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Investigation upon the phenotypic modulation of histiocytic cells

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by
Vanessa Bono Contioso
Barcelona (Spain)

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Supervisor: Prof. Dr. med. vet. W. Baumgärtner, Ph.D.

Supervision Group: Prof. Dr. med. vet. W. Baumgärtner, Ph.D.
Prof. Dr. med. vet. L. Haas
Prof. Dr. med. vet. A. Moritz

1st Evaluation: Prof. Dr. med. vet. W. Baumgärtner, Ph.D.
Department of Pathology, University of Veterinary Medicine Hannover, Germany

Prof. Dr. med. vet. L. Haas
Institute of Virology, University of Veterinary Medicine Hannover, Germany

Prof. Dr. med. vet. A. Moritz
Small Animal Clinic, Justus-Liebig University Gießen, Germany

2nd Evaluation: Prof. Dr. Andrea Gröne, Ph. D.
Department of Pathobiology, University of Utrech, Netherlands

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For all those who care for me, no matter where they are

To my loving family and wonderful friends
«Any man could, if he were so inclined, be the sculptor of his own brain»

Santiago Ramón y Cajal
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Abbreviation list

APC: antigen presenting cells
bp: base pair
°C: degree Celsius
CD: cluster of differentiation
cDNA: complementary deoxyribonucleic acid
CDV: canine distemper virus
CDV-Ond: canine distemper virus strain Onderstepoort.
CNS: central nervous system
CO₂: carbon dioxide
COL: collagen
Cy3: indocarbocyanine green fluorochrome
DMSO: dimethyl sulfoxide
DNA: deoxyribonucleic acid
ECM: extracellular matrix
EGF: epidermal growth factor
FASL: fas ligand
FAK: focal adhesion kinase
FGF: fibroblast growth factor
GF: growth factor
HS: histiocytic sarcoma
ICAM: intercellular adhesion molecule
IGF: insulin-like growth factor
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>IL:</td>
<td>interleukin</td>
</tr>
<tr>
<td>kDa:</td>
<td>kilodalton</td>
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<tr>
<td>MAPK:</td>
<td>mitogen-activated protein kinase</td>
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<tr>
<td>mg:</td>
<td>milligram</td>
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<tr>
<td>MHC:</td>
<td>major histocompatibility complex</td>
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<tr>
<td>min:</td>
<td>minute</td>
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<tr>
<td>ml:</td>
<td>milliliter</td>
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<tr>
<td>MMP:</td>
<td>matrix metalloproteinase</td>
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<tr>
<td>mRNA:</td>
<td>messenger ribonucleic acid</td>
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<tr>
<td>MS:</td>
<td>multiple sclerosis</td>
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<tr>
<td>MT:</td>
<td>membrane-type</td>
</tr>
<tr>
<td>MV:</td>
<td>measles virus</td>
</tr>
<tr>
<td>NEAs:</td>
<td>non essential aminoacid solution</td>
</tr>
<tr>
<td>N-WASP:</td>
<td>neural Wiskott-Aldrich syndrome protein</td>
</tr>
<tr>
<td>OVs:</td>
<td>oncolytic viruses</td>
</tr>
<tr>
<td>PBS:</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR:</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PDGFR:</td>
<td>platelet-derived growth factor receptor</td>
</tr>
<tr>
<td>PKC:</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>RECK:</td>
<td>reversion-inducing-cysteine-rich protein with Kazal motifs</td>
</tr>
<tr>
<td>RNA:</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RT-PCR:</td>
<td>reverse transcription-polymerase chain reaction</td>
</tr>
<tr>
<td>RT-qPCR:</td>
<td>reverse transcription-real time quantitative PCR</td>
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</table>
**TCR:** T cell receptor

**TdT:** terminal deoxynucleotidyl transferase

**TGF-α:** transforming growth factor-alpha

**TGF-β:** transforming growth factor-beta

**TIMP:** tissue inhibitors of metalloproteinases

**TNF-α:** tumor necrosis factor alpha

**TUNEL:** TdT-mediated dUTP nick end labeling

**µl:** microliter

**µm:** micrometer

**VEGF:** vascular endothelial growth factor

**WHO:** World Health Organization
Chapter 1

Introduction
Chapter 1: Introduction

Macrophage function in health and disease

Myelomonocytic cells including macrophages, which belong to the innate immune system, are involved in various functions in health and disease (Hunter et al., 2009). The main components of the mononuclear phagocyte system are blood monocytes, tissue macrophages and dendritic cells (Chow et al., 2011). Furthermore, several other tissue specific cell types including microglia, Kupffer cells and dermal Langerhans cells exist, which have similar functions to tissue macrophages and dendritic cells (Prinz et al., 2014; Varol et al., 2015; Ju and Tacke, 2016).

Macrophages play a key role in maintaining tissue homeostasis and various different diseases ranging from autoimmune disorders to inflammation and cancer (Murray and Wynn, 2011). They are main components of inflammatory reactions, mainly dealing with phagocytosis of pathogens / foreign material, and resorption of dead or senescent cells (Linehan and Fitzgerald, 2015). Furthermore, macrophages are an important source of various cytokines and chemokines to obtain tissue homeostasis, orchestrate inflammatory reactions and influence regeneration and repair processes (Murray and Wynn, 2011; Arango Duque and Descoteaux, 2014).

Moreover, several proliferative disorders arising from macrophages and dendritic cells exist in man as well as in animals like dogs and cats (Dalia et al., 2014; Moore, 2014).
1.1 Canine histiocytic tumors

Canine histiocytic proliferative disorders include a wide spectrum of diseases with different biologic behaviors (Affolter and Moore, 2002; Moore, 2014). They can be classified as reactive disorders, such as cutaneous and systemic histiocytosis, and neoplasms, such as cutaneous histiocytoma and localized or disseminated histiocytic sarcoma (HS), the latter was previously called malignant histiocytosis (Figure 1; Moore, 1984; Moore and Rosin 1986; Affolter and Moore, 2000; Moore et al., 2006; Abadie and Hédan, 2009; Moore, 2014). These disorders are characterized by proliferation of histiocytes and their precursors (Wellman et al., 1988; Hayden et al., 1993; Cline, 1994; Hammer, 2001; Weiss, 2001; Coomer et al., 2008, Constantino-Casas et al., 2010). Under behavioral aspects, cutaneous histiocytomas are defined as benign unlike localized and disseminated histiocytic sarcomas that are considered malignant disorders often having guarded to poor prognosis (Affolter and Moore, 2002; Fulmer and Mauldin, 2007; Moore, 2014).

Histiocytes are a subset of leukocytes with antigen-presenting-like properties which derive from a common CD34+ bone marrow stem cell precursor (Fulmer and Mauldin, 2007; Day, 2008). These stem cells give rise to promonocytes that mature in the bone marrow to monocytes, which then briefly circulate in the blood before entering the tissues to complete the maturation process (Cline, 1994). Blood monocytes differentiate to tissue macrophages, which when activated, have abundant, often vacuolated cytoplasm and a bean-shaped nucleus (Day, 2008). Furthermore, recent studies discuss the existence of yolk-sack derived tissue macrophages which do not develop from circulating monocytes and which possess the possibility of local self-renewal (Varol et al., 2015).
Currently both, monocyte/macrophages and Langerhans/dendritic cells, which arise from a common multilineage hematopoietic precursor cell and differentiate depending on the cytokine influence and growth factor milieu, are included in the term histiocyte. Differentiated macrophages and dendritic cells share many of the same surface antigens, including CD11c, CD18, CD45, CD54 (ICAM-1), and some surface receptors for immunoglobulin and complement molecules, but each has also unique antigens (Steinman, 1991; Cline, 1994; Janeway et al., 1999; Moore et al., 2006; Fulmer and Mauldin, 2007).

Localized and disseminated histiocytic sarcoma are included, together with cutaneous histiocytoma, cutaneous (reactive) histiocytosis, systemic (reactive) histiocytosis and granulocytic sarcoma in the solid myeloid proliferations subsection of the WHO classification of hematopoietic tumors of domestic animals (Valli et al., 2002). In veterinary medicine malignant histiocytic disorders were firstly thought to affect uniquely Bernese Mountain dogs, later related breeds where also found sporadically affected or genetically predisposed to suffer from histiocytic disorders, but afterwards cases of unrelated breeds where described and even other species like rodent, feline or bovine cases have been reported (Hayden et al., 1993; Anjiki et al., 2000; Yamate et al., 2001, Affolter and Moore, 2006; Friedrichs and Young, 2008; Ide et al., 2009).
**Disseminated histiocytic sarcoma** (formerly termed “malignant histiocytosis”) is an aggressive multisystem disease characterized by the presence of multiple tumors in several organ systems (Moore, 2014). Frequent primary sites are spleen, lung and bone marrow (Moore and Rosin, 1986). Later on lesions are observed in lymphnodes and liver and, subsequently, other organs are affected (Affolter and Moore, 2002; Moore et al., 2006; Fulmer and Mauldin, 2007). Most disseminated histiocytic sarcomas present with widespread lesions in several organ systems (Affolter and Moore, 2002). Since it is impossible to determine whether proliferations represent metastases from a primary site or arose all simultaneously, the currently accepted terminology refers to both conditions as disseminated histiocytic sarcoma (Fulmer and Mauldin, 2007). The disease is characterized by infiltration of neoplastic histiocytes and is most frequently observed in middle-aged dogs (often with a familiar association; Schwens et al., 2011). The most common clinical signs in dogs suffering
from disseminated histiocytic sarcoma are anorexia, lethargy and weight loss (Fulmer and Mauldin, 2007). Intervertebral lesions have resulted in ataxia and paraparesis (Affolter and Moore, 2002; Fulmer and Mauldin, 2007). Circling, seizures, hyperaesthesia and proprioceptive deficits may also be shown due to neurological involvement (Thio et al., 2006). Hematological investigations at the time of diagnosis reveal anemia and thrombocytopenia in more than 50% of the cases (Abadie and Hédan, 2009). Clinical outcome reflects the very aggressive behavior and poor prognosis of this disease (Moore and Rosin, 1986; Abadie and Hédan, 2009).

Histologically, localized and disseminated histiocytic sarcomas display identical morphologic features (Affolter and Moore, 2002). The infiltratively growing, poorly demarcated tumors often possess a pleomorphic appearance of neoplastic cells varying from individualized large cells to multinucleated giant cells (Moore, 2014). A marked anisokaryosis is often observed (Affolter and Moore, 2002). No differences are noted in the phenotype between localized and disseminated histiocytic sarcomas. Both are of leukocytic origin based on the expression of the antigens CD45 and CD11a/CD18 (Affolter and Moore, 2002). Tumor cells are negative for CD3 and CD79 (Abadie and Hédan, 2009). Histiocytic sarcomas constantly express molecules involved in antigen presentation including CD1b, CD1c, and MHC class II (Affolter and Moore, 2002). Adhesion molecules like the β2-integrin adhesion molecule CD11c and the intercellular adhesion molecule ICAM-1 are strongly expressed by these tumors (Affolter and Moore, 2002). Histiocytic sarcoma cells do not express CD4, which is upregulated upon activation of dendritic antigen presenting cells (APC), as seen in canine reactive histiocytosis (Affolter and Moore, 2002). Other surface molecules are either broadly expressed by leukocytes, such as CD44, or inconsistently expressed by the tumor cells, such as CD45RA or the adhesion molecules CD11b, CD49d, and ICAM-3 (Affolter and Moore, 2002). These
observations confirm the myeloid dendritic cell origin of these tumor cells displaying a CD1+, CD11c+, MHC class II+, ICAM-1+ phenotype (Affolter and Moore, 2002). Morphologically, histiocytic sarcomas often resemble other sarcomas and therefore immunophenotyping of neoplastic cells is necessary to confirm the cellular origin (Figure 2; Moore et al., 1996; Affolter and Moore, 2002).
The five entities englobed in canine histiocytic disorders are:

**Canine cutaneous histiocytomas (1)**, characterized by being epitheliotropic lesions of the skin whose proliferative cells are CD1a+, CD11c+, Thy-1(CD90)- and CD4-Langerhans cells.

**Canine reactive histiocytosis (2)**, including a cutaneous (2a) and a systemic (2b) form, being both characterized by cellular infiltrates of CD1+, CD11c+, Thy-1+, CD4+, myeloid APC, CD8+, TCR α/β+ T cells, and neutrophils.

**Canine histiocytic sarcomas (3)**, which appear either as a localized form (3a) affecting a single organ with or without draining lymph node/s (In/s) metastasis, or as a disseminated form (3b) with multiorgan lesions. Tumor cell populations involved equally express a CD45+, CD11a/CD18+ leukocyte phenotype, CD1b+, CD1c+ and MHC class II+ antigen and are consistently CD4-, CD44+ and ICAM-1+. They inconsistently express CD45RA, ICAM-3 and Thy-1, and tumor cells consistent of macrophage origin are CD1- and CD14+, whereas those of myeloid dendritic APC origin are CD1+, CD14-.
1.2 Canine histiocytic sarcoma cell lines

Few tumor cell lines derived from canine histiocytic sarcoma have been established (Wellman et al., 1988; Sakai et al., 2003; Azakami et al., 2006). They include DH82, CCT and CHS-1 to CHS-7 cell lines (Wellman et al., 1988; Sakai et al., 2003; Azakami et al., 2006). They can form xenograft tumors in mice with similar morphological and immunohistochemical features as the original tumor (Sakai et al., 2003; Azakami et al., 2006).

The DH82 cell line was established from collected femoral bone marrow cells from a ten years old male Golden Retriever that was histopathologically diagnosed with a disseminated histiocytic sarcoma (Wellman et al., 1988). The diagnosis was based on typical histological findings including an infiltrative growth of large, neoplastic histiocytes in various organs such as spleen, liver and lymph nodes (Wellman et al., 1988). In culture, DH82 cells are large round cells which grow as a loosely adherent monolayer and that possess abundant cytoplasm with variable numbers of vacuoles and cytoplasmic granules (Wellman et al., 1988). Furthermore, DH82 cells have cytoplasmic pseudopods and projections and are able to phagocytose latex particles highlighting their macrophage-like appearance and function (Wellman et al., 1988). Similar to macrophages DH82 cells express a panel of cytokines and chemokines such as tumor necrosis factor alpha (TNF-α), interleukin (IL)-1, IL-5, IL-6, IL-8, IL-10 and IL-12 (Gröne et al., 1999; Barnes et al., 2000). Also several matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) have been shown to be produced by DH82 cells on a molecular as well as on a functional level (Barnes et al., 2000; Puff et al., 2009). DH82 cells have been widely used to perform immunological, comparative or specific oncological studies (Barnes et al., 2000; Bird et al., 2008; Hafeman et al., 2010; Divino et al., 2010; Ouedraogo et al., 2010) but also for
cultivation, cloning, isolation and molecular characterization of intracellular pathogens (Brouqui et al., 1994; Harrus et al., 2003; Aguirre et al., 2004) and for replication and study of viruses (Hines and Maury, 2001; von Messling et al., 2001; Puff et al., 2009).

1.3 Canine distemper virus

Canine distemper virus (CDV) is an enveloped, non-segmented, negative-stranded RNA morbillivirus, closely related to measles virus (MV) belonging to the Paramyxoviridae family (Wyss-Fluehmann et al., 2010; de Vries et al., 2015). In dogs, CDV causes a systemic infection with spread to multiple organs including the central nervous system (CNS; Wiener et al., 2007). Brain infection with CDV can induce a chronic demyelinating disease in dogs which is considered morphologically to be a model for multiple sclerosis (MS) in humans (Miao et al., 2003; Vandevelde and Zurbriggen, 2005; Alldinger et al., 2006; Sips et al., 2007; Beineke et al., 2008; Orlando et al., 2008; Techangamsuwan et al., 2009; Wyss-Fluehmann et al., 2010; Lempp et al., 2014). All canids may be susceptible to CDV but susceptibility of Felidae, Hyaenidae, Mustelidae, Procyonidae, Ursidae and Viverridae family members has also been documented (Deem et al., 2000; Céspedes et al., 2010). Furthermore, occasional infections of non-human primates and peccaries with CDV have been described (Beineke et al., 2015).

Several different CDV strains are available as vaccine strains including the Rockborn strain, the Onderstepoort strain and the Lederle strain (Harder and Osterhaus, 1997). These strains have been attenuated culturally by passaging on canine kidney cells, hen eggs or chicken fibroblasts (Harder and Osterhaus, 1997). The Onderstepoort strain of CDV was derived from the so-called Green’s distemperoid virus (Green and Carlson, 1945) which had been isolated from a natural distemper case and serially
passaged in ferrets. The ferret-passaged virus was then adapted to chicken eggs and passaged in this system numerous times, after which it was called OP-CDV (CDV-\textit{Ond}; Haig, 1948). CDV-\textit{Ond} is considered to be completely apathogenic (Appel and Gillespie, 1972) and, in contrast to other CDV strains, establishes a cytolytic infection in culture and spreads by budding. It easily infects a variety of cell lines (Stettler et al., 1997). Onderstepoort CDV infection of primary dog brain cell cultures induces massive cell-cell fusion subsequently leading to cell destruction (Zurbriggen et al., 1995). CDV-Ond can lead to single cell necrosis and syncytium formation in Vero cells, both interpreted as cytopathogenic effects (Hirayama et al., 1986; Guo and Lu, 2000). As \textit{in vivo} CDV has a broad cell tropism \textit{in vitro}, being able to infect, grow efficiently and propagate in canine histiocytic sarcoma cells like DH82 cells and CCT cells (Gröne et al., 2002; Yamaguchi et al., 2005).

1.4 Oncolytic viruses and tumor therapy

Oncolytic viruses (OVs) are developed as anticancer drugs (Cassady et al., 2016). They propagate nearly selectively in tumor tissue and destroy it without causing excessive damage to normal non-transformed cells (Russell and Peng, 2007; Workenhe and Mossman, 2014). In addition to direct lytic effects, oncolytic viruses may induce an anti-tumor immune response (Figure 3; Sinkovics and Horvath, 2000; Parato et al., 2005; Schuster et al., 2006; Sinkovics and Horvath, 2006; Väha-Koskela et al., 2007).
Figure 3. Infection and killing of tumor cells by an oncolytic DNA virus (Parato et al., 2005; modified)

**A.** Oncolytic virus targeted tumor cell infection as a consequence of viral interaction with tumor cells over-expressing specific cell-surface receptors;

**B.** The virus binds to the cell surface receptor and subsequent internalization takes place resulting in virus entry and viral genome release into the cells. Replication and viral gene expression proceed either in the cell cytoplasm (e.g. vesicular stomatitis virus) or in the nucleus and cytoplasm (e.g. adenovirus), differing due to virus species-specificity. The tumor cell is responsible for viral gene expression and viral protein synthesis. Viral gene expression and replication activates cellular antiviral defences (e.g. apoptosis) that could be deficient or inactive in transformed cells. Viral protein expression may trigger immune-mediated lysis of infected cells by T-cells (CD8+) after recognition of viral peptide epitopes presented by MHC class I on their surface. Following virus assembly, release of virus progeny and finally cell lysis occurs, being the newly formed viruses able to infect adjacent cells and repeat the cycle.
Genetically engineered, recombinant, naturally occurring and attenuated viruses of different families, including adenoviruses, herpesviruses, polyomaviruses, poxviruses, parvoviruses, reoviruses, orthomyxoviruses, paramyxoviruses, rhabdoviruses, coronaviruses, picornaviruses, togaviruses and retroviruses are used for viral oncolysis either as models or in clinical trials (Väha-Koskela et al., 2007; Kirn et al., 2008; Shi et al., 2009; Hartkopf et al., 2011; Lapp et al., 2014).

Measles virus and canine distemper virus, both belonging to the family Paramyxoviridae, are investigated in various types of cancer including human breast cancer, multiple myeloma, ovarian cancer, malignant lymphoma and glioma often associated with a tumor regression and or prolonged survival times in mouse xenograft models (Grote et al., 2001; Peng et al., 2001; Myers et al., 2005; Suter et al., 2005; Künzi et al., 2006; McDonald et al., 2006; Iankov et al., 2010; Peng et al., 2009).

The mode of action of paramyxovirus-induced oncolysis is not fully elucidated until now. However, it has been shown, that canine distemper virus can induce apoptosis in canine neoplasic lymphoid cells, canine histiocytic sarcoma cells (CCT cells) and human cervical carcinoma cells (HeLa cells) in vitro (Suter et al., 2005; Yamaguchi et al., 2005; Del Puerto et al., 2011).

Therefore, viral oncolysis represents an interesting alternative for tumors unresponsive to conventional therapeutic approaches.

1.5 In vitro models for tumor invasion

Most cancer-associated deaths are not caused by the primary tumor itself but by tumor cell invasion and metastasis (Masuda et al., 2016). To form metastases, tumor
cells have to cross the basement membrane and this interaction initiates further steps of the metastatic cascade (Stetler-Stevenson et al., 1993). To facilitate studies of this interaction, several in vitro models, mimicking basement membranes have been established (Katt et al., 2016). Most often a transwell system using a reconstituted basement membrane (Matrigel) as a barrier between upper and lower chamber is used (Albini and Noonan, 2010). In this model the attachment of neoplastic cells to the basement membrane as well as degradation of this barrier and migration into the lower chamber, all important steps of metastases have been studied, often focusing on matrix modulating enzymes (Poincloux et al., 2009). Furthermore, similar models are used to evaluate the effect of anti-neoplastic drugs (Katt et al., 2016).

1.6 Adhesion molecules

The first step for migration of tumor cells is acquisition of an invasive phenotype (Makrilia et al., 2009). Therefore neoplastic cells need adhesion molecules and enzymes to favour cell attachment to physical barriers like basement membranes, local proteolysis and migration (Stetler-Stevenson et al., 1993). Molecules involved in these processes include integrins, cadherins and matrix metalloproteinases (Price and Thompson, 2002). Most of the afore-mentioned processes take place in so called “invadopodia”, specialized cellular protrusions with an actin-based core (Paz et al., 2014). The complex interactions of cytoskeletal proteins, receptors, signal cascades, enzymes and extracellular matrix in invadopodia are depicted exemplarily in figure 4.
AFAP-110 (actin filament associated protein 1); Arp2/3 (actin related protein 2/3 complex); Cdc42 (cell division cycle 42); ECM (extracellular matrix); EGFR (epidermal growth factor receptor); Fak (focal adhesion kinase); IQGAP (IQ motif containing GTPase activating protein); N-WASP (neuronal Wiskott–Aldrich Syndrome protein); PIP2 (Phosphatidylinositol 4,5-bisphosphate); PIP3 (Phosphatidylinositol (3,4,5)-trisphosphate); PKC (protein kinase C); Pyk (proline-rich tyrosine kinase); Rap1 (Ras-related protein 1); SCR (src proto-oncogene); Tks4 (tyrosine kinase substrate with four Src homology 3 domains); Tks5 (tyrosine kinase substrate with five Src homology 3 domains);

Red arrows (negative feedback regulation); Blue arrows (regulation)
Integrins represent a family of transmembrane cell surface receptors that interact between cells or cells and extracellular matrix components (Price and Thompson, 2002). These interactions result in complex signalling cascades leading, which include regulation of matrix metalloproteinase expression and activity (Maity et al., 2011). Therefore, integrins are considered as important factors for tumor invasion and metastasis, representing interesting targets for cancer therapy (Maschler et al., 2005; Stefanidakis and Koivunen, 2006).

Many other, important molecules involved in tumorigenesis, invasion and metastasis belong to the immunoglobulin superfamily, forming multiple subgroups such as antigen receptors growth factor and cytokine receptors and cellular adhesion molecules (Paschos and Bird, 2009).

Molecules of CD1 family, representing cell surface receptors, are regularly expressed on antigen presenting cells (Blumberg et al., 1995). Therefore it can be used, together with other surface markers, to identify neoplasms like histiocytic sarcomas derived from myeloid-dendritic antigen-presenting cells (Affolter and Moore, 2002).

Similar to CD1, CD11a, CD11b, CD11c and CD11d are expressed on different cell types including macrophages (CD11b), interstitial and epithelial dendritic cells (CD11c; Fulmer and Mauldin, 2007). CD18 is often also useful for demonstrating the histiocytic origin of cells (Affolter and Moore, 2002). Furthermore CD11 and CD18 form an adhesion complex, which is necessary for the adherence of leukocytes to the endothelium of blood vessels in the process of leukocyte emigration (Harlan, 1993).

CD14 is expressed on blood monocytes and macrophages whereas dendritic cells are negative for this marker (Fulmer and Mauldin, 2007).
**CD44**, a transmembrane glycoprotein, which is expressed on many different cell types including histiocytic sarcoma cells (DH82 cells), predominantly serves as a hyaluronic acid receptor (Alldinger et al., 1999; Jordan et al., 2015). It has been shown, that CD44 has important functions in various physiological and pathological processes such as angiogenesis, astrocyte migration, cell proliferation, immune response including lymphocyte homing, tumor cell invasion and metastasis (Hertweck et al., 2011; Dzwonek and Wilczynski, 2015).

The common leukocyte antigen (**CD45**) is specifically expressed by hematopoietic cells (Tchilian and Beverley, 2006). Furthermore CD45 can be expressed by histiocytic sarcomas in man as well as in dogs (Zhang et al., 2008; Rossi et al., 2009).

**CD54** (intercellular adhesion molecule, ICAM-1) represents a surface molecule, which can be expressed on many different cell types including leukocytes, endothelial and epithelial cells (Roebuck and Finnegan, 1999; Zecchinon et al., 2006). Interestingly, both localized and disseminated histiocytic sarcomas of dogs express this protein at the cell surface (Affolter and Moore, 2002).

**CD80** (B7-1) and **CD86** (B7-2) are typically present on antigen-presenting cells (Sharpe and Freeman, 2002). Immunophenotyping of a histiocytic sarcoma in a young dog revealed an occasional expression of CD80 whereas CD86 could not be detected in this case (Mastrorilli et al., 2012).
1.7 Matrix metalloproteinases in different disorders

Matrix metalloproteinases (MMPs) represent a large group of different enzymes, grouped according to their structure and ability to degrade extracellular matrix components like collagen and gelatin (Somerville et al., 2003). Beside their functions in regulating extracellular matrix composition MMPs interact with growth factors, cytokines and some of them have the possibility to cleave cell-cell-adhesions (Somerville et al., 2003). This multitude of functions lead to the involvement of MMPs in many diseases which range from viral encephalitis like canine distemper encephalitis (Miao et al., 2003; Gröters et al., 2005) to canine myxomatous mitral valve disease (Moesgaard et al., 2014) and mammary tumors (Aresu et al., 2011).

1.8 Role of metalloproteinases and their inhibitors in tumorigenesis and invasion

As described above, MMPs are essential for extracellular matrix remodeling and are therefore important factors for invasive growth of neoplasms, metastasis and angiogenesis (Figure 5, Vihinen and Kähäri, 2002). For invasion and tumor cell migration, specialized cellular protrusions, called “invadopodia” are produced by cancer cells establishing cell-matrix interactions (Cmoch et al., 2014). Invadopodia are actin-rich structures which also contain cortactin (Clark et al., 2007). The latter has been shown to regulate MMP secretion and therefore invasiveness of tumor cells (Clark et al., 2007). In many tumor types an increased expression of MMPs correlates with a poorer prognosis for affected individuals (Sun et al., 2014; Wang et al., 2014; Aparna et al., 2015). These processes are impeded by inhibitors of matrix metalloproteinases such as tissue inhibitors of matrix metalloproteinases (TIMPs) and reversion-inducing protein with Kazal motifs (RECK; Noda et al., 2003; Cruz-
Munoz and Khokha, 2008; Nagini, 2012). Often, a reduced expression of RECK correlates with a poorer prognosis (Zhou et al., 2014). However, in some studies an elevated expression of TIMPs, especially TIMP-1, correlates with a worsened prognosis (Grunnet et al., 2013; Chang et al., 2015).

Matrix metalloproteinases are not only produced by tumor cells but also by tumor infiltrating inflammatory cells, mainly macrophages (Galdiero et al., 2013; Deryugina and Quigley, 2015).

Figure 5. Pivotal roles of MMPs in tumorigenesis and invasion (Gialeli et al., 2011; modified)

COL-IV (collagen IV); COL-XVIII (collagen XVIII); EGF (epidermal growth factor); FASL (Fas ligand); FGF (fibroblast growth factor); GF (growth factor); IGF (insulin-like growth factor); IL (interleukin); MMP (matrix metalloproteinase); TGF-α (transforming growth factor alpha); VEGF (vascular endothelial growth factor);
1.9 Aim of the study

Histiocytic sarcomas represent tumors with a poor prognosis in affected individuals. Therefore one aim of the study was to evaluate an in vitro model of histiocytic sarcomas using DH82 cells. Since DH82 cells are a permanent cell line, the cell passage used can differ from early to late passages. The present study analyzes the expression of cell surface molecules, mainly adhesion molecules, which represent important factors early in the metastatic cascade, in different passages of histiocytic sarcoma cells in order to determine inter-passage variances which might be crucial to interpretation of the results and transferability of the results to in vivo mechanisms.

The second aim of the study was to evaluate the expression and distribution of cortactin in DH82 cells with or without canine distemper virus infection in vitro. Viral oncolysis, in this case using canine distemper virus, might represent an elegant approach for tumor therapy namely in neoplasms, which do not respond well to conventional therapies. Cortactin, a cytoskeletal protein necessary for cell migration and therefore also important in early tumor invasion and metastasis steps, is known to be overexpressed in many tumor types, often associated with a poor prognosis. Therefore a down-regulation of this protein using viral oncolysis might improve the prognosis.

The third aim of the study was to evaluate microglia, resident central nervous system histiocytes, regarding their expression of matrix metalloproteinases and their inhibitors in different intracranial diseases. Beside their important function in tumor cell invasion and metastasis matrix metalloproteinases and their inhibitors represent important modulators in opening of the blood brain barrier and inflammatory cell migration to a chemotactic stimulus.
Chapter 2
Chapter 2

Passage-dependent morphological and phenotypical changes of a canine histiocytic sarcoma cell line (DH82 cells).

Heinrich, F., V. B. Contioso, V. M. Stein, R. Carlson, A. Tipold, R. Ulrich, C. Puff, W. Baumgärtner and I. Spitzbarth

DH82 cells represent a permanent macrophage cell line isolated from a dog with histiocytic sarcoma (HS) and are commonly used in various fields of research upon infection and cancer, respectively. Despite its frequent use, data on cell surface antigen expression of this cell line are fragmentary and in part inconsistent. We therefore aimed at a detailed morphological and antigenic characterization of DH82 cells with respect to passage-dependent differences. Cellular morphology of early (≤ 13) and late (≥ 66) passages of DH82 cells was evaluated via scanning electron microscopy. Moreover, cells were labelled with 10 monoclonal antibodies directed against CD11c, CD14, CD18, CD44, CD45, CD80, CD86, MHC-I, MHC-II, and ICAM-1 for flow cytometric analysis. Early passage cells were characterized by round cell bodies with abundant small cytoplasmic projections whereas later passages exhibited a spindle-shaped morphology with large processes. The percentage of CD11c-, CD14-, CD18-, CD45-, and CD80 positive cells significantly decreased in late passages whereas the expression of CD44, CD86, MHC-I, MHC-II and ICAM-1 remained unchanged. DH82 cells represent a remarkably heterogeneous cell line with divergent antigenic and morphologic properties. The present findings have important implications for future studies, which should consider distinct characteristics with regard to the used passage.
Keywords: DH82 cells; Flow cytometry; Histiocytic sarcoma; Macrophage cell line; Passage-dependency; Scanning electron microscopy

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Author contributions

FH and VBC performed the experiments. FH, VBC, VMS, RC and AT analyzed the data. FH drafted the manuscript. RU and CP helped drafting the manuscript. WB was involved in the study design, helped drafting the manuscript and obtained the funding. IS was involved in study planning and concept design and helped drafting the manuscript. All authors were involved in writing and critically revising the manuscript and had final approval of the submitted version.
Chapter 3
Chapter 3

Persistent morbillivirus infection leads to altered cortactin distribution in histiocytic sarcoma cells with decreased cellular migration capacity

Pfankuche, V. M.\textsuperscript{1,4}, M. Sayed-Ahmed\textsuperscript{1}, V. B. Contioso\textsuperscript{1}, I. Spitzbarth\textsuperscript{1,4}, K. Rohn\textsuperscript{2}, R. Ulrich\textsuperscript{1,4}, U. Deschl\textsuperscript{3}, A. Kalkuhl\textsuperscript{3}, W. Baumgärtner\textsuperscript{1,4,*} and C. Puff\textsuperscript{1}

Abstract

Histiocytic sarcomas represent rare but fatal neoplasms in humans. Based on the absence of a commercially available human histiocytic sarcoma cell line the frequently affected dog displays a suitable translational model. Canine distemper virus (CDV), closely related to measles virus, is a highly promising candidate for oncolytic virotherapy. Therapeutic failures in patients are mostly associated with tumour invasion and metastasis often induced by misdirected cytoskeletal protein activities. Thus, the impact of persistent CDV infection on the cytoskeletal protein cortactin, which is frequently overexpressed in human cancers with poor prognosis, was investigated \textit{in vitro} in a canine histiocytic sarcoma cell line (DH82). Though phagocytic activity, proliferation and apoptotic rate were unaltered, a significantly reduced migration activity compared to controls (6 hours and 1 day after seeding) accompanied by a decreased number of cortactin mRNA transcripts (1 day) was detected. Furthermore, persistently CDV infected DH82 cells showed a predominant diffuse intracytoplasmic cortactin distribution at 6 hours and 1 day compared to controls with a prominent membranous expression pattern ($p \leq 0.05$). Summarized, persistent CDV infection induces reduced tumour cell migration associated with an
altered intracellular cortactin distribution, indicating cytoskeletal changes as one of the major pathways of virus-associated inhibition of tumour spread.

Author contributions

VMP, MS and VBC performed the experiments. VMP, IS and RUL analyzed the microarray data. VMP drafted the manuscript. WB was involved in the study design, helped drafting the manuscript and obtained the funding. CP was involved in study planning and concept design and helped drafting the manuscript. All authors were involved in writing and critically revising the manuscript and will have final approval of the submitted version.
Persistent morbillivirus infection leads to altered cortactin distribution in histiocytic sarcoma cells with decreased cellular migration capacity

Vanessa M. Pfankuche¹,⁴, Mohamed Sayed-Ahmed¹, Vanessa Bono Contioso¹, Ingo Spitzbarth¹,⁴, Karl Rohn², Reiner Ulrich¹,⁴, Ulrich Deschi³, Arno Kalkuhl³, Wolfgang Baumgärtner¹,⁴,* , Christina Puff¹

¹ Department of Pathology, University of Veterinary Medicine Hannover, Bünteweg 17, 30559 Hannover, Germany
² Institute for Biometry, Epidemiology and Information Processing, University of Veterinary Medicine Hannover, Bünteweg 2, 30559 Hannover, Germany
³ Department of Non-Clinical Drug Safety, Boehringer Ingelheim Pharma GmbH&Co KG, Biberach (Riß), Germany
⁴ Center for Systems Neuroscience, University of Veterinary Medicine Hannover, Hannover, Germany.

*corresponding author

**Corresponding author:**

Prof. Dr. Wolfgang Baumgärtner, Ph.D.
Department of Pathology
University of Veterinary Medicine Hannover
Bünteweg 17
30559 Hannover
Germany
Tel. 0049-511-953-8620

e-mail: wolfgang.baumgaertner@tiho-hannover.de
Abstract

Histiocytic sarcomas represent rare but fatal neoplasms in humans. Based on the absence of a commercially available human histiocytic sarcoma cell line the frequently affected dog displays a suitable translational model. Canine distemper virus (CDV), closely related to measles virus, is a highly promising candidate for oncolytic virotherapy. Therapeutic failures in patients are mostly associated with tumour invasion and metastasis often induced by misdirected cytoskeletal protein activities. Thus, the impact of persistent CDV infection on the cytoskeletal protein cortactin, which is frequently overexpressed in human cancers with poor prognosis, was investigated in vitro in a canine histiocytic sarcoma cell line (DH82). Though phagocytic activity, proliferation and apoptotic rate were unaltered, a significantly reduced migration activity compared to controls (6 hours and 1 day after seeding) accompanied by a decreased number of cortactin mRNA transcripts (1 day) was detected. Furthermore, persistently CDV infected DH82 cells showed a predominant diffuse intracytoplasmic cortactin distribution at 6 hours and 1 day compared to controls with a prominent membranous expression pattern (p ≤ 0.05). Summarized, persistent CDV infection induces reduced tumour cell migration associated with an altered intracellular cortactin distribution, indicating cytoskeletal changes as one of the major pathways of virus-associated inhibition of tumour spread.
Introduction

Neoplastic disorders still represent one of the most common causes of death in humans as well as in companion animals such as dogs and cats\textsuperscript{1,2}. Furthermore, despite a wide range of therapeutic approaches including surgery, chemo- and radiotherapy, many tumour types still possess a guarded to poor prognosis\textsuperscript{3,4}. One example of such a neoplasm, with comparable short survival times in humans and dogs represents the histiocytic sarcoma\textsuperscript{3,5,6}. This highly aggressive tumour type, occurring in a localised or disseminated variant, has a limited response to different conventional therapies including chemo- and radiotherapy, highlighting the need for new therapeutic approaches to overcome the current limitations of a palliative care in most cases\textsuperscript{3,5,7,8}. Median survival of human patients suffering from histiocytic sarcoma with greatest tumour dimensions of more than 3.5 cm for example is not exceeding 6 months regardless of the therapy\textsuperscript{4}. A promising new approach to overcome restricted therapeutic alternatives might be oncolytic virotherapy, based on the ability of several viruses to destroy cancer cells by simultaneous wide protection of non-transformed tissue\textsuperscript{9}. For this purpose, members of many different virus families are currently investigated thoroughly in human medicine, resulting in several clinical trials\textsuperscript{10}. Measles virus, a member of the family \textit{Paramyxoviridae}, yielded promising results as a potential oncolytic virus by inducing the regression of human lymphoma-xenografts in immuno-deficient nude-mice\textsuperscript{11}. A closely related, veterinary relevant virus is represented by canine distemper virus (CDV)\textsuperscript{12}. It is an enveloped, negative orientated, single-stranded RNA virus, containing six structure proteins\textsuperscript{12}. CDV is capable of infecting canine lymphoid cell lines, histiocytic sarcoma cell lines, such as DH82 cells, and neoplastic lymphocytes \textit{in vitro}, commonly inducing apoptosis of tumour cells\textsuperscript{13,14}. Recent studies suggest a decreased invasive and metastatic potential of persistently CDV-infected DH82 cells compared to non-
infected controls\textsuperscript{15}. However, the field of oncolytic virotherapy is still in its infancy and many questions remain to be asked and answered. Especially the function and mode of action of different viruses still remain largely unknown\textsuperscript{9}. The lack of a commercially available human histiocytic sarcoma cell line in contrast to its canine counter-part and the close relationship between measles and canine distemper virus highlight the present study design as a suitable translational model for further research and possible future therapeutic interventions of this devastating disease in humans\textsuperscript{6,16}.

A hallmark of many malignant neoplasms represents their ability to metastasize\textsuperscript{17}. For this process as well as for many other developmental and functional mechanisms including invasion of adjacent tissues, cell motility represents one main basic requirement\textsuperscript{18}. Cell motility is mostly based on changes in the cytoskeleton, which is crucially depending on members of the actin family\textsuperscript{19,20}. The actin cytoskeleton is critical for various aspects of the cell motility process, including polarisation, leading edge protrusion and cellular contraction\textsuperscript{21}. One member of the actin family, frequently overexpressed in multiple human tumours, represents cortactin\textsuperscript{21,22}. Cortactin is an actin-binding protein and a substrate of the Src-kinase, being involved in mechanisms, such as cell migration, invasion, synaptogenesis, endocytosis, intercellular contacts and host-pathogen interactions\textsuperscript{23}. The over-expression of cortactin in many different types of tumours is accompanied by an increased cell-migration activity and metastatic potential resulting in a worsened prognosis\textsuperscript{22}. Cell migration often depends on the ability of cells to form actin-rich protrusions, called podosomes or invadopodia\textsuperscript{23-27}. Invadopodia selectively appear in invasive cancer cells in comparison to non-invasive neoplasms and possess the ability to degrade the extracellular matrix. A decreased release of matrix metalloproteinases, which are important regulators of extracellular matrix metabolism, is reported for cells with a selective inhibition of cortactin\textsuperscript{28}. In addition, a decreased number of invadopodia has
been documented in head and neck squamous cell carcinoma cells treated with cortactin inhibiting RNA29.

The aim of the present study was to determine the impact of CDV infection on cell migration of canine histiocytic sarcoma cells (DH82 cells) with special emphasis on cortactin expression, gene regulation and possible functional implications.

**Results**

**Persistent CDV infection does not alter cellular proliferation, apoptosis and phagocytosis**

The persistent CDV infection of DH82 cells was ascertained using immunofluorescence for CDV nucleoprotein. Persistently CDV infected (strain Onderstepoort; CDV-Ond) DH82 cells exhibited a median percentage of infected cells of 94.15% at 1d post seeding (minimum 92.99%; maximum 98.36 %), 96.02% at 3d post seeding (minimum 93.95%; maximum 97.98 %) and 94.58% at 5d post seeding (minimum 93.26%; maximum 98.16%), whereas no CDV-immunoreactivity was detected in non-infected controls at any time point.

Cumulative population doubling assay, performed for 4 weeks, revealed no significant differences in the proliferation rates of non-infected and persistently CDV-Ond-infected DH82 cells ($p \geq 0.05$; Figure 1a). Similarly, the apoptotic rate as determined by cleaved caspase 3 immunofluorescence, showed no significant difference at 1 day post seeding (1d; $p \geq 0.05$; Figure 1b). A median of 0.80% (minimum 0.73%; maximum 0.87%) of non-infected and 0.78% (minimum 0.73%; maximum 0.94%) of persistently CDV-Ond-infected DH82 cells was observed. Furthermore, typical macrophage functions, examined by scanning and transmission electron microscopy performing latex bead phagocytosis assay, were retained by
Persistently CDV-Ond infected DH82 cells independent of time point post seeding (Figure 2).

**Persistent CDV infection reduces the migration capacity of DH82 cells**

At 6 hours post seeding (6h) in median 1411 non-infected DH82 cells reached the lower compartment of the transwell system (minimum 1290 cells; maximum 2719 cells). In contrast, persistently CDV-Ond infected DH82 cells displayed a median of 587 migrated cells at 6h (minimum 348 cells; maximum 677; p ≤ 0.05; Figure 1c), indicating a major impact of virus infection on cell migration. This was substantiated by the observation that at 1d after seeding in median 8995.5 migrated non-infected DH82 cells were found in the lower compartment (minimum 5806 cells; maximum 14502 cells), whereas only a median of 3068.5 persistently CDV-Ond infected DH82 cells (minimum 2328 cells; maximum 3441 cells) were migrated (p ≤ 0.05; Figure 1c).

**Microarray analysis reveals significant differences in the expression of invadopodia associated genes in DH82 cells by persistent CDV infection**

A microarray dataset of non-infected and persistently CDV-Ond infected DH82 cells was analysed for changes by investigating the expression of a manually generated list of 77 literature based genes, which are known to be involved in invadopodia formation and function. Persistent CDV infection caused a significant difference (Mann Whitney U Test p ≤ 0.05 and fold change ≤ -2 or ≥ 2) in the expression of a total number of 12 unique canine gene symbols (5 down- and 7 up-regulated) out of 77 canine gene symbols (Table 1). Differentially expressed down-regulated genes comprised CTTN (cortactin), CAV1 (caveolin 1), PTK2 (PTK2 protein tyrosine kinase 2), TGFB2 (transforming growth factor, beta 2) and IQGAP2 (IQ motif containing GTPase activating protein 2) with fold changes ranging from -6.52 to -3.13 with
cortactin as the top hit of down-regulated genes associated with invadopodia. Differentially expressed up-regulated genes comprised PDGFC (platelet derived growth factor C), MMP14 (matrix metallopeptidase 14), PIK3CG (phosphoinositide-3-kinase, catalytic, gamma polypeptide), FSCN1 (fascin homolog 1, actin-bundling protein), PIK3CD (phosphoinositide-3-kinase, catalytic, delta polypeptide), PDGFA (platelet-derived growth factor alpha polypeptide) and PLAUR (plasminogen activator, urokinase receptor) with fold changes ranging from 6.58 to 2.02.

To substantiate these findings, the number of cortactin mRNA transcripts was determined using real-time quantitative PCR. At 1d post seeding, persistently CDV-Ond infected DH82 cells possessed significant lower numbers of cortactin mRNA transcripts compared to non-infected controls (p ≤ 0.001; Figure 3).

Cortactin displays a predominantly, diffuse distribution in persistently CDV-Ond infected DH82 cells at early time points

More than 90% of non-infected and persistently CDV-Ond infected DH82 cells were immunopositive for cortactin at all time points investigated (1d, 3d, 5d after seeding; p ≥ 0.01; Table 2). Interestingly, the intracellular cortactin distribution differed in persistently CDV-Ond infected DH82 cells at different time points compared to non-infected cells (Table 3; Figure 4). At 6h and 1d post seeding a significantly higher number of non-infected DH82 cells displayed a cortical cortactin expression compared to persistently CDV-infected DH82 cells (p ≤ 0.01). In addition, the percentage of cells with a cortical cortactin expression was higher in non-infected controls at all time points investigated, compared to persistently CDV-Ond infected DH82 cells, albeit not reaching the level of significance at 3 and 5 days post seeding (3d; 5d; p ≥ 0.05). Furthermore the percentage of cells with a cortical cortactin expression decreased over time in culture, independently of the virus-infection.
Discussion

The hallmark of many malignant tumours is their ability to invade the adjacent tissue and to form metastases\textsuperscript{30}. Often these factors dramatically influence the prognosis for the affected individual. For invasion and metastasis neoplastic cells need the ability to migrate through the adjacent extracellular matrix, which is commonly mediated by the formation of invadopodia\textsuperscript{31}. Therefore inhibition of invadopodia formation with consecutive reduction of tumour cell migration represents an exciting new possibility for improving prognosis and survival time. One possibility of targeting invadopodia include the administration of different drugs often targeting pathways like the Src signalling, platelet derived growth factor signalling pathway and metalloprotease activity\textsuperscript{32}. However, effective therapeutic drugs targeting these pathways, for example the Src signalling, are often lacking or only useful at an early developmental stage\textsuperscript{32}, demonstrating the need of new treatment strategies like viral oncolysis.

Viral oncolysis, especially with morbilliviruses like measles virus and canine distemper virus, might represent an elegant method to overcome the common limitations of chemotherapy since many studies have shown that these viruses predominantly infect neoplastic cells while largely sparing non-transformed tissue\textsuperscript{10}. Furthermore, despite the existence of several genetically modified virus strains, many life-attenuated vaccine strains, often known and well tolerated since decades, have been shown to exert oncolytic activity \textit{in vitro} and \textit{in vivo}\textsuperscript{10,33}.

While depicting very similar proliferation and apoptotic rates as well as retaining phagocytic activity as a typical macrophage function, DH82 cells persistently infected with CDV (vaccine strain Onderstepoort) and non-infected controls differed significantly in their migratory capacity with significantly lower numbers of migrated virus-infected DH82 cells compared to non-infected controls in the present study.
Interestingly, measles virus has been shown to interact with the Src-kinase pathway, which is also involved in invadopodia formation\textsuperscript{34}. In addition, canine distemper virus is known to interfere with the actin cytoskeleton\textsuperscript{35-37}. Therefore the present study focused on the influence of CDV-infection upon the cytoskeleton, namely constituents of invadopodia formation.

Microarray analysis of genes, associated with invadopodia formation revealed a modulation of several genes. Interestingly, a significant, more than six-fold down-regulation of cortactin in persistently infected DH82 cells compared to controls was observed, which was further substantiated by using quantitative PCR. Cortactin represents an actin regulator required for invadopodia formation and also a substrate of the Src-kinase\textsuperscript{38,39}. Furthermore, several studies demonstrated a correlation between a high cortactin expression and a poor prognosis in several types of human neoplasms such as oesophageal squamous cell carcinoma, pancreatic and colorectal adenocarcinoma and laryngeal carcinoma\textsuperscript{40-43}. This implicates that a strategy to reduce the cortactin expression in other malignant neoplasms might also be helpful to improve prognosis and survival time. However, the influence of modulating the cortactin expression in histiocytic sarcomas has not been investigated so far, despite the fact that histiocytic sarcoma cells link both, a cell type which typically is able to migrate and a malignant transformation of the cells. The present study was conducted using histiocytic sarcoma cells, a tumour type with comparable poor prognosis in humans and dogs\textsuperscript{3-5,44}. The canine tumour cell line (DH82 cells) was chosen as a translational model with benefits for both, humans and dogs, since no permanent human histiocytic sarcoma cell line is commercially available\textsuperscript{16,45}. Data subsequently could be used for further \textit{in vivo} and \textit{in vitro} studies in the canine translational model and their extrapolation to the human counter-part with respect to tumour type and morbillivirus to be applied.
In contrast to the significant down-regulation of cortactin in persistently CDV-Ond infected DH82 cells on a molecular level, the number of cortactin expressing cells did not differ significantly between persistently CDV-Ond infected DH82 cells compared to controls on the protein level at any time point investigated. Similarly it has been described for different breast cancer cell lines, that the expression level of cortactin does not directly correlate with the ability of cells to form invadopodia and to migrate\textsuperscript{46}, stressing the importance of the subcellular localisation of cortactin.

The present study revealed significant differences in the intracellular localisation of cortactin between non-infected and persistently CDV-Ond infected DH82 cells at early time points (6h and 1d post seeding). At these time points cortactin was mainly located beneath the cell membrane and within cellular processes in non-infected cells, whereas persistently CDV-Ond infected DH82 cells exhibited a diffuse cytoplasmic distribution. As a membrane-associated cortactin expression is attributed to an “active” state with invadopodia formation and migration\textsuperscript{47} the diffuse distribution in persistently CDV-Ond infected DH82 cells might explain their reduced locomotion.

A similar observation, also emphasising the importance of the intracellular cortactin localisation, has been made in human fibrosarcoma cells, where the total amount of cortactin was unchanged by treatment with alpha-tocopheryl phosphate, whereas a delocalisation of cortactin from cell membrane and invadopodia to the cytoplasm occurred accompanied by reduced cell motility\textsuperscript{47}. The enhanced diffuse cytoplasmic distribution of cortactin at later time points (3d and 5d post seeding) in both CDV-Ond infected and non-infected DH82 cells was attributed to the increased confluence of cultures, since a contact inhibition of cell migration associated with cell density has been described for many cell types including cancer cells\textsuperscript{48-50}.

Summarised, persistent CDV-Ond infection of canine histiocytic sarcoma cells reduced the cellular migration capacity \textit{in vitro}, associated with a diminished cortactin
accumulation at the cell periphery. This might indicate a reduced metastatic potential of CDV infected DH82 cells *in vivo*. However, the latter has to be substantiated in further *in vivo* studies. Additionally, this canine model of viral oncolysis might represent an interesting translational method for this rare human tumour since CDV represents the canine counterpart of the closely related measles virus.

**Materials and methods**

**Cell culture**

DH82 cells are a permanent canine histiocytic sarcoma cell line, obtained from the European Collection of Cell Cultures, originally isolated from a Golden Retriever. DH82 cells were cultivated as formerly described. Passage 10 of non-infected and passage 141 of persistently CDV-infected DH82 cells were used for the present experiments. Persistently CDV-infected DH82 cells were generated as described. Cells were periodically harvested, frozen and stored in liquid nitrogen.

**Cell doubling assay**

To assess cellular proliferation, the cumulative population doubling (CPDs) was determined. During continuous passages, cells were seeded at same numbers into 25 cm² tissue culture flasks (Nunc GmbH & Co. KG, Thermo Scientific, Langenselbold, Germany) and counted at each, weekly passage over 4 weeks. The population doubling (PD) was calculated according to the following formula: "PD = log_{10} (cells harvested - initial cell number) / log_{2}". The cumulative population doubling was performed by adding the population doubling of each passage to that of the previous passage.

**Migration assay and cytospin preparation**
The migration ability of non-infected and persistently CDV-Ond infected DH82 cells was assessed by transwell migration assays. Cells were seeded on uncoated 24-well Millicell cell culture inserts with a pore diameter of 8µm (Merck KGaA, Darmstadt, Germany) at a density of 200,000 cells/well in minimal essential medium (MEM) with Earle's salts (PAA, Cölbe, Germany), 1% penicillin/streptomycin (P/S; PAA) and 1% non-essential amino acids (NEAA; Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany). The lower chamber additionally contained 10% foetal calf serum (FCS; PAA) as a chemoattractant. The transwell systems were incubated at 37°C, 5% CO₂ in a water-saturated atmosphere for 6 and 24 hours, respectively. Migrated cells were scraped, re-suspended and cytospin preparations were performed. After Pappenheim staining all migrated cells were counted.

**Immunofluorescence**

Non-infected and persistently CDV-Ond infected DH82 cells were stained for cleaved caspase 3 (Asp175; rabbit polyclonal; diluted 1:900; Cell Signaling Technology, Inc., Danvers, USA) with a secondary Cy3-conjugated goat-anti-rabbit IgG (H+L) antibody (1:100; Jackson ImmunoResearch Laboratories, Hamburg, Germany) to determine the number of apoptotic cells 1d after seeding. Nuclear staining was performed with bisbenzimide (Hoechst 33258; Sigma-Aldrich Chemie GmbH). Briefly, cells were seeded in quadruplicates at a density of 30,000 cells/cm² on 96 Microwell Nunc plates (Nunc GmbH & Co. KG, Thermo Scientific) and maintained under standard conditions. Cells were fixed with 4% paraformaldehyde at 1d after seeding and immunofluorescence was performed according to a 2 day protocol with minor variations. The number of cortactin positive cells (1d, 3d and 5d) and the intracellular cortactin distribution (6h, 1d, 3d and 5d) were determined using a polyclonal anti-cortactin IgG
antibody (H-191; rabbit polyclonal; diluted 1:100; Santa Cruz Biotechnology, California, USA). To verify the CDV infection of persistently CDV-Ond infected DH82 cells on a cellular level double-labelling with an anti-CDV nucleoprotein antibody (D110; mouse monoclonal; 1:100; kind gift from Prof. Dr. A. Zurbriggen, University of Bern, Switzerland) was performed. As secondary antibodies a Cy3-conjugated goat-anti-rabbit IgG (H+L; dilution 1:100; Jackson ImmunoResearch Laboratories) and an Alexa Fluor 488-conjugated AffiniPure goat-anti-mouse IgG (H+L) antibody (dilution 1:200; Jackson ImmunoResearch Laboratories) were used. Cells were seeded in quadruplicates at a density of 30000 cells/well on 96 Microwell Nunc plates (number of cortactin positive cells) and at a density of 50000 cells/well in 8 well lab-Tek chamber slides (intracellular cortactin distribution; Nunc GmbH & Co. KG, Thermo Scientific).

**Laser scanning confocal microscopy**

The intracellular cortactin distribution was analysed in detail by laser scanning confocal microscopy using the Leica TCS SP5 AOBS with a tandem-scanner and the Leica Application Suite Advanced Fluorescent Lite 2.0.2 build 2038 (Leica, Biberach, Germany). To evaluate the intracellular cortactin distribution three-dimensionally, 0.5µm thick z-stacks were recorded. The staining pattern was categorized as cell-membrane / protrusion accentuated (cortical) or diffuse cytoplasmic. Cells displaying a cortical cortactin staining in at least one half (≥ 50%) of the cellular circumference in at least one z-stack were defined as cells with a cortical staining pattern, whereas all others were described as diffusely cytoplasmic. 30 cells of each well (n=4) and each condition were analysed per time point and classified.
Phagocytosis assay

Phagocytic activity of non-infected and persistently CDV-Ond infected DH82 cells was determined using polystyrene latex beads (0.8µm; Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) in a dilution of 1:100 in MEM (PAA) containing 1% P/S (100 units/ml, 100 mg/ml; PAA), 10% FCS (PAA) and 1% NEAA (Sigma-Aldrich Chemie GmbH). 250µl of this solution were added to each well in a 24 well plate (Nunc GmbH & Co. KG, Thermo Scientific) or 4 well lab-Tek chamber slides (Nunc GmbH & Co. KG, Thermo Scientific) containing non-infected and persistently CDV-Ond infected DH82 cells 1d, 3d and 5d post seeding. Cells were incubated for 3 hours at 37°C and 5% CO₂ in a water-saturated atmosphere. Afterwards, cells were washed twice with phosphate buffered saline (PBS) to remove excess latex beads. The presence of phagocytized particles was determined by scanning and transmission electron microscopy⁵⁴.

Transmission electron microscopy

Embedding and sample processing were performed as described before⁵⁵,⁵⁶. Transmission electron microscopic analysis was performed by a transmission electron microscope (EM C 10A, Zeiss, Jena, Germany) at 60kV.

Scanning electron microscopy

Embedding and sample processing was performed as described previously⁵⁷. Slides were examined with a scanning electron microscope (DSM940, Zeiss).

RNA isolation and cDNA synthesis

RNA isolation was performed as described previously¹⁵. RNA concentration was ascertained by measuring the optical density at 260nm. The Omniscript kit (Qiagen
N. V., Venlo, The Netherlands) with RNase Out (Invitrogen™ GmbH, Darmstadt, Germany) and random hexamers (Random Primers, Promega, Fitchburg, USA) was used for reverse transcription of total RNA into complementary DNA (cDNA) following the manufacturers’ protocol.

**Primer design**

Primers used in this study were designed with Primer3 software, Beacon Designer version 2.1 software (Premier Biosoft International, Palo Alto, USA) or taken from the literature. Primer oligonucleotides were purchased from Eurofins MWG Operon (Ebersberg, Germany).

**RT-PCR**

RT-PCR was performed using a PTC200 thermocycler (Biozym, Hessisch Oldendorf, Germany) under the following conditions: 94°C for 1min, 40 cycles at 94°C for 1min, 58°C (GAPDH) or 59°C (cortactin) for 2min, 72°C for 1min and 72°C for 5min. Amplification was performed using AmpliTaq DNA Polymerase (Applied Biosystems Applera Deutschland GmbH, Darmstadt, Germany) in 1x GeneAmp 10x PCR Buffer II (Applied Biosystems Applera Deutschland GmbH) with 1.25 mM MgCl₂, 0.2 mM dNTP mix (Applied Biosystems Applera Deutschland GmbH) and 300 nM of each primer [Cortactin: Forward: 5’-GACTGGGAGACTGACCCTGA-3’; Reverse: 5’-ACACCAAACCTTGCTCCTAAA-3’; 320 base pairs (bp); GenBank accession number: XM_005631371; glyceraldehyde-3-phosphate-dehydrogenase (GAPDH): Forward: 5’-AAGGTCGGAGTCAACGGATT-3’; Reverse: 5’-GCAGAAGAAGCAGAGATGATG-3’; 365 bp; GenBank accession number: AB038240]. PCR products were analysed by agarose gel electrophoresis.
Real-time quantitative PCR

Real time quantitative PCR (RT-qPCR) was performed as described\textsuperscript{15}. In addition to cDNA samples, tenfold serial dilutions of purified, agarose gel extracted (NucleoSpin Extract II Kit, Macherey-Nagel GmbH & Co. KG, Düren, Germany) RT-PCR products ranging from $10^2$ to $10^8$ copies per sample were used as templates to generate standard curves. The plates contain duplicates of serially diluted samples for the standard curves and a no template control in duplicate. The reaction was quantified using SYBR-Green I in a reaction volume of 25 µl. RT-qPCR with Sybr Green I (1:40000) was performed under the following conditions: 95°C for 10min; 95°C for 30sec, 60°C (cortactin) or 64°C (GAPDH) for 1min and 72°C for 30sec, repeated 40 times and 72°C for 1min. Amplification was performed using 0.05 U/µl SureStart Taq DNA Polymerase in 1x Core PCR buffer with 2.5 mM MgCl$_2$, 8.0% glycerol, 3% dimethyl sulphoxide (DMSO), 150nM of each primer [Cortactin: Forward: 5’-TTTCAAGAACCACCAGACCCTCAA-3’; Reverse: 5’-CAAACTTTCCCGCATAACCATG-3’; 79 bp; GenBank accession number: XM_00563137; GAPDH: Forward: 5’-GTCATCAACGGGAAGTCCATCTC-3’; Reverse: 5’-AACATACTCAGCACCAGCATCAC-3’; 84 bp; GenBank accession number: AB038240], 30nM Rox as reference dye and 200µM dNTP mix.

Relative gene expression was normalised against the housekeeping gene GAPDH.

Analysis of differentially expressed genes of published microarray data

For molecular characterisation of the potential influence of a persistent CDV infection on cellular motility, a data set obtained from a global gene expression analysis was used. Briefly, 4 replicates of non-infected and persistently CDV-Ond infected DH82 cells were analysed at 1d post seeding. RNA isolation was performed as described above and hybridized to Affymetrix Canine Genome 2.0 Arrays. Data sets are
deposited in the ArrayExpress database (http://www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-3942.

The present study focused on a list of manually selected genes associated with invadopodia (supplemental table S1) according to the literature^{25,39,59-62}.

**Statistical analysis**

Analysis of data not otherwise specified was performed using SAS Enterprise Guide (SAS-version 9.3; SAS Institute Inc, Cary, USA). The assumption of normality was tested using the Kolmogorov-Smirnov test and visual assessment of qq-plots of model residuals. In case of rejection of normal distribution, distribution-free nonparametric methods were applied. For descriptive statistics median and range were calculated. Used statistical procedures included the non-parametric Wilcoxon-Mann-Whitney two-sample-test for the analysis of the cell doubling assay, migration assay, immunofluorescence and RT-qPCR. Intracellular cortactin distribution was analysed using a three-way ANOVA. Proc mixed was used for the linear model. The level of significance was set at $p \leq 0.05$ or $p \leq 0.01$ for data with low ranges, respectively.

For microarray data, independent pair-wise Wilcoxon-Mann-Whitney tests (IBM SPSS Statistics version 20; IBM Corporation, Armonk, USA) were applied in order to compare the gene expression of non-infected and persistently CDV-Ond infected DH82 cells^{63}. Significantly differentially expressed invadopodia-associated genes between persistently CDV-Ond infected and non-infected DH82 cells were selected employing a $p$-value $\leq 0.05$ cut-off combined with a $\geq 2.0$ or $\leq -2.0$ fold change filter.
Acknowledgements
The authors are thankful to Bettina Buck, Thomas Feidl, Claudia Hermann, Kerstin Rohn, Kerstin Schöne, Caroline Schütz, Anuschka Unold and Danuta Waschke for excellent technical support. Vanessa Maria Pfankuche was financially supported by the Akademie für Tiergesundheit e. V. (Bonn, Germany). Mohamed Sayed-Ahmed received financial support from the Ministry of Higher Education (Cairo, Egypt). This study was in part supported by the German Research Foundation (Researcher’s group 1103; grants BA815/10-2 and UL421/1-2 and the Gesellschaft zur Förderung Kynologischer Forschung e.V (GkF, Bonn, Germany).

Author contributions
VMP, MS and VBC performed the experiments. VMP, IS and RUL analysed the microarray data. VMP drafted the manuscript. WB was involved in the study design, helped drafting the manuscript and obtained the funding. CP was involved in study planning and concept design and helped drafting the manuscript. All authors were involved in writing and critically revising the manuscript and had final approval of the submitted version.

Competing financial interests
The authors declare no conflict of interest.

Supporting Information
Supplemental table S1
References:


Figure 1:
Overview of CDV induced changes on cell mechanisms including cell doubling, apoptosis and migration. (a) Persistent CDV-Ond infection of DH82 cells has no influence on cell proliferation as demonstrated by cells lacking a significant difference (\( p \geq 0.05 \)) in the cell doubling assay of non-infected and persistently infected DH82 cells. (b) Immunofluorescence of non-infected and persistently CDV infected DH82 cells reveals no significant difference (\( p \geq 0.05 \)) in the percentage of cleaved caspase 3 positive cells indicating a similar apoptotic rate following persistent CDV-Ond infection. Median, minimum and maximum percentages of immunopositive cells are presented. (c) Transwell migration assay of non-infected and persistently CDV-Ond infected DH82 cells reveals a significant difference (depicted by the asterisk, \( p \leq 0.05 \)) in the number of migrated cells 6h and 1d after seeding, thus indicating a reduced migratory activity of DH82 cells following persistent CDV-Ond infection. Median, minimum and maximum of counted cells are presented.

Figure 2:
Scanning and transmission electron microscopy was performed on non-infected and persistently CDV-Ond infected DH82 cells demonstrating comparable latex bead phagocytosis in both cell lines. Transmission electron microscopy reveals intracytoplasmic latex beads (arrows), the CDV nucleocapsid in persistently CDV-Ond infected DH82 cells (nc), the nuclei (n) and cellular protrusions (\( ^* \)). (a) Scanning electron microscopy on non-infected DH82 cells. (b) Scanning electron microscopy on persistently CDV-Ond infected DH82 cells. (c) Transmission electron microscopy on non-infected DH82 cells. (d) Transmission electron microscopy on persistently CDV-Ond infected DH82 cells.
Figure 3:
RT-qPCR confirms the significant difference in the relative cortactin gene expression in non-infected and persistently CDV-Ond infected DH82 cells as demonstrated by microarray analysis. The relative cortactin gene expression is calculated by normalisation against the housekeeping gene GAPDH. The relative percentage of target-specific gene expression was calculated as follows: $X/Y \times 100 = \text{normalised target specific gene expression}$, where $X = \text{target-specific gene expression level}$ and $Y = \text{housekeeping gene (GAPDH) expression level}$. (significance ($p \leq 0.05$) is highlighted by an asterisk).

Figure 4:
(a) Confocal laser microscopy of non-infected and persistently CDV-Ond infected DH82 cells demonstrates significant differences in the intracellular cortactin distribution (significance is highlighted by asterisks). Depicted are differences in cortical cortactin distribution between non-infected and persistently CDV-Ond infected DH82 cells at the same time point (Fig. 4 b-i). Immunofluorescence of non-infected (Figs. 4b, d, f, h) and persistently CDV-Ond infected (Fig. 4sc, e, g, i) DH82 cells, 6h (Fig. 4b,c), 1d (Fig. 4d,e), 3d (Fig. 4f/g) and 5d (Fig. 4h/i) after seeding (red: cortactin; blue: nuclear staining with bisbenzimide) highlights the differences in the cortactin distribution pattern and the tendency of both cell types to exhibit a diffuse cytoplasmic cortactin distribution at later time points.
Table 1:
Persistent CDV infection causes a significant difference (Wilcoxon-Mann-Whitney test \( p \leq 0.05 \) and fold change \( \leq -2 \) or \( \geq 2 \)) in the expression of a total number of 12 unique canine gene symbols (5 down- and 7 up-regulated) out of 77 manually selected literature based genes (supplemental table S1), which are known to be involved in invadopodia formation and function. Microarray analysis of these genes, revealed a significant, more than six-fold down-regulation of cortactin in persistently CDV-Ond infected DH82 cells compared to controls.

<table>
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<tr>
<th>Canine gene symbol</th>
<th>Gene Title (Canis familiaris)</th>
<th>p-value</th>
<th>Fold change</th>
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<td>CTTN</td>
<td>cortactin</td>
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<td>-6.522</td>
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<td>-3.382</td>
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Table 2:
The table depicts the percentage of cortactin expression in non-infected (DH82) and persistently CDV-Ond infected (DH82pi) DH82 cells at different time points post seeding. The values are shown as median (minimum; maximum). Statistically significant differences (p ≤ 0.05) between DH82 and DH82pi cells were not observed.

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<td></td>
<td></td>
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<td>3d</td>
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<td>DH82</td>
<td>95.13%</td>
<td>96.26%</td>
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<tr>
<td></td>
<td>(94.66%; 96.82%)</td>
<td>(95.88%; 97.72%)</td>
<td>(98.27%; 98.57%)</td>
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<tr>
<td>DH82pi</td>
<td>98.09%</td>
<td>96.46%</td>
<td>98.39%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(95.15%; 99.17%)</td>
<td>(95.21%; 97.13%)</td>
<td>(97.71%; 98.83%)</td>
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<td>p-value</td>
<td>≥ 0.01</td>
<td>≥ 0.01</td>
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Table 3:
The table depicts the percentage of cortical cortactin expression in non-infected (DH82) and persistently CDV-Ond infected (DH82pi) cells at different time points post seeding. The values are shown as median (minimum; maximum). Statistically significant differences in the percentage of cells with a cortical cortactin expression between DH82 and DH82pi cells are highlighted in grey ($p \leq 0.05$).

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<td>(36.67%; 90.00%)</td>
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<td>DH82pi</td>
<td>45.00%</td>
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<td>(13.33%; 63.33%)</td>
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<td>p-value</td>
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</table>
Figure 1

(a) Cell doubling assay

(b) Apoptotic rate (1 d)

(c) Migration assay (6 h)

(d) Migration assay (24 h)

- Persistently CDV-Ond infected DH82 cells
- Non-infected DH82 cells
Figure 2
Figure 3

Relative cortactin gene expression [in % of GAPDH]

RT-qPCR (1 d)

Non-infected DH82 cells
Persistently CDV-Orf infected DH82 cells
Figure 4
## Supplemental table S1:

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<th>Canine gene symbol</th>
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Chapter 4
Chapter 4:
Variations on brain microglial gene expression of MMPs, RECK, and TIMPs in inflammatory and non-inflammatory diseases in dogs.

Stein, V. M., C. Puff, S. Genini, V. B. Contioso, W. Baumgärtner and A. Tipold

Matrix metalloproteinases (MMPs), MMP inhibitors (TIMPs, tissue inhibitors of matrix metalloproteinases), and the membrane-anchored glycoprotein RECK (reversion-inducing cysteine-rich protein with Kazal motifs) contribute to the pathogenesis of many CNS diseases. To assess the potential pathogenetic roles of microglial MMP, TIMP, and RECK generation in extracellular matrix breakdown, opening of the blood brain barrier (BBB) and subsequent recruitment of leukocytes in the CNS, twenty-four dogs suffering from spontaneously occurring different intracranial and extracranial (control group) diseases were examined. Microglia cells were isolated ex vivo by density gradient centrifugation and their expressions of MMP-2, MMP-9, MMP-12, MMP-13, MMP-14, TIMP-1, TIMP-2, and RECK were examined via quantitative real-time polymerase chain reaction (qPCR). Zymography on CNS tissues in selected cases was performed to assess differences at the protein level. Dogs were grouped in different disease categories according to histopathological examinations, in groups with or without inflammatory reactions, and in groups with/without contrast enhancement in advanced diagnostic imaging as a function of BBB breakdown. The results showed a significant up-regulation of MMP-9 in dogs with inflammation in the nervous system compared to dogs with non-inflammatory diseases. An increased expression of MMP-9 might lead to a facilitated invasion of white blood cells. Furthermore, down-regulation of MMP-13 was found in dogs with contrast enhancement. Zymographical data reflected MMP-2 qPCR data. In conclusion,
differential expression of MMPs and their inhibitors, but not of RECK, which might crucially influence the pathogenesis of a given disease, could be demonstrated in canine microglia. This reflects a further pathway in the microglial repertoire to respond to various disease conditions in the CNS, a characteristic that might be of particular relevance as a target for specific treatments.

Keywords: Microglia; Matrix metalloproteinase (MMP); Tissue inhibitors of matrix metalloproteinases (TIMPs); RECK (reversion-inducing cysteine-rich protein with Kazal motifs); Quantitative real-time polymerase chain reaction (qPCR); mRNA expression; Zymography

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Author contributions

VMS, CP, VBC and WB performed the experiments. VMS, CP, SG, WB and AT analyzed and discussed the data. VMS drafted the manuscript. VMS and AT were involved in the study design, helped drafting the manuscript and obtained the funding. All authors were involved in writing and critically revising the manuscript and had final approval of the submitted version.
Chapter 5

Discussion
Chapter 5: Discussion

Diseases involving histiocytic cells represent a broad spectrum of disorders from inflammatory conditions to malignant transformations (Moore, 2014; Dunster, 2016). However, a common attribute of many histiocytic cells is their ability to migrate through the surrounding microenvironment and to express or secrete factors, which modulate it (Maridonneau-Parini, 2014). To analyze disorders of histiocytic cells in a “reduced” complexity, *in vitro* studies could be performed. However, this requires detailed knowledge of the *in vitro* system used. It is well known, that long-term culture of cells might lead to morphological or functional changes of the cultured cells (Bala et al., 2011; Witek et al., 2016). For example, the embryonic cardiomyocyte cell line H9C2 developed morphological changes with increasing passage number and react different to toxic substances (Witek et al., 2016). Comparable to these studies, morphological variations had been demonstrated in DH82 cells with increasing passage number (Heinrich et al., 2015). In early passages, DH82 cells displayed a roundish morphology whereas late passages exhibited an elongated to spindle-shaped appearance (Heinrich et al., 2015). Independent of the cellular morphology which might be less important for cell function, a marked decrease in the mean percentage of DH82 cells expressing typical histiocytic surface markers like CD11c, CD14, CD18, CD45 and CD80 was noted in late passages compared to early ones (Heinrich et al., 2015). This might indicate a markedly decreased reduction of typical histiocyctic functions such as antigen recognition and presentation, cellular adhesion and phagocytosis (Lavin and Merad, 2013, Heinrich et al., 2015). Taken together, the present results highlight the importance of an adequate passage number to ensure reproducibility of *in vitro* findings. However, a detailed study regarding specific functional capabilities of DH82 cells at different passages was not performed and has to be clarified in further investigations.
In addition to the aforementioned functions of histiocytic cells, many of them can migrate through the extracellular matrix (Wiesner et al., 2014). Beside this migration in inflammatory disorders and tissue remodeling after injury, macrophage infiltration takes place in many tumors (tumor associated macrophages; Wiesner et al., 2014). Furthermore, neoplastic cells invade and migrate through the extracellular matrix as an early event in metastasis formation (Samatov et al., 2015). DH82 cells represent both, tumor cells derived from a disseminated tumor and histiocytic origin (Wellman et al., 1988). Therefore, they represent the interesting, unique possibility to combine the analysis of typical histiocytic functions with investigation of the pathogenesis of the most dreaded consequence of a malignant tumor – metastasis. Migration of histiocytic cells is mostly mediated either by podosomes (in non-neoplastic cells) or by invadopodia (in transformed cells; Linder and Wiesner, 2015). A main structural component of both, podosomes and invadopodia, is the cytoskeletal protein cortactin, which is commonly overexpressed in various tumor types, often associated with a poorer prognosis for the affected individual (Cai et al., 2010; Folio et al., 2011; Gang et al., 2013). In the present study, the cortactin expression and distribution as well as the migratory activity of non-infected and persistently canine distemper virus infected DH82 cells were evaluated (Pfankuche et al., in prep.). The persistent canine distemper virus infection was chosen as a model for viral oncolysis with the advance of >90% infected cells in the persistently infected cultures. The aim was to inhibit invadopodia formation and consecutive cell migration (Pfankuche et al., in prep.). This would be an elegant alternative to improve prognosis and survival time of cancer patients. The so far existing drugs targeting pathways like the Src signaling, which also would lead to a reduced invadopodia formation are hitherto lacking or in early developmental stages (Eckert and Yang, 2011). Interestingly, although the persistent canine distemper virus infection led to a reduced migration rate compared to non-
infected controls, the number of cortactin positive cells was not significantly different (Pfankuche et al., in prep.). Similar findings are described for breast cancer cell lines where the invasive potential was not associated with the total amount of cortactin but with membranous cortactin localization (Bowden et al., 1990). At early time points after seeding, analogous results were obtained in the present study. At these time points, non-infected DH82 cells exhibited a pronounced membrane-associated cortactin expression whereas persistently canine distemper virus infected DH82 cells showed a diffuse cortactin distribution (Pfankuche et al., in prep.). The latter is attributed to an “inactive” state of the cell with reduced invadopodia formation and concurrent reduced locomotion (Saitoh et al., 2009). Taken together, a canine distemper virus infection of histiocytic sarcoma cells decreased the migration capacity in vitro and is associated with a diffuse cytoplasmic cortactin distribution. Although this might indicate a reduced metastatic potential in vivo, the latter has to be substantiated in further studies.

Another important histiocytic cell type, involved in retention of tissue homeostasis, represent microglia (Aguzzi et al., 2013). These cells are, like other histiocytic cells, involved in various diseases, ranging from neuroinflammation to gliomas, where they often represent a large population of tumor-infiltrating cells comparable to tumor associated macrophages in other neoplasms (Könnecke and Bechmann, 2013). Furthermore, comparable to other histiocytic cells, they can produce matrix metalloproteinases (MMPs) and their inhibitors (tissue inhibitors of metalloproteinases, TIMPs) constitutively and after stimulation (Crocker et al., 2006; Könnecke and Bechmann, 2013; Lee and Kim, 2014). However, these enzymes are expressed at low levels in normal central nervous system, while they are often up-regulated upon stimulation and in various pathological conditions (Yong et al., 2001; Brkic et al., 2015). In the present study the transcript number of MMP-2, MMP-9,
MMP-12, MMP-13, MMP-14, TIMP-1 and TIMP-2 as well as of the reversion inducing cysteine-rich protein with kazal motifs (RECK), another MMP inhibitor, was analyzed in microglia isolated from dogs with central nervous system disorders including intracranial tumors and inflammation. Interestingly, the different disease categories were not necessarily associated with a specific MMP and TIMP pattern, suggesting a relatively stereotypic microglial reaction pattern (Stein et al., 2011). However, an increased number of MMP-9 transcripts was demonstrated in inflammatory lesions (either primary or tumor-associated) compared to animals of the group “other intracranial diseases” (Stein et al., 2011). The latter includes dogs with hydrocephalus, meningomyelocele, head trauma and cerebellar abiotrophy (Stein et al., 2011). This up-regulation of MMP-9 might facilitate infiltration of leukocytes since microglia are considered as main mediators of neuroinflammation (Bai et al., 2014). Summarized, microglia represent a source for MMPs and their inhibitors, which are differently expressed upon stimulation but lack a disease-specific transcript pattern.
Chapter 6

Summary
Chapter 6: Summary

Investigation upon the phenotypic modulation of histiocytic cells

Vanessa Bono Contioso

Histiocytic cells represent an important, heterogeneous cell family, involved in innate immune response and maintaining of tissue homeostasis. However, beside these physiological functions, proliferative disorders originating from histiocytic cells could occur, the most devastating one being the disseminated histiocytic sarcoma. This malignant tumor exhibits a poor prognosis in both, humans and dogs. Since the analysis of pathogenetic mechanisms remains difficult in tissue sections, in vitro studies are one way to overcome these limitations. One possibility represents the permanent canine histiocytic sarcoma cell line (DH82 cells) originally isolated from a dog with disseminated histiocytic sarcoma. The poor prognosis of individuals suffering from histiocytic sarcoma is often the result of ineffective therapies highlighting the need for alternative treatment strategies. An interesting approach might be the oncolytic virotherapy, in which viruses are used to destroy tumor cells while largely sparing non-transformed tissues. Canine distemper virus (CDV), a morbillivirus closely related to the often studied measles virus, might be a promising candidate of such an oncolytic virus. Besides direct elimination of neoplastic cells, oncolytic viruses can modify the tumor microenvironment and / or the function of transformed cells. Metastasis is one of the most common, fatal consequences of tumors. For this process different steps are required including modification of the cytoskeleton to allow cell migration, regulation of adhesion molecules and degradation of the extracellular matrix for example by matrix metalloproteinases.
The aims of the present study were (i) to analyze the expression of cell surface molecules in different passages of DH82 cells to identify possible inter-passage variances which might be crucial to interpretation and transferability of the results to in vivo mechanisms and (ii) to determine the expression and distribution of cortactin, an important molecule needed for cell migration, in DH82 cells with or without persistent canine distemper virus infection.

Furthermore, the third aim of the study was to investigate the expression of matrix-metalloproteinases and their inhibitors in canine microglia, specialized central nervous system histiocytic cells, in different intracranial diseases.

Comparable to studies in other cell types, DH82 cells revealed a different morphology at early and late passages, and, more intriguingly, a down-regulation of typical histiocytic surface markers such as CD11c, CD14, CD18, CD45 and CD80. The latter might be crucial for result interpretation obtained by using different passages in experimental setups.

Beside the expression of cell surface molecules, many histiocytic cells, including DH82 cells, are able to migrate by rearrangement of the cytoskeleton. Remarkably, DH82 cells persistently infected with CDV displayed a reduced migratory capacity compared to non-infected controls, which might account for a reduction in the metastatic potential. Evaluation of cortactin revealed the same percentage of cells expressing this protein independent of the infection status. However, non-infected DH82 cells possessed a membrane-accentuated distribution at 1 and 3 days post seeding whereas persistently CDV-infected cells showed a diffuse cytoplasmic expression. The latter is described as being the “inactive” state, which might account for the reduced migration.
Microglia, resident central nervous system histiocytic cells, are able to modify their microenvironment by producing MMPs and their inhibitors. In different intracranial diseases the expression of MMPs and their inhibitors is up-regulated. However, this reaction seems to be widely independent of the underlying cause with the result that no disease-specific expression pattern could be recognized.

In conclusion, histiocytic cells and their migration ability as well as their modification of the surrounding microenvironment, play an important role in different diseases ranging from intracranial disorders to malignant histiocytic neoplasms. However, in in vitro studies, the passage number used, has to be taken into account in interpretation of the results.
Chapter 7

Zusammenfassung
Chapter 7: Zusammenfassung

Studien über die phänotypische Modulation histiozytärer Zellen

Vanessa Bono Contioso


Ähnlich wie bei Studien anderer Zelltypen zeigten DH82-Zellen eine verschiedene Morphologie in frühen und späten Passagen. Wichtiger ist jedoch, dass es bei späten Passagen zu einer Herabregulierung typischer, histiozytärer Oberflächenmarker wie CD11c, CD14, CD18, CD45 und CD80 kam. Diese können entscheidend bei der Interpretation der Befunde sein, die durch Untersuchung verschiedener Passagen zustande kamen.

Neben der Expression von Zelloberflächenmolekülen besitzen viele histiozytäre Zellen die Fähigkeit zur Migration durch Reorganisation des Zytoskeletts.
Bemerkenswerterweise ist die Migrationsfähigkeit bei persistierend CDV-infizierten DH82-Zellen verglichen mit nicht-infizierten Zellen herabgesetzt, was für eine Reduktion des Metastasierungs potentials sprechen könnte. Die Cortactin-Analyse ergab einen gleichen Prozentsatz exprimierender Zellen, unabhängig davon, ob sie eine CDV-Infektion aufwiesen oder nicht. Nichtsdestotrotz zeigte sich 1 und 3 Tage nach Aussaat eine randständige Expression bei nicht-infizierten Zellen, wohingegen persistierend CDV-infizierte Zellen eine diffuse, zytoplasmatische Verteilung aufwiesen. Letztere ist als „inaktive“ Form beschrieben, die für die verminderte Migration verantwortlich sein könnte.

Mikroglia sind ortsständige, histiozytäre Zellen des zentralen Nervensystems, die ihre Umgebung durch die Produktion von MMPs und ihren Inhibitoren modifizieren können. Bei verschiedenen, intrakraniellen Erkrankungen war die Expression von MMPs und ihren Inhibitoren aufreguliert, wobei dies weitgehend unabhängig von der zugrundeliegenden Erkrankung erfolgte, so dass kein krankheitsspezifisches Expressionsmuster festgestellt werden konnte.

Zusammengefasst handelt es sich bei histiozytären Zellen um Zellen, die durch ihre Migrationsfähigkeit und ihre Modifikation des umgebenden Mikromilieus eine wichtige Rolle bei verschiedenen Erkrankungen, von intrakraniellen Läsionen bis zu malignen, histiozytären Neoplasien, spielen. Dabei sollte bei in vitro-Studien die Passagezahl bei der Interpretation der Ergebnisse berücksichtigt werden.
Chapter 8

References
Chapter 8: References


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Chapter 9

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Chapter 9: Acknowledgements

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