-1</td>
<td>Microglia / macrophages</td>
<td>Lectin (L 3759; Sigma)</td>
<td>None / 1:300</td>
<td>-</td>
</tr>
</tbody>
</table>

GaM-b, goat anti-mouse; GaR-b, goat anti-rabbit; RaRt-b, rabbit anti-rat; b, biotinylated; CDV-NP, canine distemper virus-nucleocapsid protein; APC, antigen presenting cells; GFAP, glial fibrillary acidic protein; BS-1, lectin from *Bandeiraea simplicifolia*.

The quantity of different cell subsets within the white pulp determined by leukocyte-specific immunohistochemistry was evaluated semi-quantitatively (immunoreactivity score) as described previously [38]. In brief, 10 randomly chosen areas of the white pulp were counted in follicles, PALS and marginal zones. The immunoreactivity score was
determined as follows: 1 = 1-25% positive cells, 2 = 26-50% positive cells, 3 = 51-75% positive cells, and 4 = 76-100% positive cells. The immunoreactivity index was calculated by multiplying the cellularity score by the immunoreactivity score [38].

To determine virus load and distribution in splenic follicles, PALS and marginal zones by CDV-specific immunohistochemistry, the following scale was used: 0 = no virus detected, 1 = few infected cells, 2 = some infected cells, 3 = numerous infected cells; 4 = almost all cells infected [38].

Cytokine expression analysis by reverse transcriptase-quantitative polymerase chain reaction

Primer design
Primers for the generation of standards and primers for measuring the quantity of specific cytokines are listed in tables 2 and 3. PCR primer sequences for detecting glyceraldehyde-3-phosphate dehydrogenase (GAPDH), elongation factor-1α (EF-1α), hypoxanthine-guanine phosphoribosyltransferase (HPRT), tumor necrosis factor (TNF), transforming growth factor-β (TGF-β), interleukin (IL)-2, IL-6, IL-8, and IL-10 as well as for CDV were taken from the literature [42,44,54–57]. Primers were designed using Beacon Designer software version 2.1 (Premier Biosoft International, Palo Alto, CA) or the primer-blasting tool of basic local alignment search tool (BLAST). All primers were purchased from Eurofins MWG Operon (Ebersberg, Germany).

Nucleic acids isolation
Total RNA was isolated from spleens using the RNeasy Mini Kit (Qiagen) as previously described [44,54]. For the generation of serial standards dilution, total RNA was extracted from the canine macrophage cell line DH82 (for GAPDH, EF-1α, TGF-β, TNF, IL-6, IL-8, and IL-10), persistently CDV-infected DH82 cells (for CDV and HPRT) and a canine lymph node (for IL-2) using TRIZOL (Invitrogen). To isolate total RNA from spleen tissue of CDV-infected dogs and control animals, 10 sections (thickness 25µm)
from frozen spleen tissue were cut on a cryostat (Microm, Heidelberg, Germany). Total RNA was extracted using the RNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions. RNA concentrations were calculated by measuring the optical density at 260 nm (GeneQuant™ pro, Amersham Biosciences Europe GmbH, Freiburg, Germany). Subsequently, total RNA was reversely transcribed to complementary DNA using the Omniscript kit (Qiagen) with RNase Out (Invitrogen) and Random Primers (Promega, Mannheim, Germany) following the manufacturers’ instructions.

**Polymerase chain reaction for the generation of standard dilutions**

For the production of standards, PCR was performed using a Biometra TProfessional basic thermocycler (Biometra GmbH, Göttingen Germany), as described before [44,54,57]. Annealing temperature was adjusted to 50°C (IL-2), 56°C (HPRT), 57°C (TGF-β), 58°C (TNF, IL-6), 59°C (GAPDH, IL-10, CDV), 60°C (EF-1α, IL-8) for two minutes, and amplification was achieved using AmpliTaq DNA Polymerase (Applied Biosystems, Carlsbad, CA) in 1 x GeneAmp PCR Buffer II (Applied Biosystems), with 1.25 mmol/L MgCl₂, 0.2 mmol/L dNTP mix (Biosystems, Carlsbad, CA), and 300 nmol/L of each primer. Polymerase chain reaction products of standards were subsequently analyzed by agarose gel electrophoresis and extracted using NucleoSpin Extract II Kit (Macherey-Nagel, Düren, Germany) for production of a standard dilution from 10² to 10⁸ copies per microliter.

**Reverse transcriptase-quantitative polymerase chain reaction**

RT-qPCR and data analysis were performed using the Mx3005P QPCR System (Agilent Technologies, Waldbronn, Germany) [44,54,57,58]. In addition to the standard dilution, complementary DNA of spleen samples and negative controls were measured in duplicate on the same run. Quantification was carried out in 25μl of Brilliant SYBR Green qPCR Core Reagent Kit (Agilent Technologies). Amplification was performed using 0.05 U/μl SureStart Taq DNA Polymerase in 1 x Core PCR buffer, with 2.5 mmol/L (CDV, GAPDH, TNF, IL-6, IL-10) or 5 mmol/L (EF-1α, HPRT, IL-2, IL-8, TGF-β) MgCl₂, 8.0% glycerol, 3% dimethyl sulfoxide (4% for TNF-α, IL-2), 150 nmol/L of each
primer (Table 4), 30 nmol/L Rox as reference dye, and 200 μmol/L dNTP mix. Specificity of the products was assessed by melting curve analysis. Calculated copy numbers of each gene were normalized to an amount of 100ng of transcribed RNA and gene expression values were normalized against the three housekeeping genes, GAPDH, EF-1α, and HPRT, using the software geNorm (Ghent University Hospital Center for Medical Genetics; available at http://medgen.ugent.be/~jvdesomp/genorm/) [59]. In brief, the software detects the most stable reference genes of which the geometric means were used to calculate a normalization factor for the genes of interest.

**Statistics**

To determine distribution of data, a Shapiro-Wilk test and visualization assessment were performed. If data were not normally distributed, non-parametrical tests (Mann-Whitney U-test) were applied and data between two groups were compared. For statistical analyses and graph development, SPSS software was used.
Table 3. Gene expression analyzed by polymerase chain reaction

<table>
<thead>
<tr>
<th>Gene / primer direction</th>
<th>Primer sequence</th>
<th>Genebank accession no., position</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH S₁</td>
<td>AAGGTGGATGTCACGGATT</td>
<td>AB038240, 7-26</td>
</tr>
<tr>
<td>GAPDH AS₁</td>
<td>GCAGAAGAGCAGAGATGATG</td>
<td>AB038240, 371-351</td>
</tr>
<tr>
<td>EF-1α S₁</td>
<td>AGCCCTTCGCTGCTCTTC</td>
<td>X03558, 784-803</td>
</tr>
<tr>
<td>EF-1α AS₁</td>
<td>CAGACACATTCTTGACATTGAAGC</td>
<td>X03558, 1002-979</td>
</tr>
<tr>
<td>HPRT S₁</td>
<td>TAAAAGTAATTGGTGGAGAT</td>
<td>CFU16661, 2-21</td>
</tr>
<tr>
<td>HPRT AS₁</td>
<td>ATTATACTGCGCACGTACAGC</td>
<td>CFU16661, 123-105</td>
</tr>
<tr>
<td>IL-2 S³</td>
<td>ACCTCAACTCTGCCCACAAT</td>
<td>D30710, 14-33</td>
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<tr>
<td>IL-2 AS³</td>
<td>GCACCTCCTCCAGGTGTTTG</td>
<td>D30710, 302-283</td>
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<tr>
<td>IL-6 S¹</td>
<td>TCTCCACACACGCTCTTCC</td>
<td>U12234, 68-87</td>
</tr>
<tr>
<td>IL-6 AS¹</td>
<td>TTCTTGTCAGCAGGCTTCC</td>
<td>U12234, 385-366</td>
</tr>
<tr>
<td>IL-8 S¹</td>
<td>ACTTCAAGCTGTGCTTGGC</td>
<td>U10308, 10-29</td>
</tr>
<tr>
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<td>GGCCACTGTCAATCACTTCC</td>
<td>U10308, 181-162</td>
</tr>
<tr>
<td>IL-10 S¹</td>
<td>CCTGGGTTGCAAGCCCTGTC</td>
<td>U33843, 235-255</td>
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<tr>
<td>IL-10 AS¹</td>
<td>ATGCCGCTCTTCACCTGCTCC</td>
<td>U33843, 446-427</td>
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<tr>
<td>TNF S¹</td>
<td>CCAAGTGACAGCGCTAGGC</td>
<td>Z70046, 32-51</td>
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<tr>
<td>TNF AS¹</td>
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<td>TGF-β S¹</td>
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<td>CAGGGCAGAAGTTAGGTGT</td>
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</tr>
<tr>
<td>CDV S²</td>
<td>ACAGGATTGTGAGAGCCATAT</td>
<td>AF378705, 769-789</td>
</tr>
<tr>
<td>CDV AS²</td>
<td>CAAGATAACCATGTGACGG</td>
<td>AF378705, 1055-1035</td>
</tr>
</tbody>
</table>

¹ [44]; ² [54]; ³ [57]. S = sense; AS = antisense; bp = base pair; EF-1α = elongation factor-1α; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; HPRT = hypoxanthine-guanine phosphoribosyltransferase; IL = interleukin; TGF-β = transforming growth factor-β; TNF = tumor necrosis factor.
Table 4. Gene expression analyzed by reverse transcriptase quantitative polymerase chain reaction

<table>
<thead>
<tr>
<th>Gene / primer direction</th>
<th>Primer sequence</th>
<th>Genebank accession no., position</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH S&lt;sup&gt;1&lt;/sup&gt;</td>
<td>GTCATCAACGGGAAGTCCATCTC</td>
<td>AB038240, 196-218</td>
</tr>
<tr>
<td>GAPDH AS&lt;sup&gt;1&lt;/sup&gt;</td>
<td>AACATACTCAGACACGACATC</td>
<td>AB038240, 279-257</td>
</tr>
<tr>
<td>EF-1α S&lt;sup&gt;1&lt;/sup&gt;</td>
<td>CAAAAACGACCCCAATGG</td>
<td>AY195837, 770-789</td>
</tr>
<tr>
<td>EF-1α AS&lt;sup&gt;1&lt;/sup&gt;</td>
<td>GGCCTGGATGTCTAGGATA</td>
<td>AY195837, 837-818</td>
</tr>
<tr>
<td>HPRT S&lt;sup&gt;1&lt;/sup&gt;</td>
<td>GAGATGACCTCTCAACTTTAATGAAA</td>
<td>CFU16661, 17-44</td>
</tr>
<tr>
<td>HPRT AS&lt;sup&gt;1&lt;/sup&gt;</td>
<td>GGGAAGCAAAGGTCTATGTGAG</td>
<td>CFU16661, 105-86</td>
</tr>
<tr>
<td>IL-2 S&lt;sup&gt;2&lt;/sup&gt;</td>
<td>CCAACTCTCCAGAGTCTCAC</td>
<td>D30710, 196-216</td>
</tr>
<tr>
<td>IL-2 AS&lt;sup&gt;2&lt;/sup&gt;</td>
<td>TCTGCTAGACATTGAAGGTTGTGA</td>
<td>D30710, 276-252</td>
</tr>
<tr>
<td>IL-6 S&lt;sup&gt;1&lt;/sup&gt;</td>
<td>TGATGCCACTTCAATATGCTACCA</td>
<td>U12234, 156-180</td>
</tr>
<tr>
<td>IL-6 AS&lt;sup&gt;1&lt;/sup&gt;</td>
<td>TCAGTGCAGAGATTTTGCCGAGGA</td>
<td>U12234, 244-221</td>
</tr>
<tr>
<td>IL-8 S&lt;sup&gt;1&lt;/sup&gt;</td>
<td>AAGAAGTGAGAGTGGTGGGATGGGA</td>
<td>D28772, 184-203</td>
</tr>
<tr>
<td>IL-8 AS&lt;sup&gt;1&lt;/sup&gt;</td>
<td>TTATACACTGGCAGTTGGGATGGG</td>
<td>D28772, 149-130</td>
</tr>
<tr>
<td>IL-10 S&lt;sup&gt;1&lt;/sup&gt;</td>
<td>GGTGGGAGCCAGCCGACACCAG</td>
<td>U33843, 49-70</td>
</tr>
<tr>
<td>IL-10 AS&lt;sup&gt;1&lt;/sup&gt;</td>
<td>AAGAAGATCTCCACCACCCAAGGGA</td>
<td>U33843, 168-144</td>
</tr>
<tr>
<td>TNF S&lt;sup&gt;1&lt;/sup&gt;</td>
<td>GGAGCTGACAGACAAACCAGCATTGA</td>
<td>Z70046, 133-155</td>
</tr>
<tr>
<td>TNF AS&lt;sup&gt;1&lt;/sup&gt;</td>
<td>GGAAGGGCCACCTTGGGCCCTT</td>
<td>Z70046, 223-204</td>
</tr>
<tr>
<td>TGF-β S&lt;sup&gt;1&lt;/sup&gt;</td>
<td>TGTCGCTACCCCTCAGACACCAG</td>
<td>NM_001003309.1, 592-611</td>
</tr>
<tr>
<td>TGF-β AS&lt;sup&gt;1&lt;/sup&gt;</td>
<td>AGCCCTCGACTTCCCCAATCCTCA</td>
<td>NM_001003309.1, 706-687</td>
</tr>
<tr>
<td>CDV S&lt;sup&gt;3&lt;/sup&gt;</td>
<td>GCTCTTGGGTCATGAGTTTCTGCC</td>
<td>AF378705, 954-973</td>
</tr>
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1<sup>[44]</sup>; 2<sup>[54]</sup>; 3<sup>[57]</sup>. S = sense; AS = antisense; bp = base pair; EF-1α = elongation factor-1α; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; HPRT = hypoxanthine-guanine phosphoribosyltransferase; IL = interleukin; TGF-β = transforming growth factor beta; TNF = tumor necrosis factor.
Chapter 3: Regulatory T cells in CDV infection

Results

Characterization of white matter lesions in the cerebellum of canine distemper virus infected dogs

Detection of canine distemper virus in brain lesions
The CDV-NP was detected in all investigated cerebellar lesions by immunohistochemistry. The virus amount was most prominent in SII lesions (Fig. 1). In agreement with previous studies [37,53], with disease progression, the number of CDV-infected cells significantly decreased in SIII lesions compared to non-inflammatory SII lesions (p=0.019).

Results of statistical analyses (comparison between controls and infected animals) are listed in table S1.

Phenotypical analyses of inflammatory responses in brain lesions
Progressive hypercellularity was observed in white matter lesions during the disease course. According to the scheme described in material and methods, 28 brain lesions were classified as acute non-inflammatory (SI; Fig. 2), 17 plaques as subacute non-inflammatory (SII) and 25 plaques as subacute to chronic inflammatory (SIII; Fig. 2). A significant infiltration of CD3+ T cells was found in all lesion types (SI-SIII), whereas a significant number of infiltrating Foxp3+ cells was found only in advanced inflammatory brain lesions (SIII; Fig. 3B), indicative of delayed infiltration of Treg. With the onset of active demyelination (presence of LFB+ gitter cells) and perivascular lymphohistiocytic inflammation in SIII lesions, the amount of CD3+ T cells significantly increased compared to SI (p≤0.001) and SII lesions (p=0.006), respectively (Fig. 3A). Similar to Foxp3+ Treg, CD79α+ B cells were found only in SIII lesions (Fig. 3C). As a consequence of continuous leukocyte infiltration and glial activation, MHC II was significantly up-regulated in all lesion types (SI-SIII) compared to control brains (Fig. 3D).
In addition, a significantly elevated MHC II expression was found in SIII compared to SI lesions (p=0.001). A similar kinetic was observed for BS-1+ macrophages/microglia (Fig. 3E), while the number of GFAP+ astrocytes was only transiently significantly increased in acute (SI) and subacute non-inflammatory lesions (SII) compared to the controls.

Results of statistical analyses (comparison between controls and infected animals) are listed in table S1.

Results demonstrate a virtual lack of Treg in predemyelinating lesions (SI) and early demyelinating lesions (SII) during the acute and subacute phase of canine distemper leukoencephalitis, which is in agreement with previous reports about dominating proinflammatory properties of early infiltrating T cells and resident glial cells in canine distemper leukoencephalitis [42].
Chapter 3: Regulatory T cells in CDV infection

To testify the hypothesis that the delayed onset Treg infiltration in the brain is a consequence of peripheral Treg depletion, phenotypical changes and the associated cytokine expression in the spleen, as a lymphoid organ consistently affected in systemic distemper, were investigated [38].

Characterization of immunological changes in the spleen of canine distemper virus infected dogs

Detection of canine distemper virus in the spleen

The quantity and distribution of CDV protein in the spleen were determined by immunohistochemistry. In addition, the amount of virus RNA was measured by RT-qPCR. Similar to the brain, virus load in the spleen was highest during early infection phases. Using immunohistochemistry, CDV-infected cells were found in animals with early brain lesions (SI and SII) with most prominent virus expression in SII group animals.
Figure 3. Phenotyping of white matter lesions in the cerebellum of canine distemper virus infected dogs. A) CD3\(^+\) T cells show a significant increase in acute (SI) and subacute non-inflammatory lesions (SII) compared to controls (C). Note prominent CD3-expression in subacute to chronic inflammatory lesions (SIII). B) In comparison to CD3\(^+\) T cells, significant accumulations of CD79α\(^+\) B cells and C) Foxp3\(^+\) regulatory T cells are only observed in advanced inflammatory brain lesions (SIII). D) Continuous increase of MHC II-expression and E) BS-1\(^+\) macrophages/microglia with disease progression F) GFAP\(^+\) astrocytes are significantly increased in acute non-demyelinated lesions (SI) and subacute non-inflammatory demyelinating lesions (SII), followed by a decrease in advanced demyelinating lesions (SIII).  = significant difference to controls (C). ▲ = significant difference to SI. • = significant difference to SII.

With disease progression, in animals showing subacute to chronic demyelinating lesions (SIII group animals), the virus load was significantly reduced and restricted mainly to germinal centers. Results were confirmed by RT-qPCR, demonstrating that virus RNA in the spleen was most abundant in dogs with SI and SII brain lesions (Fig. 4), while only small quantities of CDV-RNA were detected in animals with advanced disease phases (SIII group animals).
Results of statistical analyses (comparison between controls and infected animals) are listed in table S1.

**Morphological evaluation and phenotypical characterization in the spleen**

Lymphoid depletion, which was most prominent in the PALS and follicles, was found in all infected animals (Fig. 5). In order to detect more subtle changes associated with virus infection, phenotypical analyses were performed. As expected, CD3$^+$ T cells in the PALS and CD79α$^+$ B cells in follicles were predominantly affected. The degree of CD3-depletion in the PALS was identical in all stages (SI-SIII; Fig. 6A). Similarly, Foxp3$^+$ Treg were significantly decreased in all groups (SI-SIII) compared to control spleens.

![Figure 4. Detection of canine distemper virus (CDV) in the spleen by reverse transcriptase-quantitative polymerase chain reaction.](image)

The amount of viral RNA in the spleen is elevated in animals with acute (SI) and subacute non-inflammatory brain lesions (SII) compared to controls (C). In animals with subacute to chronic inflammatory lesions (SIII) only small amounts of CDV-RNA are detected $*$ = significant difference to controls (C).

However, in contrast to CD3$^+$ T cells, Foxp3$^+$ Treg were totally absent in the spleen of SI and SIII group animals, and only very few individual Foxp3$^+$ cells were observed in SII group animals (Fig. 6B). Follicular CD79α$^+$ B cells were significantly depleted in all
infected animals (SI-SIII) compared to controls. Suggestive of B cell repopulation, a statistical tendency (p=0.054) of increased CD79α+ B cell numbers in the spleen of SIII compared to SI group animals was observed (Fig. 6C). L1+ macrophages were significantly depleted in the splenic marginal zone of SII group animals (Fig. 6D). Associated with lymphoid depletion a reduced expression of MHC II in all compartments of the white pulp (follicle, PALS and marginal zone) was detected in all CDV-infected dogs. However, a significant increase of total MHC II+ cells in SIII group compared to SII, was found in follicles (p=0.029; Fig. 6E), PALS (p=0.040; Fig. 6F) and marginal zones (p=0.021; data not shown), indicative of partial repopulation of lymphoid organs in advanced stages of canine distemper as previously described [38].

Results of statistical analyses (comparison between controls and infected animals) are listed in table S1.
Figure 5. Phenotypical changes in the spleen of canine distemper virus infected dogs. Marked lymphoid depletion in SII group animals is associated with a decrease of CD3⁺ T cells (A, A’), Foxp3⁺ regulatory T cells (B, B’), CD79α⁺ B cells (C, C’), L1⁺ macrophages (D, D’) and MHC II⁺ antigen presenting cells (E, E’). * = central artery. F = follicle. P = PALS. MZ = marginal zone. Scale bars = 200μm.
Figure 6. Phenotypical changes in the spleen of canine distemper virus infected dogs. A) Depletion of CD3\(^+\) T cells and B) Foxp3\(^+\) regulatory cells in the periarteriolar lymphoid sheath (PALS) in infected animals. C) Decrease of CD79α\(^+\) B cells in splenic follicles. Note statistical tendency (▲, p=0.054) of increased CD79α\(^+\) B cell numbers in SIII group animals compared to SI group animals. D) Significant drop of L1\(^+\) macrophages in marginal zones of SII group animals. E, F) MHC II\(^+\) cells are depleted in the spleen of infected dogs. Note significant increase of MHC II expression in follicles and PALS of SIII animals, indicative of lymphoid repopulation. * = significant difference to control (C). • = significant difference to SII group.

Cytokine expression in the spleen

Expression of IL-2 mRNA was significantly reduced in the spleen of SI and SII group animals compared to control animals, as a presumed consequence of reduced T cell function (Fig. 7A). Strikingly, despite reduced IL-2 transcription, gene expression of TNF was significantly elevated in the spleen of SI and SII group dogs compared to controls (Fig. 7B) indicative of peripheral M1 polarization of macrophages and/or Th1 biased
immune responses of residual lymphocytes. Gene expression of IL-6, IL-8, IL-10 and TGF-β showed no statistical significant changes at different disease phases compared to the controls.

Results of statistical analyses (comparison between controls and infected animals) are listed in table S1.

![Figure 7](image_url)

**Figure 7. Cytokine expression in the spleen of canine distemper virus infected animals.** A) Expression of interleukin (IL)-2 mRNA is significantly decreased in non-inflammatory stages (SI, SII) compared to controls (C). B) Significantly increased tumor necrosis factor (TNF) mRNA expression in SI group animals and statistical tendency (p=0.052) of cytokine expression in SII group animals. * = significant difference compared to control (C).

**Discussion**

The comparative analysis of immune responses in the brain and spleen demonstrates the occurrence of a delayed CNS-infiltration of Treg in canine distemper, associated with immune dysregulation in peripheral lymphoid organs. The paucity of functional Treg is implicated in immune mediated demyelinating disorders, including experimental autoimmune encephalomyelitis (EAE) and MS [60–62]. Thus, an inadequate immune
homeostasis as a consequence of Treg depletion is assumed to allow excessive glial and T cell responses during the predemyelinating and early demyelinating phase of CDV-induced leukencephalitis as previously described [36,42].

Manipulation of Treg in the periphery has significant consequences for the fate of viral infection in the brain. In agreement with the idea of a misbalanced CNS immune response in canine distemper, insufficient expansion of peripheral Treg leads to strong immunopathology in acute severe dengue fever and West Nile virus infection [63,64]. The protective role of Treg against an overwhelming immune response in the CNS also becomes obvious in animal models of stroke and EAE [65,66] and in human immunodeficiency virus-1-associated neurodegeneration, where they reduce astrogliosis and microglia-mediated inflammation [67]. Furthermore, functional deficits or reduced numbers of Treg are suspected to contribute to demyelination in acute MS lesions [68]. Silencing of microglia by Treg is mediated by IL-4 which leads to reduced production of toxic factors such as nitric oxide [69]. Moreover, interaction with Treg induces a M2 phenotype of microglia/macrophages which exhibit immunomodulatory properties and potentially promote regeneration in the injured CNS [70,71]. Accordingly, the observed absence of CNS-infiltrating Treg is in agreement with the previous findings of low anti-inflammatory cytokine expression [42] and release of myelinotoxic molecules by microglia in early distemper brain lesions [43].

Interestingly, the increase of Foxp3+ Treg in the CNS during advanced stages of canine distemper coincides with the persistent infection, which corresponds to findings in experimental measles virus infection of mice [5,30]. Different explanations about the origin of Treg in the brain during chronic CDV-infection have to be considered. Since prominent depletion of Treg was observed in the spleen of chronically infected dogs, brain infiltration might result from an accumulation of residual Treg in other peripheral organs. In addition, it has been shown that the inflammatory environment critically influences the balance of Treg versus effector T cell differentiation which might lead to local induction and expansion of Foxp3+ Treg within the brain of CDV-infected animals.
For instance, virus-specific CD4⁺ T cells can acquire a Treg phenotype including Foxp3 expression in the CNS during mouse hepatitis virus infection [72]. Moreover, neurons and activated astrocytes are able to induce Treg as a mechanism for decreasing excessive inflammation and demyelination in EAE [73,74]. However, as observed in the present study, Treg seem to be unable to terminate neuroinflammation in chronic demyelinating lesions of CDV-infected dogs, indicative of an insufficient amount or dysfunction of these cells. Comparably, large numbers of brain-infiltrating Treg are present in EAE, but their function is impaired by the elevated expression of proinflammatory molecules, such as IL-6 and TNF [85, 86]. As demonstrated in previous studies, the presence of these cytokines in the cerebrospinal fluid is associated with disease exacerbation in CDV-infected dogs [77]. Thus, it is tempting to speculate that the pro-inflammatory milieu in the canine brain inhibits the neuroprotective efficacy of Treg in advanced stages. Alternatively, in spite of a potential beneficial effect upon immune mediated brain damage in chronic distemper, immunosuppression by Treg might contribute to viral persistence triggering progressive myelin loss as observed in coronavirus-induced demyelination of mice [78].

Similar to human measles, a striking feature of CDV-infection of dogs is a marked loss of immune cells and impaired immune response which favors viral persistence in lymphoid organs and CNS [4,38,79,80]. Lymphoid depletion in canine distemper is induced by necrosis and apoptosis directly mediated by the virus as well as by virus independent mechanisms, probably by an over-activation of the innate immune response [79]. Accordingly, the observed increased TNF expression might enhance splenic depletion by induction of lymphocyte apoptosis in acutely infected dogs. Moreover, TNF has been shown to downregulate the suppressive function of Treg by decreasing Foxp3 expression [81]. Thus, the pro-inflammatory microenvironment might contribute to the observed depletion of Foxp3⁺ cells in the spleen and, in addition, potentially foster Th1-biased immune responses and/or M1 polarization of macrophages [82,83]. Such primed immune cells lead to antiviral immunity, but might also account for
early lesion development by promoting excessive neuroinflammation in canine distemper [36,37,42].

As shown in previous studies, the low number of inflammatory cells in early non-inflammatory CNS lesions coincided with the degree of peripheral lymphoid depletion, whereas CNS-infiltration of inflammatory cells during the chronic demyelinating phase is associated with reconstitution of lymphoid organs [38]. Partial repopulation in chronically infected dogs in the present survey was demonstrated by an increase of MHC II⁺ lymphoid cells in splenic T and B cell areas, associated with a statistical trend of elevated numbers of CD79α⁺ B cells in follicles. Following measles virus infection of children, an early Th1 response is quickly succeeded by a prolonged Th2 or mixed Th1/Th2 response [84]. Th2 cytokine predominance after resolution of measles rash produces an environment favoring B cell maturation which facilitates humoral immunity important for lifelong protection against reinfection, while depressing macrophage activation and Th1 responses which are required for combating pathogens [85]. The importance of a robust Th1 immunity for virus elimination has also been demonstrated in CDV-infected ferrets, a model for morbillivirus-induced immunosuppression [86]. Taken together, B cell immunity and Th1/Th2 imbalance rather than mounting Treg responses might contribute to prolonged immune alteration in canine distemper, which contrasts with the currently discussed role of Treg in human measles [24,25]. However, further studies of advanced disease stages are needed to depict further similarities and differences between canine distemper and human measles.

Interestingly, according to the currently discussed role of B cells and antibodies in the pathogenesis of demyelinating disorders [47,75,76] an increased intrathecal antibody production is suspected to accelerate myelin destruction in chronic canine distemper [89]. Thus, besides its impact on virus-specific immunity, observed reconstitution of splenic B cells and recruitment of peripheral CD79α⁺ B cells to the brain might also represent an initiator for CNS-restricted plasma cell differentiation and subsequent myelin loss in dogs during the chronic distemper phase. Referring to this, a
compartmentalization of immune responses in the brain with local antigen presentation, plasma cell formation and antibody production behind a closed blood brain barrier is currently discussed for progressive MS [90,91].

In conclusion, for the first time the interplay between Treg in peripheral lymphoid tissue and brain of CDV-infected dogs has been demonstrated. Results of the present study are indicative of inadequate immunoregulation which represents a potential prerequisite for early lesion development. As suggested for MS [75], the development of novel therapies targeting Treg represent a promising strategy in canine CNS disorders. However, further studies are needed to determine disease phase-specific and probably ambiguous functions of Treg in the pathogenesis of canine distemper, which might also have relevance for the understanding of human demyelinating diseases.

Acknowledgements

The authors would like to thank Danuta Waschke, Bettina Buck and Petra Grünig for their excellent technical support during the laboratory work and Dr. Karl Rohn for statistical analyses. This study was supported by the German Research Foundation (FOR 1103, BA 815/10-2 and BE 4200/1-2).
References


Chapter 3: Regulatory T cells in CDV infection


Supporting Information

Figure S1. Histological grading of lymphoid depletion in the spleen. A) White pulp of control animal with normal architecture of white pulp. B) Mildly depleted spleen with reduced size of follicle. C) Moderately depleted spleen, characterized by continuous reduction of white pulp size and hypocellular follicle. D) Severely depleted spleen with nearly complete loss of white pulp architecture. Bar size 200μm.
Table S1: Results of statistical analyses (comparison between controls and infected animals)

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Table S1. Bold values display significant changes compared to control, ↑= significantly increased, ↓ = significantly decreased compared to control; ↑* = statistical tendency compared to control; CD3 = T cells; Foxp3 = regulatory T cells; CD79α = B cells; L1 = macrophages; MHC II = antigen presenting cells; IL = interleukin; TNF = tumor necrosis factor; TGF-β = transforming growth factor-β
Chapter 4: Canine dendritic cells

Species-specific properties and translational aspects of canine dendritic cells

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Abstract

Dogs are affected by spontaneously occurring neoplastic and inflammatory diseases which often share many similarities with pathological conditions in humans and are thus appreciated as important translational animal models. Dendritic cells (DCs) represent the most potent antigen presenting cell population. Besides their physiological function in the initiation of primary T cell responses and B cell immunity, a deregulation of DC function is involved in immune-mediated tissue damage, immunosuppression and transplantation complication in human and veterinary medicine. DCs represent a promising new target for cancer immunotherapy in dogs. However, the therapeutic use of canine DCs is restricted because of a lack of standardized isolation techniques and limited information about dog-specific properties of this cell type. This article reviews current protocols for the isolation and in vitro generation of canine monocyte- and bone
marrow-derived DCs. DCs of dogs are characterized by unique morphological features, such as the presence of cytoplasmic projections and periodic microstructures. Canine DCs can be discriminated from other hematopoietic cells also based on phenotypic properties and their high T cell stimulatory capability in mixed leukocyte reactions. Furthermore, the classification of canine DC-derived neoplasms and the role of DCs in the pathogenesis of selected infectious, allergic and autoimmune diseases, which share similarities with human disorders, are discussed. Future research is needed to expand the existing knowledge about DC function in canine diseases as a prerequisite for the development of future therapies interfering with the immune response.

Keywords: dendritic cells, cytokines, phenotyping, functional assays, translational research
Chapter 5: Dendritic cells in canine distemper virus infection

Canine distemper virus infection leads to reduced antigen presenting function of monocyte-derived dendritic cells

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Abstract

Canine distemper virus (CDV) shows a profound lymphotropism that causes immunosuppression and increased susceptibility of affected dogs to opportunistic infections. Similar to human measles virus, CDV is supposed to inhibit terminal differentiation of dendritic cells (DCs), responsible for disturbed repopulation of lymphoid tissues and diminished antigen presenting function in dogs. In order to testify the hypothesis that CDV-infection leads to an impairment of co-stimulatory functions of professional antigen presenting cells, canine DCs have been generated from peripheral blood monocytes in vitro and infected with CDV. Virus infection was confirmed and quantified by transmission electron microscopy, CDV-specific immunofluorescence and virus titration. Phenotypical changes of cultured cells were determined by flow cytometry. In addition, apoptotic changes and cellular damage were quantified by the terminal desoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling-method and lactate dehydrogenase assay, while cell proliferation was determined by 5-bromo-2'-deoxyuridine-incorporation. Results demonstrated a significant time dependant increase of the infectivity rate of DCs at 24, 72 and 120 hours post infection (hpi). As observed by flow cytometry at 120 hpi, CDV-infection of canine DCs led to a down-regulation of co-stimulatory molecules CD80 and CD86 as well as of the major histocompatibility complex class II, indicative of disturbed antigen presenting properties. As a potential mechanism to evade host immune responses, infected DCs showed no evidence of apoptosis or cell lysis at 24, 72 and 120 hpi. Similarly, cell proliferation rate of infected cells was unaffected at any investigated time points. These data suggest that CDV-infection of DCs plays a role for pathogenesis of long lasting immune alterations and virus persistence in canine distemper.
1. Introduction

Canine distemper virus (CDV), a morbillivirus, causes persistent infection of peripheral lymphoid organs and the central nervous system of carnivores (Krakowka 1982; Beineke et al. 2009). Similar to measles virus (MV) infection of human beings clinical findings in CDV-infected dogs include fever, rash, respiratory signs, and lymphopenia. Affected dogs show an increased susceptibility to opportunistic infections as a consequence of generalized lymphoid depletion and profound immunosuppression, respectively (von Messling et al. 2006; Beineke et al. 2009; Sellin et al. 2009). Several pathogens, including human herpesvirus type 1 as well as human and feline immunodeficiency viruses, target DCs and have evolved strategies to modulate their cytokine expression and antigen presenting capacity, thereby promoting virus immune evasion and persistence (Steinman and Banchereau, 2007; Tompkins and Tompkins 2008). Additionally, the interaction between different viruses and DCs causes an alteration of endocytosis, vesicle trafficking and immunological synapse formation, and apoptosis induction of DCs (Klagge and Schneider-Schaulies 1999; Rinaldo and Piazza 2004; Harman et al. 2006; Schneider-Schaulies and Dittmer 2006; Cunningham et al. 2010). Immunosuppressive effects of MV is supposed to be a consequence of disturbed function of antigen presenting cells, including DCs (Oldstone et al. 1999; Hahm et al. 2005; Trifilo et al. 2006; Zilliox et al. 2006). Moreover, following infection of the respiratory tract, DCs might mediate viral transport to secondary lymphoid organs in measles patients (Steinman 2007; Abt et al. 2009). MV-infection of DCs has been demonstrated in vitro and in animal experiments using cotton rats, transgenic mice and macaques. However, until now, DC infection by MV has not been confirmed in human beings in vivo (Hahm et al. 2005; de Swart et al. 2007; Griffin 2010).

During the chronic disease stage of canine distemper, DCs seem to serve as the primary host cells for the virus, which might promote viral persistence in lymphoid organs (Wünschmann et al. 2000). This process is assumed to inhibit terminal differentiation of DCs, responsible for disturbed repopulation of lymphoid tissues and
diminished antigen presenting function in dogs, as suggested for MV-infection (Kerdiles et al. 2006; Schneider-Schaulies and Dittmer 2006). In addition, CDV-infection of thymic epithelial DCs may result in compromised maturation of T cells, promoting the release of immature, potentially autoreactive lymphocytes, demonstrating a potential central role of DCs in CDV-induced immunosuppression and immunopathology (Wünschmann et al. 2000). However, until now, the effect of CDV upon the function of canine DCs has not been investigated. Thus, the aim of the present study was to investigate the susceptibility of canine DCs for CDV and to testify the hypothesis that CDV-infection leads to an impairment of co-stimulatory functions of canine DCs in vitro.

2. Materials and Methods

2.1 Generation of monocyte derived dendritic cells

Monocyte dendritic cells (moDCs) were generated as previously described, with minor modifications (Yoshida et al. 2003; Ibisch et al. 2005; Bonnefont-Rebeix et al. 2006). Briefly, 20ml of fresh heparinized blood was taken from clinically healthy dogs (n=16). Peripheral blood mononuclear cells (PBMC) were obtained by density gradient centrifugation (500xg) using Histopaque®-1077 (Sigma-Aldrich, Germany) at room temperature for 30 minutes. PBMC were carefully collected from the interface, washed twice with phosphate buffered saline (PBS) + 0.02% EDTA, counted and adjusted to a concentration of 2x10^6 cells/ml, seeded in RPMI 1640 medium (PAA, Austria) supplemented with 100UI/ml penicillin, 100µg/ml streptomycin and 10% fetal calf serum (FCS) and incubated for 24h at 37°C and 5%CO₂. Subsequently, non-adherent cells were removed by gentle washing with PBS and adherent cells were incubated under standard conditions for 6 more days, supplemented with 10.6µg/ml recombinant human (rh) GM-CSF (RND Systems, MN, USA) and 20µg/ml recombinant canine (rc) IL-4 (RND Systems, MN, USA). Fresh medium was added every third day. During medium
change, non-adherent cells were collected, centrifuged, and supernatant (SNT) was discarded. Diluted cell pellet was transferred back to the culture flask.

2.2 **Immuophenotyping of dendritic cells**

Antibodies used for phenotypic analyses were either cell surface markers specific for dog or cross-reacting with canine antigens. Monoclonal antibodies CD1a, CD11c, CD14, CD80, CD86, and major histocompatibility complex (MHC) II were used for labeling monocytes and DCs, respectively, while CD3- and CD21-specific antibodies were used for labeling T cells and B cells, respectively. IgG1 and IgG2a were used as isotype controls. All antibodies used for phenotypic analysis are listed in table 1. Monocytes and DCs were stained as already described (Stein et al. 2004). Briefly, after the cultivation period cells were collected, centrifuged (250xg, 4°C, 10 minutes) and washed. Non-specific antibody binding was blocked by pretreatment of cells with 10 mg/ml human normal IgG (Globuman Berna, Switzerland). Primary antibodies and isotype controls were incubated for 30 minutes at 4°C and afterwards centrifuged (250xg, 20°C, 10 minutes) and washed twice with cell wash solution (BD Dickinson, Germany). Cells labeled with conjugated markers (CD14, CD21 and IgG2a) were resuspended in FACS flow solution (BD Dickinson, Germany) and stored at 4°C until use. Non-conjugated primary antibodies were incubated with the secondary antibody (goat anti-mouse pycoerythrin; Jackson ImmunoResearch, Dianova, Germany) for 30 minutes at 4°C, followed by centrifugation (250xg, 20°C, 10 minutes) and two washing steps. Subsequently, cells were resuspended in FACS flow solution and analyzed immediately. Cells were gated using forward scatter height (FSC-H) and side scatter height (SSC-H) not exceeding 2% positive staining with serotypes. Phenotyping of cells was performed using the FACSCalibur flow cytometer (Becton Dickinson, CA, USA) and data were analyzed with FlowJo software (Tree star, OR, USA). Monocytes were analyzed at day one in culture after the cell adherence and DCs were analyzed at day seven in culture.
**Table 1.** List of primary and secondary antibodies and isotype controls used for immunophenotyping of dendritic cells.

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Target cells</th>
<th>Producer/clone no.</th>
<th>Dilution</th>
<th>Secondary antibodies</th>
<th>Isotype control</th>
</tr>
</thead>
<tbody>
<tr>
<td>MHC II</td>
<td>APC</td>
<td>P.F. Moore, (University of Davis, CA, USA) / (CA2.1C12)</td>
<td>1:6</td>
<td>GaM PE</td>
<td>IgG1</td>
</tr>
<tr>
<td>CD11c</td>
<td>APC</td>
<td>Abd Serotec (Oxford, UK) / (CA11.6A1)</td>
<td>1:6</td>
<td>GaM PE</td>
<td>IgG1</td>
</tr>
<tr>
<td>B7-1 (CD80)</td>
<td>APC</td>
<td>P.F. Moore, (University of Davis, CA, USA) / (CA24.5D4)</td>
<td>1:6</td>
<td>GaM PE</td>
<td>IgG1</td>
</tr>
<tr>
<td>B7-2 (CD86)</td>
<td>APC</td>
<td>P.F. Moore, (University of Davis, CA, USA) / (CA24.3E4)</td>
<td>1:6</td>
<td>GaM PE</td>
<td>IgG1</td>
</tr>
<tr>
<td>CD21 PE</td>
<td>B cells</td>
<td>Abd Serotec (UK) / (CA2.1D6)</td>
<td>1:6</td>
<td>-</td>
<td>IgG1</td>
</tr>
<tr>
<td>CD3</td>
<td>T cells</td>
<td>Abd Serotec (UK) / (MCA17.74)</td>
<td>1:50</td>
<td>GaM PE</td>
<td>IgG1</td>
</tr>
<tr>
<td>CD1a</td>
<td>APC</td>
<td>Abcam (UK) / (NA1/34-HLK)</td>
<td>1:50</td>
<td>GaM PE</td>
<td>IgG2a</td>
</tr>
<tr>
<td>CD14 PE</td>
<td>Monocytes, dendritic cells</td>
<td>Abcam (UK) / (TÜK4)</td>
<td>1:6</td>
<td>-</td>
<td>IgG2a</td>
</tr>
<tr>
<td>IgG1</td>
<td>-</td>
<td>Abd Serotec (UK) / (MCA928)</td>
<td>1:6</td>
<td>GaM PE</td>
<td>-</td>
</tr>
<tr>
<td>IgG2a PE</td>
<td>-</td>
<td>Souther Biotech (AL, USA) (1080-09)</td>
<td>1:6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>GaM PE</td>
<td></td>
<td>Jackson ImmunoResearch, Dianova (Germany)</td>
<td>1:100</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

APC = antigen presenting cells; GaM-PE = goat anti-mouse pycoerythrin

2.3 **Canine distemper virus infection of monocyte derived dendritic cells**

moDCs were harvested after an incubation period of seven days for the infection experiment. Cells were centrifuged and washed in RPMI 1640 medium (FCS free), seeded at the density of $0.25 \times 10^4$ cells/ml in 6-well plates (Nunc™, Sigma-Aldrich, Germany) and infected with the CDV strain R252 at multiplicity of infection (MOI) 0.1.
After an incubation period of 2 hours under standard conditions (37°C, 5% CO₂), cells were centrifuged and washed twice with PBS to remove unbound virus. Subsequently, fresh conditioned medium containing cytokines (rhGM-CSF, rcIL-4) and 10% FCS was added to the culture. Medium was changed every third day as described above. Infectivity rate of the cells was investigated at 24, 72 and 120 hours post infection (hpi).

2.4 Immunofluorescence

For the detection of infected cells and quantification of the infectivity rate, respectively, cells were labeled using a monoclonal mouse anti-CDV-specific antibody (D110; dilution 1:200; A. Zurbriggen, Switzerland). Briefly, cells were transferred to a glass slide by cytopsin centrifugation (250xg, 5 minutes). After fixation with paraformaldehyde 4% for 30 minutes at room temperature, cells were washed with phosphate buffered saline Triton X (PBST). Non-specific blocking was performed with goat and horse serum (5% each) for 20 minutes. Subsequently, cells were incubated with the primary antibody (dilution 1:100) for 4 hours at room temperature, followed by incubation with the secondary antibody (goat anti-mouse Cy3; 1:100; Jackson, ImmunoResearch, Dianova, Germany) for 1 hour at room temperature in a dark chamber. For counterstaining cells were incubated with bisbenzimidine (1:100; Sigma-Aldrich, Germany) for 15 minutes at room temperature. The percentages of CDV-infected cells were determined at 24, 72 and 120 hpi in duplicates (n=4 for each time point) by immunofluorescence microscopy (Olympus IX-70, Olympus Life Science Europe GmbH, Germany).

2.5 Virus titration

At 24, 72 and 120 hpi, the SNT was harvested to calculate the 50% log₁₀ tissue culture infectious dose/ml (TCID₅₀/ml) of cell free SNT of CDV-infected cells. Briefly, SNT was centrifuged at 300xg at 4°C, aliquoted and stored at -80°C until used. SNT was diluted logarithmically from 10⁰ to 10⁻⁸ in RPMI 1640 medium containing 10% FCS and titrated
in 96-well microtiter plates (Nunc™, Sigma-Aldrich, Germany) containing Vero.dogSLAM cells (1.5x10^4 cells/well). After an incubation period of five days under standard conditions cells were examined and evaluated for presence of cytopathogenic effects. The TCID_{50} was calculated as described (Frisk et al. 1999; Techangamsuwan et al. 2009). All samples were evaluated in triplicates (n=5; for each time point).

2.6 Proliferation assay

The proliferation rate was determined in control samples (non-infected DCs) and CDV-infected DCs at 24, 72 and 120hpi in duplicates by the 5-bromo-2'-deoxyuridine (BrdU)-assay (In Situ Proliferation Kit, FLUOS, Roche, Switzerland) following the manufacturer’s instruction. Briefly, infected and non-infected cells were incubated with 10μM BrdU for 12 hours (37°C, 5%CO_2). After centrifugation (200xg, 5 minutes) cells were washed and fixated for 30 minutes (4°C) with ethanol fixative (3 volumes of glycine solution [50mM, pH 2.0] with 7 volumes of ethanol). Subsequently, cells were denaturated with 4M HCl for 20 minutes at room temperature. Denaturation was stopped by adding 2ml PBS and centrifugation (300xg, 10 minutes). Non-specific antibody binding was blocked with goat and horse serum (5% each) for 20 minutes and incubation with 50μl anti-BrdU-FLUOS-antibody for 45 minutes at room temperature. Cells were counterstained by bisbenzimidine (1:100, Sigma-Aldrich, Germany) for 15 minutes at room temperature. For the detection and quantification of proliferating cells, ten randomly chosen areas (40x magnification; n=4 for each time point) were evaluated by immunofluorescence microscopy (Olympus IX-70; Olympus Life Science Europe GmbH, Germany).

2.7 Detection and quantification of apoptosis

Apoptotic cells were identified by the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL)-method (ApopTag® Plus Peroxidase In Situ
Apoptosis Detection Kit, Chemicon, MA, USA) according to the manufacturer's instructions (Urhausen et al. 2011). Briefly, infected and non-infected DCs were transferred to glass slides by cytospin centrifugation (250xg, 5 minutes). Cells were fixated with 1% paraformaldehyde for 10 minutes at room temperature and post-fixated with ice cold (-20°C) ethanol:acetic acid (2:1) for 5 minutes. Subsequently, endogenous peroxidase was quenched with 3% H$_2$O$_2$ for 5 minutes followed by 2 washing steps in PBS. After short incubation (10 seconds) in washing equilibration buffer, the terminal desoxynucleotidyl transferase (TdT) enzyme (working strength TdT enzyme) was added and samples were incubated in a humidified chamber for 1 hour. Reaction was stopped by transferring the slides into working strength stop/wash buffer and three subsequent washing steps. Afterwards, anti-dioxigenin conjugate was applied and slides were incubated at room temperature for 30 minutes. Following cytospin slides were washed four times in PBS and incubated at room temperature in diaminobenzidine-tetrahydrochloride peroxidase substrate for 5 minutes. Mayer's hematoxylin was used for nuclear counterstaining following dehydration in a series of graded alcohols. Slides were mounted under coverslip and ten randomly chosen areas (40x magnification; n=4 for each time point) were investigated by light microscopy. Mouse spleen tissue served as a positive control, while DC cytospin slides that lacked TdT enzyme was used as negative controls.

2.8 Lactate dehydrogenase assay

For detecting cell lysis, a lactate dehydrogenase assay (LDH; CytoTox 96® Non-radioactive Cytotoxicity Assay assay Promega, WI, USA) was performed according to manufacturer`s instructions. Briefly, after centrifugation of cells, SNT from non-infected and CDV-infected cells at 24, 72, and 120 hpi were carefully collected and stored at -80°C until further use. 50μl of SNT and 50μl of substrate mix were added to a 96-well microtiter plate, and incubated for 30 minutes. Reaction was stopped with 1M acetic acid (stop solution) and absorbance was measured at 490nm using an ELISA reader.
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(Fluorostat Optima, BMG LABTECH, Germany). Data (n=4 for each time point) were analyzed using the Optima data analysis software (BMG LABTECH, Germany).

2.9 Transmission electron microscopy

Isolated monocytes at day one and generated DCs at day seven in culture were centrifuged (250xg, 4°C, 10 minutes) and collected in 1.5ml tubes, while non-infected and CDV-infected DCs at 24, 72, and 120hpi in 6-well plates (Nunc™, Sigma-Aldrich, Germany) were centrifuged (250xg, 4°C, 10 minutes). Subsequently cells were fixated with 2.5% glutaraldehyde and incubated overnight at 4°C. Post-fixation was performed in 1% aqueous osmium tetroxide and after 5 washes in cacodylate buffer (5 minutes each) samples were dehydrated through series of graded alcohols and embedded in Epon 812 medium. Semi-thin sections were cut at microtome (Ultracut Reichert-Jung, Leica Microsystems, Germany) and stained with uranyl citrate for 15 minutes. After eight washing steps samples were incubated with lead citrate for 7 minutes. Ultra-thin sections were cut with a diamond knife (Diatome, PA, USA) and transferred to copper grids. Samples were examined by a transmission electron microscope (EM 10C, Zeiss, Germany).

2.10 Statistical analyses

To determine the distribution of data a Shapiro-Wilk test and visualization assessment were performed. For not normally distributed values (phenotypic analyses, LDH assay) a non-parametric test (Mann-Whitney U-test) was used. For normally distributed data (evaluation of semi-thin sections, infectivity rate, BrdU-assay, TUNEL-assay) a student’s T-test was performed. For statistical analyses and visualization of data the SPSS software (IBM, NY, USA) was used.
1 Results

3.1 Generation and characterization of monocyte derived dendritic cells in culture

3.1.1. Morphological characterization

After seven days in culture in the presence of rcIL-4 and rhGM-CSF, isolated PBMC showed a typical DC-like morphology with long cytoplasmic processes as demonstrated by phase contrast microscopy (Fig.1A). In agreement with this, counting of different cell types on semi-thin sections at day one in culture revealed that the majority of cells represented monocytes (p=0.001) and only few lymphocytes and neutrophils were detected (Fig.2A). In contrast, at day seven in culture the majority of generated cells showed a DC-like morphology (Fig.1B) with long cytoplasmic processes, while the amount of monocyte-like cells was comparatively low (p=0.001; Fig.2B). Comparing day one and day seven a significant decrease of monocytes (p=0.001) and lymphocytes (p=0.031) was observed over time (data not shown). At day one in culture individual cells were identified as neutrophils, while no neutrophils were observed at day seven. DC differentiation in vitro was confirmed by transmission electron microscopy which revealed a typical DC-like morphology, including long cytoplasmic processes, abundant golgi apparatus formation, only few lysosomes and the presence of periodical microstructures as a distinct ultrastructural feature of canine monocyte derived DCs (Fig.3).

3.1.2. Flow cytometry

Phenotypical analyses of PBMC (day one) and moDCs (day seven) were performed by flow cytometry (Fig.4). The percentage of gated cells was determined to characterize culture purity and the geometrical mean fluorescent intensity (GMFI) for the quantification of surface marker expression of monocytes and DCs, respectively. The majority of cultured cells at day one and day seven expressed CD14 and CD11c, indicative of monocytic origin. Moreover, CD3+ T cells showed a significant decrease at day seven compared to day one in culture (p=0.01). An increased percentage of cells
expressing CD86 at day seven compared to day one in culture was noticed (p=0.03), while no statistical differences were found for CD1a, CD11c, CD14, CD21, CD80 and MHC II (Fig.5). Analysis of GMFI revealed a significant up-regulation of the co-stimulatory molecules CD80 (p=0.012) and CD86 (p=0.018) as well as an increased surface expression of CD1a (p=0.001) and CD14 (p=0.001) of cells at day seven in culture compared to cells at day one in culture, indicative of DC differentiation (Fig.6,7).

Figure 1. Generated canine dendritic cells. A) Phase contrast microscopy B) Light microscopy. Bar sizes = 20μm.

Figure 2. Morphological characterization of cells in culture at day one and seven. A) At day one the majority of cells showed monocyte morphology, while only few cells represented lymphocytes and neutrophils. No dendritic cells were observed at this day B) The majority of cells showed a characteristic DC morphology, while only a small percentage of cells represented monocytes. □□ = significant increase (p≤0.05) compared to all other cell types; ▲ = significant increase (p≤0.05) compared to lymphocytes and neutrophils. Box and whisker plots display median and quartiles with maximum and minimum values.
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Figure 3. Periodic microstructure in a monocyte derived dendritic cell. Wasp nest-like structure representing a periodical microstructure (arrowhead) in the cytoplasm as a distinct ultrastructural marker for canine monocyte derived dendritic cells. Magnification 25,000x.

Figure 4. Flow cytometric analyses of canine monocytes and dendritic cells in culture. A) Flow cytometric analysis of cultured cells: dot plots showing gated monocytes at day one and B) dendritic cells at day seven in culture. FSH-H = forward scatter height; SSC-H = side scatter height

3.2 Canine distemper virus infection of monocyte derived dendritic cells

3.2.1. Virus detection by immunofluorescence and transmission electron microscopy

In order to determine the ability of CDV to infect canine DCs and to quantify the infectivity rate, respectively, moDCs were infected at seven days in culture. Infections
were stopped at 24, 72 and 120hpi and cell cultures were investigated by immunofluorescence (Fig.8A). CDV-infected cells were detected as early as 24 hours post infection. A significantly higher infectivity rate was found at 72 (p=0.03) and 120hpi (0.023) compared to 24hpi, indicative of a time dependant increase (Fig.9). CDV infection was confirmed by electron microscopy, which revealed the presence of virus nucleocapsid in DCs (Fig.8B).

Figure 5. Phenotypical analyses by flow cytometry of cells at day one and day seven in culture. Percentage of cells expressing A) CD1a, B) CD3, C) CD11c, D) CD14, E) CD21, F) CD80, G) CD86, and H) MHC II at day one and seven. Significant differences (p≤0.05) between time points are labeled with asterisks. Box and whisker plots display median and quartiles with maximum and minimum values.
Figure 6. Expression of different markers measured by flow cytometry in monocytes and generated dendritic cells. During the period of culture, monocytes at day one showed a significantly lower levels of co-stimulatory molecules, CD80 and CD86, and CD1a. MHC II- and CD11c-expression exhibited no changes. CD14 was higher expressed in dendritic cells at day seven compared to monocytes at day one. Significant differences ($p \leq 0.05$) between time points are labeled with asterisks.

Figure 7. Histograms of monocytes and dendritic cells in culture. A) Increased expression of CD80 in dendritic cells at seven days. B) Increased expression of CD86 in dendritic cells at day seven. C) Increased expression of CD1a in dendritic cells at seven days in culture. Filled tinted curve = isotype control; thin line = monocytes at day one; thick black line = dendritic cells at seven days in culture.
Figure 8. Detection of canine distemper virus (CDV) in dendritic cells by immunofluorescence and transmission electron microscopy. A) Infected dendritic cell (DC) at 72 hour post infection labeled with a CDV-specific antibody (red color). Nuclear staining with bisbenzimidine (blue color); Bar size = 20μm. B) Transmission electron microscopy showing CDV-infected dendritic cell at 120 hours post infection. Note accumulation of viral nucleocapsid in the cytoplasm (arrow) and DC-specific periodical microstructure (arrowhead). Magnification 50,000x.

Figure 9. Phenotypical analyses by flow cytometry of non-infected and canine distemper virus (CDV)-infected dendritic cells at 120 hours post infection. Significantly decreased expression of A) CD80, B) CD86, and C) MHC II in CDV-infected dendritic cells compared to controls. Box and whisker plots display median and quartiles with maximum and minimum values. * = significant differences (p≤0.05).
3.2.2  Virus titration

The amount of cell free virus in the SNT was determined by virus titration. All samples at 24, 72 and 120hpi were titrated in quadruplicates using the Vero.dogSLAM cells and the TCID_{50}/ml was calculated. Replicating virus was detected throughout the observation period, although no statistical difference of virus titer among the investigated time points was found probably attributed to a limited virus release of infected cells.

3.2.3.  Flow cytometry

The impact of CDV upon phenotypical properties of canine DCs was evaluated at 120hpi by flow cytometry (Fig.9). Flow cytometric analysis revealed a significant down-regulation of the co-stimulatory molecules CD80 (p=0.018) and CD86 (p=0.036) as well as of MHC II (p=0.029) of CDV-infected DCs compared to non-infected DCs (Fig.10).

![Histograms](image)

**Figure 10. Histograms of non-infected and canine distemper virus-infected dendritic cells at 120 hours post infection.** Decreased expression of A) CD80, B) CD86 and C) MHC II in CDV-infected dendritic cells compared to non-infected cells. Filled tinted curve = isotypetype control; thin line = non-infected dendritic cells; thick black line = CDV-infected dendritic cells.
3.2.4 Quantification of cell proliferation, apoptosis and lysis

To determine the possible effect of CDV upon cell division the proliferation rate of infected and non-infected DCs was measured by BrdU-incorporation. Although proliferating cells (BrdU⁺ cells) were present at 24, 72 and 120hpi, no statistical differences of proliferation rates were found between non-infected and CDV-infected DCs at any time point.

TUNEL-assay was performed to detect apoptotic changes at 24, 72 and 120hpi. Strikingly, similar apoptotic rates of infected DCs and non-infected cells were observed (data not shown). The absence of an overt apoptosis induction and cell damage following infection was confirmed by LDH-assay, which revealed no statistical differences between infected and non-infected cells at any time point (data not shown).

4 Discussion

Results of the present study demonstrate the ability of CDV to infect canine DCs and to modulate their antigen presenting properties, which has the potential to influence host innate and adaptive immune responses. Many viruses have developed strategies to alter antigen presentation or allostimulatory properties of DCs in order to evade immune responses, as described for influenza virus, HIV, and herpes simplex virus (Mikloska et al. 2001; Rinaldo and Piazza, 2004; Granucci, 2005). Furthermore, infection of DCs is supposed to account for long lasting and profound immunosuppression in measles patients (Fugier-Vivier et al. 1997; Murabayashi et al. 2002; Ohgimoto et al. 2007). Similarly, CDV-mediated modulation of DC function might represent a mechanism to suppress protective immunity, which favors persistent infection in infected dogs. In agreement with this, Wünschmann et al. (2000) reported the occurrence of CDV-infected DC-like cells within lymphoid organs in advanced stages of canine distemper. Based on these observations in vivo and the present results in vitro it is hypothesized that infection of DCs represents a mechanism of viral persistence in peripheral organs.
similar to observations in neurons and glial cells in canine distemper (Nesseler et al. 1997; Zurbriggen et al. 1998; Nesseler et al. 1999; Wünschmann et al. 2000; Beineke et al. 2009; Céspedes et al. 2010).

CDV-infection of canine moDCs leads to a down-regulation of co-stimulatory molecules (CD80 and CD86) as well as of MHC II, which might impair T cell activation in affected dogs (Björck et al. 1997). According to this, in addition to lymphopenia, remaining peripheral blood lymphocytes of dogs suffering from CDV infection show an extensively reduced mitogen-induced lymphocyte proliferation (Krakowka et al. 1975; Krakowka and Wallace, 1979). Besides reducing T cell responses, CDV inhibits IL-1 production and increases prostaglandin E₂ release, which impairs antigen presentation and subsequent B cell differentiation, plasma cell formation and immunoglobulin production in CDV-infected dogs (Krakowka et al. 1987). In addition, diminished T helper cell function as a consequence of impaired antigen presentation in persistently infected dogs might contribute also to disturbed germinal center formation and a reduced class switch from IgM to IgG (Winters et al. 1983).

Dysregulation of antigen presenting properties is supposed to account for immunosuppression in human measles (Servet-Delprat et al. 2000). Mechanisms include the down-regulation of IL-12, which leads to a failure to activate T cells by DCs (Fugier-Vivier et al. 1997; Servet-Delprat et al. 2000; Schneider-Schaulies et al. 2002). Possibly, CDV nucleoprotein acts indirectly on T cell function by modulating antigen presentation of DCs via interaction with the Fcγ receptor (CD32) and subsequent diminished IL-12 production (Schneider-Schaulies and Dittmer, 2006). Similar to the findings of the present study, MV-infection of mice causes a down-regulation of co-stimulatory molecules as well as of MHC class I and II molecules in splenic DCs (Oldstone et al. 1999; Hahm et al. 2005; Trifilo et al. 2006). However, in contrast to the present results, MV-infection of human DCs in vitro leads to an up-regulation of co-stimulatory molecules. These differences demonstrate that despite numerous similarities between the pathogeneses of human measles and canine distemper virus-
specific but also species-specific properties have to be considered for critical interpretation of data in translational research.

Human cytomegalovirus, murine cytomegalovirus and Epstein-Barr virus infect and manipulate DCs to circumvent cell death, which causes persistent infection (Rinaldo and Piazza, 2004). According to this, the observed lack of apoptosis and lysis of CDV-infected DCs represents a potential prerequisite for virus persistence in lymphoid organs in chronically infected dogs. Furthermore, DCs have a limited lifespan and defects in DC apoptosis might trigger autoimmune disease as demonstrated in transgenic FasL- and Fas-deficient mice (Cohen and Eisenberg, 1992; Kushwah and Hu, 2010). Referring to this, disturbed DC function in canine distemper is supposed to compromise maturation and selection of T cells, promoting the release of immature CD5 negative T cells, including potentially autoreactive cells (Wünschmann et al. 2000).

In conclusion, for the first time CDV infection of canine DCs has been demonstrated in vitro. Modulation of co-stimulatory molecules and reduced antigen presenting function of CDV-infected DCs might account for inhibitory effects upon the immune system of dogs. Getting insights into the interaction between viruses and DCs is fundamental to understand the pathogeneses of infectious disorders, which has implication for prevention (e.g. vaccination) and treatment strategies (Hou et al. 2007). Thus, further studies are needed to investigate the role of DCs in the pathogenesis of immunopathology and virus persistence in canine distemper.

Acknowledgment

The authors would like to thank Kerstin Schöne, Regina Carlson, Kerstin Rohn, Danuta Waschke, Bettina Buck, and Petra Grünig for their excellent technical support during the laboratory work. This study was supported by the German Research Foundation (FOR 1103, BA 815/10-2 and BE 4200/1-2).
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References:


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Chapter 6: General Discussion

Based upon the observation that immune alterations in canine distemper are a consequence of direct and indirect viral effects and thus a disturbed immunomodulation accounts for lesion development, the aims of the present study were (i) to investigate phase-dependant changes of Treg and their association with peripheral immune responses in canine distemper to testify the hypothesis that insufficient Treg numbers contribute to uncontrolled neuroinflammation and early lesion initiation in the CNS of infected dogs. Since DCs have been demonstrated to influence immune homeostasis in several infectious diseases and immunopathologies (ii) current concepts of canine DCs and their impact upon canine diseases have been reviewed. In addition, (iii) the impact of CDV upon antigen presenting function of canine DCs and the proposed role of DCs in virus persistence have been investigated in vitro.

6.1 The role of innate immunity in demyelinating disorders

Results of the present study demonstrate a lack of Treg infiltration in predemyelinating and early demyelinating lesions in CDV-infected dogs, which represents a potential cause for excessive neuroinflammation, including glial and innate immune responses, respectively. Microglia and astrocytes contribute to innate immune responses within the CNS. Together with infiltrating Natural Killer cells, granulocytes, macrophages and DCs, resident glial cells initiate responses to various stimuli and thus represent an important prerequisite for antigen-specific adaptive immune responses in neurological disorders (Gandhi et al. 2010). Similar to observations in the present survey, microglial activation can be observed in early demyelinating lesions in MS patients. However, it remains undetermined whether microglia induces primary tissue damage or have to be activated first by encephalitogenic T cells (Marik et al. 2007; Gandhi et al. 2010).

In canine distemper, microglial activation leads to bystander demyelination during the progressive disease phase (Griot et al. 1990; Botteron et al. 1992). Early signs of
activation of resident CNS cells represent the prominent up-regulation of MHC II prior to T and B cell infiltration in canine and phocine distemper (Beineke et al. 2008; Stimmer et al. 2010). Referring to this, a dominance of pro-inflammatory cytokine environment in the brain during the acute distemper phase is indicative of M1-polarization of microglia (Beineke et al. 2008; Markus et al. 2002). This hypothesis is supported by the observation of an increased expression of adhesion molecules, phagocytic activity and release of reactive oxygen species by microglia in experimentally CDV-infected dogs (Stein et al. 2004; Stein et al. 2008). In EAE and Theiler’s murine encephalomyelitis (TME), M1-type microglial cells contribute to lesion development, while M2-type cells exhibit immunomodulatory properties and attenuation of clinical signs (Mikita et al. 2011; Gerhauser et al. 2012). Thus activation of potentially neurotoxic microglial populations might represent an initiating event for subsequent immune mediated tissue damage of the cerebellar white matter in canine distemper. Similarly, a dominating role of innate immunity in acute and subacute traumatic spinal cord injury has been observed in dogs (Spitzbarth et al. 2011).

Similar to findings in canine distemper, the cerebral cortex is not primarily affected by myelin loss in TME and phocine distemper (Stimmer et al. 2010; Kummerfeld et al. 2012). The reason for region-specific differences in demyelinating disorders remains undetermined. Possible explanations for this phenomenon include topographical differences of the CNS microenvironment or functionality of glial cells, such as a reduced myelin degrading proteolytic activity of microglia as observed in C57BL/6 mice (Liuzzi et al. 1995). Functional differences of microglial cells have also been observed in different CNS regions of the dog (Stein et al. 2004; Stein et al. 2006; Stein et al. 2007; Ensinger et al. 2010). Moreover, regional differences of the vulnerability of axons might account for topographical differences of glial responses in virus-induced demyelinating disorders, such as canine distemper and TME (Tsunoda and Fujinami 2002; Seehusen and Baumgärtner 2010; Kummerfeld et al. 2012). Regional differences of myelin loss can also be observed in toxic demyelination models, such as the cuprizone model (Herder et al. 2011). While microglia and macrophages contribute to demyelination in
the corpus callosum (Biancotti et al. 2008), a delayed infiltration of CD107b+ microglial cells and CD11b-mRNA-expression can be found in the spinal cord of cuprizone treated mice (Herder et al. 2011). In contrast to the potential neurotoxic function of glial cells in canine distemper, this process might represent a reaction to toxin-induced oligodendroglial damage in the spinal cord, which potentially promotes tissue repair and remyelination (M2-type microglia; Kotter et al. 2001; Mantovani et al. 2005; Kigerl et al. 2009). In analogy to the concept of region-specific lesion development in canine distemper and human MS, recent studies have demonstrated topographical differences of de- and remyelination within the brain of cuprizone fed mice, which might be attributed to unequal densities or functions of microglia, astrocytes and oligodendocyte progenitor cells in the respective CNS areas (Skripuletz et al. 2008; Gudi et al. 2009; Skripuletz et al. 2010).

The role of microglia in neurological disorders is discussed controversially, since both detrimental and beneficial effects have been described. For instance, phagocytosis of myelin debris by microglia and macrophages represent an important prerequisite for regeneration following traumatic CNS injury (Yang and Schnaar, 2008), while excessive cytokine expression and release of reactive oxygen species by these cells enhances tissue damage (Banati and Kreutzberg, 1993; Stein et al. 2006; Ensinger et al. 2010; Stein et al. 2011). As demonstrated in canine organotypic spinal cord slice cultures and early MS lesions, an activation of microglia by phagocytosis of myelin debris leads to a pro-inflammatory and potentially neurotoxic M1-phenotype of microglia (Pinteaux-Jones et al. 2008; Spitzbarth et al. 2011). This process is supposed to be initiated by the interaction between TLR on glial cells and the released cellular compounds (damage associated molecular patterns) as a consequence of tissue damage (Kigerl et al. 2007; Kigerl and Popovich, 2009).

Besides their function in innate immunity, microglia, macrophages, and DCs play a pivotal role in the induction of specific immune responses in peripheral lymphoid organs. In TME an early migration of CD68+-antigen presenting cells from the CNS to the
cervical lymph node can be observed (Navarrete-Talloni et al. 2010). Subsequently, activated peripheral lymphocytes infiltrate the CNS and get reactivated by APCs in the CNS (McMahon et al. 2005). In agreement with this, prominent MHC II expression in early virus-induced lesions might account for antigen presenting capacity of glial cells in canine and phocine distemper (Beineke et al. 2008; Stimmer et al. 2010). However, in contrast to non-lymphotropic viruses (e.g. Theiler’s murine encephalomyelitis virus (TMEV)), pathogen-specific immune responses in peripheral lymphoid organs are not induced in MS and TME, probably as a consequence of massive lymphoid depletion as observed in the present study.

6.2 Role of regulatory T cells in neurological diseases

Treg play a key role in controlling immune responses under physiological conditions and in various systemic and CNS inflammatory diseases (Sakaguchi et al. 2006; Liesz et al. 2009). This has been demonstrated by depletion of natural Treg which leads to an activation of self-reactive T cell clones, inducing severe and widespread autoimmune inflammatory diseases (Sakaguchi et al. 2006). Moreover, functional impairment or an insufficient amount of Treg is suspected to contribute to demyelination in acute MS lesions (Fritzscheing et al. 2011). Impaired Treg function could lead to disturbed tolerance against autoantigens, resulting in autoimmunity (Viglietta et al. 2004; Baecher-Allan and Hafler, 2006; Zozulya and Wiendl, 2008). These studies demonstrate that manipulation of Treg in the periphery influences the outcome of CNS infection.

Well orchestrated immune responses against invading pathogens is essential within the CNS since also minor damage can have significant consequences for neurological functions (Lowther and Hafler, 2012). For instance, Treg reduce antiviral immunity in experimental Theiler’s murine encephalomyelitis (TME; Herder et al. 2012; Richards et al. 2012). Treg protect the CNS against an overshooting immune response, as demonstrated in EAE as well as in animal models of stroke and HIV-associated neurodegeneration. Besides dampening leukocyte responses, Treg are also able to
reduce astrogliosis and microglia-mediated inflammation (Beyersdorf et al. 2005; Liesz et al. 2009; Liu et al. 2009). For example, the absence of Treg in mouse models of stroke leads to an increased severity of tissue damage due to an excessive activation of microglia and expression of TNF and IFN-γ (Liesz et al. 2009). In mouse models of coronavirus-induced acute encephalitis, Treg help to limit immune mediated tissue damage during the acute stage, demonstrating a protective role of Treg (Anghelina et al. 2009). Moreover, during acute herpes simplex virus (HSV) infection of mice, Treg facilitate early immune responses to local viral infection by allowing a timely entry of immune cells into infected tissues, including the brain (Lund et al. 2008). In contrast, a lack of Treg in HSV-infected mice is associated with delayed infiltration of DCs and T cells, that accelerates fatal infection and increases the viral load in the CNS (Lund et al. 2008). However, although the generation of Treg in CNS inflammatory diseases is regarded as physiological process to prevent immunopathology (MacDonald et al. 2002; Sakaguchi 2003; Dittmer et al. 2004), Treg can also have detrimental effects for the host by their suppressive effects upon protective immune responses which leads to an incomplete elimination of pathogens and persistent infections, respectively (Dittmer et al. 2004; Belkaid and Rouse, 2005). For instance, in experimental MV-infection of mice, an elevated number of Treg increases viral replication in the brain, whereas transient depletion of Treg significantly reduces the number of infected neurons (Reuter et al. 2012).

In the present study, permanent depletion of Treg in the spleen of all disease stages of canine distemper has been observed, which causes a delayed infiltration of Treg infiltration in the brain in advanced stages of canine distemper. The origin of CNS-infiltrating Treg in the chronic stage of canine distemper remains unclear. It remains to determine whether Treg accumulation in the brain of investigated dogs represents a cumulative effect due to migration of residual peripheral Treg or a de novo generation of Treg within the inflamed CNS. In HIV encephalitis mouse models, Treg migrate across the blood brain barrier and are retained within neuroinflammatory sites (Gong et al. 2011). The lack of Treg observed in early stages of CDV-induced leukoencephalitis
potentially contributes to uncontrolled inflammatory responses by resident cells. Similarly, an insufficient expansion of peripheral Treg might lead to strong immunopathology in acute severe dengue fever and West Nile virus infection (Lühn et al. 2007; Lanteri et al. 2009). Interestingly, the increase of Foxp3+ Treg in the CNS during advanced inflammatory stages coincides with persistent infection, similar to findings in experimental MV infection of mice (Sellin et al. 2009; Reuter et al. 2012). Probably a compartmentalization of immune responses in the CNS as described in MS (Massacesi 2002; Meinl et al. 2008) might induce the proliferation and expansion of Foxp3+ cells locally within the brain of CDV-infected animals. In EAE, large numbers of Treg are present in the inflamed brain, but their function is impaired by the elevated expression of pro-inflammatory molecules, such as IL-6 and TNF, which counters Treg immunosuppressive effects (Pasare and Medzhitov, 2003; Korn et al. 2007). Results of the present study are indicative of inadequate immunoregulation which represents a potential prerequisite for early lesion development. However, further studies are needed to determine disease phase-specific and probably ambiguous functions of Treg in the pathogenesis of canine distemper, which might also have relevance for the understanding of human demyelinating diseases.

6.3 Role of dendritic cells in demyelinating disorders

DCs play a central role for the initiation and maintenance of both innate and adaptive immune responses to infectious agents, including bacteria and viruses (Granucci et al. 2005; Hou et al. 2007). They are distributed in the skin, mucosa, and in lymphoid organs, where they efficiently take up and process diverse microbial antigens and present them as MHC–peptide complexes to T cells (Hou et al. 2007). Despite their importance for immune homeostasis in peripheral organs, DCs have been assumed not to be important for neuroinflammation because of their absence in the CNS parenchyma (Hart and Fabre, 1981; Deshpande et al. 2007). However this view has been challenged by other studies which have shown the presence of DCs in meninges and
choroid plexus of healthy rodents, and their ability to activate naïve T cells that enter the inflamed CNS (McMenamin 1999; McMahon et al. 2005). DCs are the primary APCs directing T cell functions and therefore are extremely important in directing the immune pathology characteristic of MS (Gandhi et al. 2010). Several studies have described an accumulation of DCs in CNS during inflammation in MS and EAE pathogenesis (Bailey et al. 2007; Cudrici et al. 2007; Wu and Laufer, 2007). In EAE, DCs primed with myelin antigens in the periphery activate autoaggressive T cells and can lead to an overt autoimmune disease (Dittel et al. 1999). DCs have been shown to be functionally abnormal in MS (Wu and Laufer, 2007). For instance, DCs of MS patients exhibit an activated phenotype with increased expression of activation markers, such as CD40 and CD80, and promote a pro-inflammatory environment (Huang et al. 1999; Karni et al. 2006). Moreover, DCs seem to affect the disease course of MS, since secondary progressive MS patients show higher DC expression levels of CD80, IL-12 and TNF compared to patients with relapsing-remitting MS (Karni et al. 2006). In MS, expression of CD40 by DCs was found to be related to the increased production of IL-12 and IL-18 (Balashov et al. 1997; Karni et al. 2002). Furthermore, co-stimulatory molecules, CD80 and CD86, do not promote identical signals where expression of CD80 in DCs promotes Th1 response and is involved in the exacerbation of EAE, and is reported to correlate with disease duration, while CD86 elicits Th2 humoral response (Freeman et al. 1995; Kuchroo et al. 1995; Kouwenhoven et al. 2001). With progression of EAE, DCs tend to show a down-regulation of antigen presentation properties, characterized by reduced expression of MHC II and co-stimulatory molecules (Deshpande et al. 2007). Although, DCs in MS patients and healthy controls show no differences in expression of IL-12, an important inducer of IFN-γ and Th1 responses, respectively (Huang et al. 2001), several studies reported a down-regulation of co-stimulatory molecules of plasmacytoid DCs and moDCs of MS patients (Huang et al. 2001; Stasiolek et al. 2006).

In TME, infection rate of DCs varies between resistant and susceptible mice strains. Altered DC function is supposed to influence antiviral immunity which causes persistent infection and chronic demyelinating disease (Hou et al. 2007). In susceptible mice,
TMEV preferentially infects immature DCs, blocking their differentiation and activation, while failing to induce apoptosis. TMEV-infected DCs may also contribute to disease pathogenesis by altering the cytokine profile by T cells (Hou et al. 2007).

In the present study for the first time CDV-infection of DCs has been demonstrated. Phenotypical analyses by flow cytometry revealed an impairment of antigen presentation properties with down-regulation of co-stimulatory molecules of infected moDCs. These changes might influence peripheral immune responses which potentially reduce antiviral immunity in CDV-induced leukoencephalitis. Furthermore, the absence of apoptosis induction of CDV-infected DCs might represent a mechanism of immune evasion and viral persistence, respectively.

6.4 Interaction of dendritic cells and regulatory T cells in demyelinating and chronic viral diseases

Function of DCs strongly depends on the signal, which can induce pro-inflammatory or tolerogenic properties (Finkelman et al. 1996). The ability of DCs to induce immune tolerance depends on their origin, activation state, maturation signals and the cytokine environment at the time they encounter T lymphocytes (Janikashvili et al. 2011). The tolerogenic function of DCs is mediated by T cell deletion, induction of anergy, or the expansion of antigen-specific Treg (Steinman and Nussenzweig, 2002; Luo et al. 2007; Yamazaki et al. 2007; Darrasse-Jèze et al. 2009; Janikashvili et al. 2011). The ability of Treg to survive in the periphery requires the presence of co-stimulation through the CD28 and B7 pathway (Perrin et al. 1995; Chang et al. 1999; Tada et al. 1999; Salomon et al. 2000). In EAE, DCs support Treg-mediated immunosuppression which ameliorate autoimmune responses (Deshpande et al. 2007). Furthermore, the number of Treg in vivo is directly proportional to the number of DCs (Darrasse-Jèze et al. 2009). On the other side, a decrease of Treg has been demonstrated to induce DC proliferation (Darrasse-Jèze et al. 2009). DCs are the only APC population that can mediate peripheral activation and expansion of Treg in vivo (Walker et al. 2003; Tarbell et al.
The interaction of DCs and Treg is regulated by a feedback loop which depends on MHC expression by DCs (Fig. 8) (Darrasse-Jèze et al. 2009). DC stimulation in the presence of TGF-β induces the generation of Treg from naïve CD4+ T cells (Shevach, 2002). In general, DC-T cell contact stimulates the secretion of small amounts of IL-2 by T cells, which in conjunction with MHC II independently from DC co-stimulatory signals, is sufficient for the induction and proliferation of Treg (Walker et al. 2003; Lange et al. 2007; Zou et al. 2010). Interestingly, matured DCs, although representing potent inducer of cellular immune responses, have an higher ability to trigger expansion and activation of Treg than immature DCs, as demonstrated in in vitro studies (Walker et al. 2003; Lange et al. 2007). These tolerogenic DCs are found in vivo in Peyer’s patches, lungs, and the anterior chamber of the eye. They exhibit a mature DC phenotype but secrete IL-10 and thus trigger Treg function (Lutz and Schuler, 2002).

Co-stimulatory molecules of DCs, such as CD40, CD80 and CD86, play an important role for the interaction with Treg. Here, CTLA-4 expressed on Treg impairs antigen recognition by T cells. Figure 8 illustrates the proposed homeostatic feedback loops between DCs and Treg. Modified from Darrasse-Jèze et al. (2009).

Figure 8. Proposed homeostatic feedback loops between dendritic cells (DCs) and regulatory T cells (Treg). A) Increased numbers of DCs increase the number of Treg via MHC II expression; B) Treg are able to decrease the number of DCs due to expression of interleukin (IL)-10 and transforming growth factor (TGF)-β; C) Reduction of DCs declines the amount of Treg due to decreased MHC II expression; D) Loss of Treg induces DCs by an increased availability of Fms-related tyrosine kinase 3 ligand (Flt3L).
presentation properties of DCs (Salomon et al. 2000; Godfrey et al. 2004; Fallarino et al. 2006; Janikashvili et al. 2011). For instance, Treg can suppress the up-regulation co-stimulatory molecules CD40, CD80 and CD86 and inhibit the production of pro-inflammatory cytokines of murine DCs, which leads to a reduced ability to induce T cell activation (Chaux et al. 1997). In humans it has been reported that a Treg-mediated inhibition of DC maturation is associated with a down-regulation of CD80 and CD86 without affecting CD40 expression (Serra et al. 2003). Treg-mediated suppression of DC maturation and down-regulation of co-stimulatory molecules depends on several mechanisms, such as CTLA-4 ligation as well as the induction of indoleamine 2,3-dioxygenase (IDO), lymphocyte activation gene 3 (LAG3) and neuropilin-1 (Fallarino et al. 2006; Huang et al. 2004; Paust et al. 2004; Miyara and Sakaguchi, 2007; Sarris et al. 2008; Janikashvili et al. 2011). The precise role of LAG3, which interacts with MHC II, remains unknown, since LAG3-deficient mice fail to develop enhanced autoimmune responses unlike CTLA-4-deficient mice (Huang et al. 2004; Miyara and Sakaguchi, 2007). Furthermore, Treg can modulate DC function by the production of inhibitory cytokines such as IL-10, IL-35 and TGF-β (Miyara and Sakaguchi, 2007; Vignali et al. 2009).

As demonstrated in vitro studies, CDV modulates the expression of co-stimulatory molecules and MHC II in canine moDCs. Based on previous observations (Wünschmann et al. 2000), cells with DC-like morphology in splenic germinal centers were the only cell population to be infected with CDV during chronic stage. Moreover, after lymphoid repopulation of the spleen during chronic disease stage, Foxp3⁺ cells remained depleted. These results led to the hypothesis that impaired antigen presenting properties of DCs might contribute to an insufficient function or expansion of Treg in canine distemper.
6.5 Conclusion

In conclusion, results of the present study support the hypothesis that an inadequate immunoregulation as a consequence of Treg depletion promotes early lesion CNS development in canine distemper. These findings are in agreement with previous reports that the initiation of inflammation in myelin disorders is influenced by an immunological imbalance of the peripheral immune system (Tsunoda et al., 2005; Navarrete-Talloni et al., 2010). As suggested for human MS, the development of novel therapies targeting Treg represents a promising strategy in canine neurological disorders. However, the ambiguous functions of Treg, exhibiting both beneficial and detrimental effects upon the host immune response, have to be considered in canine CNS diseases with a confirmed (e.g. canine distemper) or suspected infectious etiology (e.g. granulomatous meningoencephalitis). Since dogs are appreciated as translational animal models for several human diseases (Kling, 2007; Spitzbarth et al., 2012), observations in canine distemper might have relevance also for human demyelinating disorders.

Getting insight into the interaction between viruses and DCs is fundamental to understand the pathogenesis of infectious disorders, which has implication for prevention (e.g. vaccination) and treatment strategies (Hou, So, and Kim 2007). For the first time, CDV infection of cultured canine DCs has been demonstrated in the present survey. Modulation of co-stimulatory molecules and reduced antigen presenting function of CDV-infected DCs might account for profound and long lasting inhibitory effects upon the immune system in canine distemper. However, further studies are needed to expand the existing knowledge about the impact of infected DCs upon leukocyte activation and polarization of immune responses in canine distemper and other canine neurological disorders, as a prerequisite for the development of future therapies.
Chapter 7: Summary

Distemper in dogs is caused by the canine distemper virus (CDV). The disease course and pathogenesis of canine distemper are similar to human measles, including fever, rash, respiratory signs, lymphopenia, and profound immunosuppression with generalized depletion of lymphoid organs during the acute disease phase. Central nervous system (CNS) infection and neurological complications can be observed frequently in infected dogs. Depending on CDV strain, host immune status and age, naturally infected dogs develop demyelinating leukoencephalomyelitis, which shares similarities with human myelin disorders. Therefore canine distemper is appreciated as a translational animal model for multiple sclerosis (MS). In virus-induced neurological diseases, an ambiguous function of regulatory T cells (Treg), with both beneficial effects by reducing immunopathology and detrimental effects by inhibiting antiviral immunity, has been described. However, the role of Treg in the pathogenesis of canine distemper has not been investigated yet. Moreover, despite the demonstration of infected dendritic cell (DC)-like cells in canine distemper, the effect of CDV upon canine DCs remains undetermined.

The aim of the first part of the present study was to testify the hypothesis that peripheral lymphoid depletion influences immunomodulatory mechanisms in the brain of CDV-infected dogs. Immunohistochemistry revealed a lack of Foxp3⁺ Treg in predemyelinating and early demyelinating lesions which was associated with the accumulation of CD3⁺ T cells, L1⁺ macrophages/microglia and GFAP⁺ astrocytes. Together with CD79α⁺ B cells, a delayed infiltration of Foxp3⁺ Treg was observed in chronic demyelinating lesions. Splenic depletion of Foxp3⁺ Treg was associated with an increased mRNA-expression of tumor necrosis factor in the acute disease phase, indicative of a pro-inflammatory microenvironment and lack of immunological counter...
regulation in peripheral lymphoid organs. In conclusion, disturbed immune regulatory mechanisms represent a potential cause for excessive neuroinflammation and early lesion development in canine distemper leukoencephalitis, as discussed for immune mediated myelin disorders such as MS.

In the second part of the thesis species-specific properties and translational aspects of canine DCs have been reviewed, including the current knowledge about in vitro generation and characterization of canine DCs. In addition, the role of DCs in the pathogenesis of selected canine neoplastic, infectious (including canine distemper), allergic and autoimmune diseases, which share similarities with human disorders and thus have significance for translational medicine, have been discussed. The impact of CDV upon professional antigen presenting cells was investigated in the third part of the thesis. Similar to human measles virus, CDV is supposed to inhibit terminal differentiation of DCs, responsible for disturbed repopulation of lymphoid tissues and diminished antigen presenting function in dogs. In order to testify the hypothesis that CDV-infection leads to an impairment of co-stimulatory functions of professional antigen presenting cells, canine DCs have been generated from peripheral blood monocytes in vitro and infected with CDV. Virus infection was confirmed and quantified by transmission electron microscopy, CDV-specific immunofluorescence and virus titration. Phenotypical changes of cultured cells were determined by flow cytometry. In addition, apoptotic changes and cellular damage were quantified by the terminal desoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling-method and lactate dehydrogenase-assay, while cell proliferation was determined by 5-bromo-2'-deoxyuridine-incorporation. Results demonstrated a significant time dependant increase of the infectivity rate of DCs at 24, 72 and 120 hours post infection (hpi). As observed by flow cytometry at 120 hpi, CDV-infection of canine DCs led to a down-regulation of co-stimulatory molecules CD80 and CD86 as well as of MHC II, indicating disturbed antigen presenting properties. As a potential mechanism to evade host immune responses, infected DCs showed no evidence of apoptosis or cell lysis at 24, 72 and 120 hpi. Similarly, cell proliferation rate of infected cells was unaffected at any
investigated time points. These data suggest that CDV-infection of DCs plays a role for pathogenesis of long lasting immune alterations and virus persistence in canine distemper.

In conclusion, results of the present study support the hypothesis that an inadequate immunoregulation as a consequence of Treg depletion promotes the development of early CNS lesion in canine distemper. These findings are in agreement with previous reports, that initiation of inflammation in myelin disorders is influenced by an immunological imbalance of the peripheral immune system. As suggested for human MS, the development of novel therapies targeting Treg represents a promising strategy in canine neurological disorders. Moreover, getting insights into the interaction between viruses and DCs is fundamental to understand the pathogenesis of infectious disorders, which has implication for prevention (e.g. vaccination) and treatment strategies. Since dogs are appreciated as translational animal models for several human diseases observations in canine distemper might have relevance also for human CNS disorders.

8 Chapter 8: Zusammenfassung


Im zweiten Teil der Arbeit wurden in einem Übersichtsartikel tierartspezifische und translationale Aspekte von kaninen DZ zusammengefasst und die Rolle der Zellen in neoplastischen, infektiösen (inklusive Hund斯塔upe), allergischen und autoimmunen Krankheiten des Hundes diskutiert. Ein Schwerpunkt war hierbei, Unterschiede und Gemeinsamkeiten zwischen kaninen und humanen Krankheiten aufzuziehen.


Chapter 9: References


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Chapter 10: Acknowledgements

I would like to thank:

- First of all, my main supervisors,
- Prof. Dr. Wolfgang Baumgartner for excellent mentorship, motivation and continues support. I appreciate his guidance toward critical thinking, organizing and giving me confidence for the future. Also, I am very grateful for a friendly and cosy working environment.
- Prof. Dr. Andreas Beineke, for continues advice and supervision, lively discussions, ideas, continues motivation, and correcting the manuscripts. Especially for his availability and openmess to discuss different ideas and encouragement for my work.
- Special thanks to my co-supervisors, Prof. Dr. Andrea Tipold and Prof. Dr. Martin Stangel, for their support, constructive comments, and thoughtful suggestions.
- Yvonne Barthel and Max Iseringhausen for their collaboration and interesting discussions that contributed to the realization of joint publication.
- Ingo Spitzbarth, Vanessa Herder, Johannes Junginger, Florian Hansmann for apprehensive reading and thoughtful corrections of the final manuscript.
- Rich Roy, for his cordial help in reading and correcting my manuscript and publications.
- Unit for Reproductive Medicine, Small Animal Clinic, Prof. Dr. Anne-Rose Günzel-Apel and Dr. Carola Urhausen, for providing the blood samples and the entire staff for their positive and friendly spirit, making me feel welcome each time I needed the samples.
- Small Animal Clinic for providing us the possibility to use their laboratories. PD Dr. Veronika Stein, for her support and advices in analysing the flow cytometry data. Regina Carlson and Arianna Maiolini, PhD, for providing me a quick and efficient help for all the technical problems in the lab. Dr. Marina Hoffmann for collecting the blood samples.
- Special thanks to coordination office of ZSN for continues support.
• Danuta Waschke, Claudia Herrmann, Kerstin Schöne, Kerstin Rohn, Petra Grünig, and Bettina Buck for their excellent technical support during the execution of this project and making the work at lab way more fun.

• Dr. Karl Rohn at the Department of Biometry, Epidemiology and Information Processing at the University of Veterinary Medicine Hannover, for helpful discussions and statistical advice.

• Office mates, Ingo Spitzbarth, for unreserved help and for the hips of answered questions, ranging from German grammar to molecular analysis, Kristel Kegler for always relaxed attitude and putting it up with my music, and Oscar for being such a well behaved and friendly dog.

• All the colleagues in the institute, Armend, Kerstin, Barbara, Florian, Vannessa, Stephi, Christina, Yanyong, Andre, Wenhui, Anika, Johannes, and many others whom I see on corridors and the others who left in meantime, for their advices, help and hints for an easier life in the lab, and with whom I shared many smokes, drinks, laughs and music.

• To my small family, Lejla, for her unconditional love, and making me happy all these years.

• To my big family, my mom and dad, Olta, Vali, Laura, Greta, and Liam, for all the hugs and support they gave me.

• Young cell scheme, European Counsel and Kosovo government, Department of Pathology, Veterinary University, Hannover, Germany, for providing me with a financial support, and German Research Foundation (FOR 1103) for financing the project.

• All the people that unintentionally I have forgot to mention.