Institute of Veterinary Pathology, Department of Veterinary Medicine

Freie Universität Berlin

Interleukin-2 Receptor Expression in Canine Cutaneous Mast Cell Tumors

Thesis submitted for the fulfillment of a

Doctor of Philosophy (Ph.D.) degree in Biomedical Sciences

at the

Freie Universität Berlin

submitted by

Anja Marina Meyer

Veterinarian from Hennigsdorf

Berlin 2013

Journal-No.: 3633

Printed with permission of the Department of Veterinary Medicine
of the Freie Universität Berlin

Dean:	Univ.-Prof. Dr. Jürgen Zentek
First Reviewer:	Prof. Dr. Robert Klopfleisch
Second Reviewer:	Univ.-Prof. Dr. Barbara Kohn
Third Reviewer:	PD Dr. Christine E. Hellweg

Descriptors (according to CAB-Thesaurus):
Dogs, immunohistochemistry, interleukin 2, mast cells, neoplasms, real time PCR, receptors

Day of Doctorate: 15.05.2013

ISBN: 978-3-86387-317-2

Choriner Str. 85 - 10119 Berlin
verlag@menschundbuch.de – www.menschundbuch.de

Aus dem Institut für Tierpathologie

des Fachbereichs Veterinärmedizin der Freien Universität Berlin

---

Interleukin-2-Rezeptor Expression in kaninen kutanen Mastzelltumoren

Inaugural-Dissertation

zur Erlangung des Doctor of Philosophy (Ph.D.)-Grades

in Biomedical Sciences

an der

Freien Universität Berlin

vorgelegt von

Anja Marina Meyer

Tierärztin aus Hennigsdorf

Berlin 2013

Journal-Nr.: 3633

Gedruckt mit Genehmigung des Fachbereichs Veterinärmedizin
der Freien Universität Berlin

Dekan:	Univ.-Prof. Dr. Jürgen Zentek
Erster Gutachter:	Prof. Dr. Robert Klopfleisch
Zweiter Gutachter:	Univ.-Prof. Dr. Barbara Kohn
Dritter Gutachter:	PD Dr. Christine E. Hellweg

Deskriptoren (nach CAB-Thesaurus):
Dogs, immunohistochemistry, interleukin 2, mast cells, neoplasms, real time PCR, receptors

Tag der Promotion: 15.05.2013

Bibliografische Information der *Deutschen Nationalbibliothek*
Die Deutsche Nationalbibliothek verzeichnet diese Publikation in der Deutschen Nationalbibliografie; detaillierte bibliografische Daten sind im Internet über <http://dnb.ddb.de> abrufbar.

ISBN: 978-3-86387-317-2
Zugl.: Berlin, Freie Univ., Diss., 2013
Dissertation, Freie Universität Berlin
D 188

Choriner Str. 85 - 10119 Berlin
verlag@menschundbuch.de – www.menschundbuch.de

In Erinnerung an meinen Opa

Contents

List of Abbreviations

5-HT1AR	**5-H**ydroxy**t**ryptamine **R**eceptor **1A**
AgNOR	**Ar**g**y**rophilic **N**ucleolar **O**rganizer **R**egions
CD25s	**S**oluble form of **CD25**
et al.	**et al**ii (latin for “and others”)
FNA	**F**ine**-N**eedle **A**spiration
HEK 293 cells	**H**uman **E**mbryonic **K**idney **293** cells
Hpf	**H**igh **P**ower **F**ield
IHC	**I**mmuno**h**isto**c**hemstry
IL-2	**I**nter**l**eukin**-2**
IL-2R	**I**nter**l**eukin**-2 R**eceptor
IL-2Rα	**I**nter**l**eukin**-2 R**eceptor (subunit) **α**
IL-2Rβ	**I**nter**l**eukin**-2 R**eceptor (subunit) **β**
IL-2Rγ	**I**nter**l**eukin**-2 R**eceptor (subunit) **γ**
JAK	**J**anus **K**inase
MAPK	**M**itogen**-A**ctivated **P**rotein **K**inase
MCT	**M**ast **C**ell **T**umor
MI	**M**itotic **I**ndex
NK cells	**N**atural **K**iller cells
NKT cells	**N**atural **K**iller **T** cells
PCNA	**P**roliferating **C**ell **N**uclear **A**ntigen
PCR	**P**olymerase **C**hain **R**eaction
PI3K	**P**hosphat**i**dylinositol **3**-**K**inase
SCF	**S**tem **C**ell **F**actor
SM	**S**ystemic **M**astocytosis
STAT	**S**ignal **T**ransducers and **A**ctivators of **T**ranscription
Treg	**Reg**ulatory **T** cells
γc	Common **γ**-**c**hain
WHO	**W**orld **H**ealth **O**rganization

List of Tables

List of Figures

List of Supplemental Material

1 Introduction

1.1 Canine Mast Cell Tumors (MCT)

The skin is the organ most commonly affected by neoplasms in dogs (Bronden, 2010). Of these, canine mast cell tumors (MCT) are the most common skin tumors with a continuously prevalence between 14 % and 21 % (Bostock, 1986; Brodey, 1970; Kaldrymidou, 2002; Rothwell, 1987). Despite the fact that normal mast cells are most frequent in the lung and gastrointestinal tract, the vast majority of canine MCT arise in the skin (London, 2003). Extracutaneous MCT are described rarely and occur in the gastrointestinal tract and infrequently in the oral cavity and the nasopharynx (Iwata, 2000; Ozaki, 2002; Patnaik, 1982).

Canine cutaneous MCT can occur as primary multiple neoplasms, which are regarded as independent events of new MCT formation rather than metastatic events (Boston, 2011). Multiple MCT in dogs account for up to 21 % of all canine MCT and are considered to have a generally good prognosis and a low rate of metastasis (Hottendorf, 1968; Mullins, 2006; Murphy, 2006). Since the clinical outcome of multiple MCT does not differ from that of single MCT, each tumor should be regarded as single entity in terms of therapeutical approaches (Boston, 2011; Murphy, 2006). Canine visceral MCT display a more malignant manifestation of canine MCT and are referred to as systemic mastocytosis (SM) or disseminated mastocytosis. SM most often involves the bone marrow, spleen, liver and lymph nodes (O'Keefe, 1987; Thamm, 2007). In the vast majority of SM cases, an undifferentiated cutaneous MCT with a metastatic spread is assumed to be the origin of SM, whereas a primary visceral origin is rare (O'Keefe, 1987; Takahashi, 2000; Thamm, 2007). Commonly these tumors have an extremely poor prognosis with most dogs dying within few months due to tumor-related disease (O'Keefe, 1987; Takahashi, 2000).

Despite differences in clinical presentation and pathomorphologic differentiation, canine MCT display the second common malignant neoplasm in dogs (Bronden, 2010). Therefore an early diagnosis and an adequately performed monitoring are necessary for these tumors.

1.1.1 Diagnostic Approach to MCT

The diagnostic workup for canine MCT displays a cascade of diagnostic steps that mainly depends on the amenability of a wide surgical excision and the presence or absence of negative prognostic factors. Macroscopically, MCT present as cutaneous or subcutaneous, partly alopecic nodules that may be ulcerated. The surrounding tissue can be edematous

and tumor manipulation can induce mast cell degranulation with subsequent erythema and wheal formation referred to as *Darrier's sign* (Thamm, 2007). Concomitant clinical symptoms include vomiting and diarrhea due to a relatively high percentage of gastrointestinal ulcers caused by elevated histamine levels (Fox, 1990; Howard, 1969).

In general, fine-needle aspiration (FNA) cytology is an easy and convenient method for a first diagnosis of canine MCT. The neoplastic mast cell appears as a round cell, with a round to oval, centrally located nucleus and different amounts of metachromatic cytoplasmic granules. These granules stain positive with Toluidine-blue and Giemsa stains but not with the often used Diff-Quick stain for cytologic specimens (London, 2003; Thamm, 2007). However, in every case of a confirmed MCT diagnosis a subsequent wide surgical excision biopsy is necessary because FNA cytology is not sufficient for tumor grading and consequent prognostication (Bostock, 1986; Thamm, 2007). In case of insufficient surgical excision or negative prognostic factors additional diagnostic steps prior to therapy include lymph node aspiration, abdominal ultrasound and a complete blood count (Thamm, 2007). Recently a staging system to determine metastatic mast cell disease in lymph node FNA specimens has been introduced (Krick, 2009). However, a precise prediction is not possible mainly due to the fact that scattered mast cells also occur in normal lymph node aspirates. Accordingly lymph node evaluation should rather be made by an incisional biopsy and histopathology (Bookbinder, 1992). Abdominal ultrasound is a sensitive method to determine metastatic spread to visceral organs. FNA should be performed either in case of ultrasonographically abnormal organs or of liver and spleen of dogs with a known grade III MCT according to Patnaik grading system (see chapter 1.1.2.1) (Stefanello, 2009). In comparison thoracic radiographs are of less importance because pulmonary metastases are very rare (London, 2003; Thamm, 2007). A bone marrow aspiration is not indicated for routine diagnostic but in cases of tumor recurrence, new tumor development or an abnormal heamogram like monocytosis, eosinophilia or thrombocytopenia. In any case, the prevalence of bone marrow involvement is very low at 4.5 % (Endicott, 2007).

1.1.2 Grading Systems and Their Prognostic Significance

Since canine MCT have been diagnosed it has been tried to categorize them according to their histological pattern and biological behavior. Three main histological grading systems were established, representing the traditionally 3-tier grading systems by Bostock (1973) and Patnaik et al. (1984) and the new 2-tier grading system by Kiupel et al. (2010). The underlying characteristics used for the grading systems could be summarized as (A) "cell features" for the Bostock grading system with cellular morphology, cellularity and nucleus-

cell-ratio, (B) “whole tumor features” for the Patnaik grading system with additional nuclear morphology, tumor extension and stromal reaction and (C) as “nuclear features” for the Kiupel grading system, only based on nuclear morphology and mitotic index (Bostock, 1973; Kiupel, 2010; Patnaik, 1984). A comparison of the currently widely used Patnaik grading system and the new 2-tier Kiupel grading system is shown in Table 1.

Table 1 Histological Grading According to Patnaik (1984) and Kiupel (2010)

	Patnaik (1984)			**Kiupel (2010)**	
Histological grade	**I**	**II**	**III**	**low-grade**	**high-grade**
Extension	dermis	dermis, subcutis, (deep tissue)	dermis, subcutis, deep tissue		
Cellularity		moderate to high	high		
Cellular morphology	monomorphic	moderately pleomorphic	pleomorphic		
Cytoplasmic granules	+++	++	+ / -		
Nuclear morphology	round	round, indented, 1 nucleolus	round, indented, ≥ 1 nucleoli	< 3 bizarre nuclei / 10 hpf	karyomegaly, ≥ 3 bizarre nuclei / 10 hpf
Multinucleated cells	-	- / +	++	< 3 / 10 hpf	≥ 3 / 10 hpf
Mitotic index	0 / hpf	0-2 / hpf	3-6 / hpf	< 7 / 10 hpf	≥ 7 / 10 hpf
Edema/ necrosis	- / +	++	+++		

hpf: high power field

1.1.2.1 Patnaik Grading System

In 1984 Patnaik et al. developed a grading system that is still today the most established and commonly used system. It is mainly based on cellular differentiation and tumor invasiveness and is composed of grade I, grade II and grade III tumors, a nomenclature that is in reverse order to that used by Bostock in 1973. Patnaik grade I tumors are confined to the dermis and consist of well-differentiated, round, monomorphic cells with intracytoplasmic granules and cells are arranged in rows or small groups separated by mature collagen. The cells contain a round nucleus without evidence of mitotic activity. Grade II tumors are moderately to highly cellular, extend to the dermis and lower subcutaneous tissue and rarely to the skeletal muscle. The cells are moderately pleomorphic with mainly round to ovoid cells but scattered

spindle and giant cells and contain intracytoplasmic granules. Cell nuclei are round to indented with single nucleoli. Occasional double nuclei and areas of edema and necrosis are present and the mitotic index (MI) is 0 to 2 per high power filed (hpf). Grade III tumors are highly cellular and composed of pleomorphic cells arranged in closely packed sheets with fine or no evident intracytoplasmic granules. The tumors usually infiltrate the subcutaneous and deep tissue and contain large areas of edema, hemorrhage and necrosis. The round to indented nuclei have one or more nucleoli and binucleated and multinucleated cells are common. The MI ranges between 3 and 6 mitotic figures per hpf (Patnaik, 1984).

1.1.2.2 Kiupel Grading System

Because of difficulties to consistently diagnose and predict the behavior of the intermediate grade II tumors, Kiupel et al. proposed in 2010 a new 2-tier grading system composed of high-grade and low-grade tumors. The diagnostic criteria are based on nuclear morphology and the number of mitotic figures with a clear definition of measurable and therefore more objective criteria. Thus a high-grade tumor is defined to fulfill at least one of the following criteria: at least 7 mitotic figures in 10 hpf, at least 3 multinucleated cells with 3 or more nuclei in 10 hpf, at least 3 bizarre nuclei in 10 hpf or the presence of karyomegaly with an at least 2-fold nuclear diameter in at least 10 % of the cells (Kiupel, 2010).

1.1.2.3 Clinical Staging

A modified clinical staging system of the World Health Organization (WHO) is used to determine the extent of MCT disease and to define adequate treatment strategies. Accordingly, canine MCT are categorized into stage 0, I, II, III and IV. Stage 0 tumors are incompletely excised tumors without regional lymph node involvement. Stage I and II are solitary tumors confined to the dermis without (I) or with (II) regional lymph node involvement. Multiple tumors or large infiltrating tumors with or without regional lymph node involvement are classified as stage III tumors whereas any tumor with distant metastasis or recurrence with metastasis is defined as stage IV tumor (Owen, 1980; Thamm, 2007).

1.1.2.4 Prognostic Significance

The histological grade is the most consistent and independent prognostic factor and highly predictive for survival, metastatic potential and clinical outcome (London, 2003; Thamm, 2007). Numerous studies demonstrated that the survival time is different between tumor

grades and significantly shorter in dogs with poorly differentiated tumors compared to well or intermediately differentiated tumors (Abadie, 1999; Bostock, 1973; Murphy, 2004; Patnaik, 1984). Considering the new 2-tier grading system, dogs with high-grade tumors also have a significantly shorter survival time; however it has to be considered that this grading system is strongly based on the MI, which is also known to be an independent prognostic factor for survival (Kiupel, 2010; Preziosi, 2004; Romansik, 2007). Other factors significantly influenced by tumor grade are local recurrence and time to metastasis (Kiupel, 2010; Murphy, 2004).

Clinical stage has been significantly associated with tumor-free interval and survival with a worsening in prognosis with advanced clinical stage (Krick, 2009; Thamm, 1999; Turrel, 1988). However, multiple dermal tumors that are categorized as stage III tumors do not necessarily have a worse prognosis than single tumors of stage 0-II (Mullins, 2006; Murphy, 2006).

1.1.3 Additional Prognostic Factors

Prognostic factors are of major importance to assess the right diagnostic workup, to try to predict the biological behavior of MCT and to direct the course of the appropriate therapy. Numerous prognostic factors have been proposed; however most of them do not display independent factors but are associated with histological tumor grade (see above). Significant prognostic factors for survival are: histological grade, clinical stage, breed, tumor location, tumor size, metastasis, local recurrence, complete surgical excision, intratumoral vessel density, mitotic index and frequency of proliferation markers (Bostock, 1986; Kiupel, 2010; Mullins, 2006; Patnaik, 1984; Preziosi, 2004; Seguin, 2006; Stefanello, 2009; Thamm, 1999; Turrel, 1988).

1.1.3.1 Breed

Several breeds develop canine MCT more frequently than others and the boxer is the most consistent predisposed breed in virtually all studies. Besides, there is a predisposition for Labrador and golden retrievers as well as terriers with Boston terriers in particular (Bostock, 1973; Cohen, 1974; Patnaik, 1984; Thamm, 1999). Other breeds often affected are schnauzers, cocker spaniels, shar-peis and Australian cattle dogs (Baker-Gabb, 2003; Miller, 1995; Patnaik, 1984; Thamm, 1999). Golden retrievers seem to be predisposed for multiple MCT and gastrointestinal MCT occur more frequently in miniature breed dogs and especially Maltese (Murphy, 2006; Ozaki, 2002; Takahashi, 2000). Despite the high incidence for

boxers, this breed tends to have well differentiated tumors and therefore often carry a better prognosis with a significant longer survival time (Bostock, 1973; Bostock, 1986).

1.1.3.2 Location and Tumor Size

Cutaneous MCT occur most frequently at the trunk, followed by the limbs and less frequently at the head and neck (Bostock, 1973; Rothwell, 1987; Simoes, 1994). The tumor location as prognostic factor is considered controversially. Tumors arising in the oral cavity, nail bed, inguinal and perianal region were found to have a worse prognosis, whereas survival time was not significantly different in another study (Sfiligoi, 2005; Thamm, 2006; Turrel, 1988). Tumors of primary visceral origin including gastrointestinal MCT have a generally poor prognosis (Takahashi, 2000). Tumors larger than 3 cm and rapid tumor growth are significantly associated with a shorter survival time (Bostock, 1973; Mullins, 2006).

1.1.3.3 Proliferation Markers

The proliferation rate of tumor cells is an indicator for tumor growth and often associated with malignant behavior. Therefore the direct measurement of dividing cells by assessing the MI per hpf is often used to evaluate the malignancy of tumors (Kuntz, 1997; Sanchez, 2007; Sarli, 2002). For canine MCT the MI is an independent prognostic factor that is significantly associated with survival (Preziosi, 2004; Romansik, 2007). Accordingly, the median survival time is 5 month for tumors with > 5 mitotic figures in 10 hpf compared with 70 month for tumors with ≤ 5 mitotic figures in 10 hpf (Romansik, 2007).

The three commonly used proliferation markers are argyrophilic nucleolar organizer regions (AgNOR), Ki-67 and proliferating cell nuclear antigen (PCNA) which are used to indirectly measure the cell proliferation in many types of tumors (Laprie, 2001; Pena, 1998; Sarli, 2002). AgNOR are areas in the nucleus that are associated with proteins for gene transcription and can be visualized by silver staining and therefore can be used for formalin-fixed and paraffin embedded biopsy specimens and FNA cytologic specimens (Kravis, 1996). PCNA and Ki-67 are proteins that are expressed during the cell cycle and are detected by immunohistochemistry. High scores for Ki-67, AgNOR and PCNA in canine MCT are significantly associated with a shorter survival time, tumor recurrence and development of metastases (Abadie, 1999; Bostock, 1989; Scase, 2006; Seguin, 2006; Webster, 2007). Ki-67 is also predictive for survival within Patnaik grade II tumors (Abadie, 1999; Scase, 2006).

1.1.3.4 KIT Receptor and *c-KIT* Mutation

Normal and neoplastic mast cells express the growth factor receptor KIT. An aberrant diffuse or perinuclear cytoplasmic protein expression of the commonly membrane bound KIT shows a correlation with tumor grade and is significantly more present in higher grade tumors (Giantin, 2012; Gil da Costa, 2007; Preziosi, 2004). It has been correlated with a poor prognosis due to local recurrence and a decrease in the disease-free interval and survival (Webster, 2004; Webster, 2008). Mutations in the encoding *c-KIT* gene occur more frequently in higher grade and thus more dedifferentiated tumors (Downing, 2002; Zemke, 2002). They are also significantly associated with a worse prognosis including an increase in local recurrence and tumor-related death and a decreased disease-free interval and survival (Webster, 2006; Webster, 2008). Because of the correlation of *c-KIT* mutations with an increase in cell proliferation they have been proposed to contribute to MCT growth (Webster, 2007).

1.1.4 Molecular Carcinogenesis

Carcinogenesis is the process of initiating and promoting cancer by transformation of normal to neoplastic cells which is caused by promotion of autonomous cell proliferation (oncogenes) or failure of growth inhibition (tumor suppressor genes). Oncogenes emerge from mutated proto-oncogenes, the normal quiescent counterpart, and lead to production of oncoproteins which often display constitutively activated growth factor receptors (Stricker, 2010).

The proto-oncogene *c-KIT* has been identified to play a role in the molecular carcinogenesis of mast cell neoplasms in men and dogs (Longley, 1999; Ma, 1999). The encoded KIT receptor is a receptor tyrosine kinase that is activated through the stem cell factor (SCF) and contributes to cell proliferation, maturation and survival. Activating mutations in the autoinhibitory juxtamembrane region lead to a constitutively activated receptor (Roskoski, 2005). In 1996 the KIT protein was identified in canine neoplastic mast cells with the property of SCF binding (London, 1996). Internal tandem duplications were identified in exon 11, the coding region of the juxtamembrane domain, and correlated with a worse prognosis (London, 1999; Webster, 2006; Webster, 2008). Despite this landmark in MCT research, it has to be considered that only 9-17 % of canine MCT carry relevant *c-KIT* mutations (Letard, 2008; Webster, 2006; Webster, 2007; Zemke, 2002). Besides the genetic alterations an aberrant cytoplasmic KIT protein expression in addition to the normal membrane signal was identified and correlated with worse prognosis in some tumors (Reguera, 2000; Webster, 2004; Webster, 2008).

Another receptor that is expressed in human and canine neoplastic mast cells is the 5-hydroxytryptamine (5-HT, serotonin) receptor 1A (5-HT1AR) and its ligand serotonin (Froberg, 2009; Ritter, 2012). Serotonin has several functions like cell proliferation, differentiation and maturation and acts as growth factor for several types of tumors (Azmitia, 2001; Siddiqui, 2005). 5-HT1AR and serotonin are expressed in both normal and neoplastic canine mast cells and the expression in canine MCT is negatively correlated with histological tumor grade (Froberg, 2009).

1.2 The Interleukin-2 Receptor (IL-2R) and Human Mastocytosis

1.2.1 Classification of Human Mastocytosis

Human neoplastic mast cell disorders consist of a variety of subtypes that are categorized according to the WHO classification system of hematopoietic tumors (Horny, 2008). There are two main entities composed of cutaneous mastocytosis, which is limited to the skin and is referred to as the equivalent to canine MCT, and systemic mastocytosis (SM), which is a disseminated condition and defined by major and minor diagnostic criteria (Andersen, 2012; Horny, 2009; Valent, 2007; Valli, 2002).

1.2.2 CD25 in Human Mastocytosis

The expression of the interleukin-2 receptor (IL-2R) subunit CD25 in neoplastic mast cells is defined as a minor diagnostic criterion for SM in humans (Horny, 2008; Valent, 2001). This was assessed after CD25 had been shown to be a marker that distinguishes neoplastic from non-neoplastic human mast cells in patients with SM by immunohistochemistry (IHC) and flow cytometry (Baumgartner, 2008; Escribano, 1998; Krokowski, 2005; Sotlar, 2004; van Daele, 2009). Accordingly, CD25 is expressed by neoplastic bone marrow, gastrointestinal and cutaneous mast cells from SM patients but not by normal and reactive mast cells in corresponding tissues (Escribano, 2001; Hahn, 2007; Hollmann, 2008). The studies, however, were restricted to develop a diagnostic tool rather than to analyze a functional role in SM carcinogenesis or to correlate it with prognosis, as it was done in other types of human cancer (Kuhn, 2005).

1.2.3 Structure and Function of the IL-2R

IL-2R signaling plays a crucial role in the adaptive immune response. It promotes differentiation of effector T cells and development of regulatory T cells (Treg) and is in charge of T cell homeostasis and regulation of T cell tolerance (Malek, 2010).

The IL-2R (Figure 1) is a cytokine receptor that consist of three subunits, namely the α-subunit (IL-2Rα) CD25, the β-subunit (IL-2Rβ) CD122 and the common γ chain (IL-2Rγ/γc) CD132 (Leonard, 1984; Sharon, 1986; Takeshita, 1992). Functional activity of the IL-2R requires complete trimeric receptor assembly, which, after binding of the ligand interleukin-2 (IL-2), displays a stable quaternary high-affinity IL-2–IL-2R complex (Boyman, 2012; Malek, 2010; Wang, 2009). Binding of IL-2 to the IL-2R complex induces phosphorylation of IL-2Rβ through Janus kinase 1 (JAK1) and IL-2Rγ through Janus kinase 3 (JAK3). Thus different intracellular signaling pathways are initiated, for instance the phosphatidylinositol 3-kinase (PI3K) pathway, the mitogen-activated protein kinase (MAPK) pathway and the signal transducers and activators of transcription (STAT) system. Associated receptor functions include cell growth and cell survival as well as cell activity and maintenance (Boyman, 2012; Malek, 2008; Wang, 2009; Zeiser, 2008). The complete trimeric receptor is generally expressed by Treg and antigen-activated T cells but expression of single subunits is evident on other cell types as well (Boyman, 2012; Letourneau, 2009; Malek, 2008).

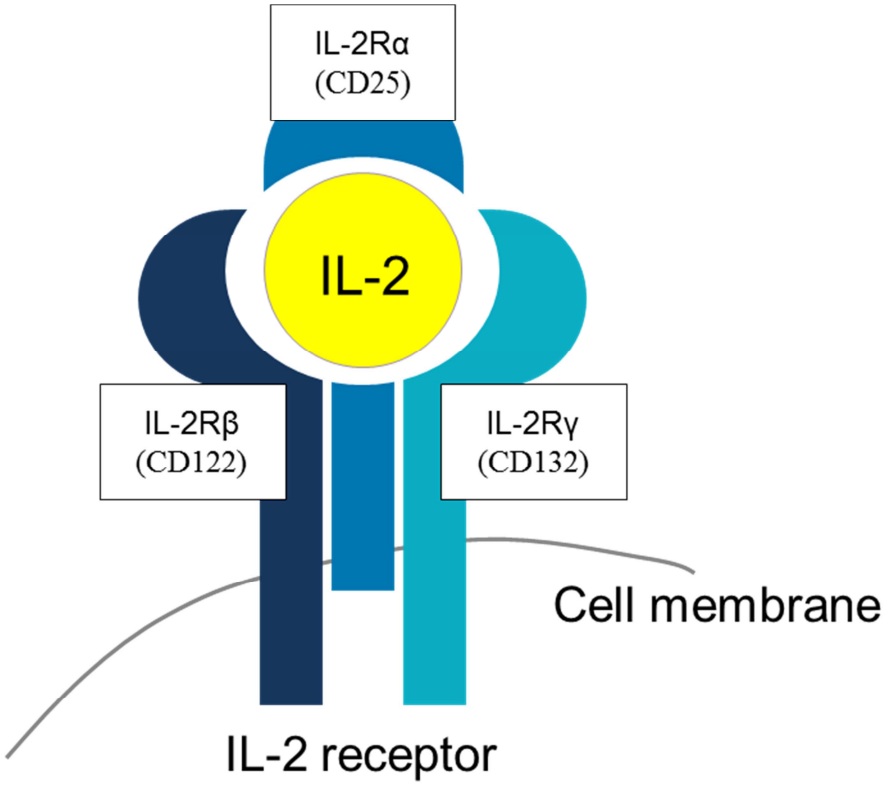

Figure 1 Schematic Structure of the Interleukin-2 Receptor

The interleukin-2 receptor is a cell surface receptor that consists of three subunits, the α-subunit CD25, the β-subunit CD122 and the common γ chain CD132. CD25 initially binds the ligand IL-2 which leads to a stable quaternary ligand-receptor complex.

1.2.4 IL-2R Subunits and the Ligand Interleukin-2 (IL-2)

The α-subunit CD25 does not participate in signaling but binds the ligand IL-2 and thus increases the affinity of the ligand–receptor interaction (Wang, 2005). Despite the expression of CD25 as part of the trimeric IL-2R there are only infrequent references of CD25 expression in lymphoid progenitor cells, antigen-presenting cells, endothelial cells and fibroblasts (Boyman, 2012). The IL-2Rβ-subunit CD122 and the common γ-chain CD132 mediate the signal transduction of the IL-2R (Nakamura, 1994). Co-expression of IL-2Rβ and the common γ-chain has been identified on fibroblast-like synoviocytes, endothelial cells, natural killer (NK) cells, natural killer T (NKT) cells, memory CD8+ T cells and some lymphoid progenitor cells. Co-expression with IL-2Rα has been found on endothelial cells, fibroblasts and occasionally on lymphoid progenitor cells (Boyman, 2012; Corrigall, 2001). The IL-2R γ-subunit or common γ-chain is necessary for IL-2R signaling (Nakamura, 1994). Apart from the IL-2R it is part of several cytokine receptors and is widely expressed on virtually all lymphoid cells and most hematopoietic cells (Kim, 2006; Letourneau, 2009; Malek, 2008).

The ligand IL-2 is a cytokine that is predominately produced and secreted by antigen-activated T cells after immunological activation. Activated dendritic cells, NK cells and NKT cells also have the ability to produce IL-2 although the biological relevance is unclear.

1.2.5 Role of IL-2R in Tumors

Single subunits, the complete receptor and/or IL-2 are also expressed by certain human tumors or cancer-derived cell lines. In addition to the most frequently affected hematopoietic tumors, including systemic mastocytosis in men, it is occasionally observed in some solid tumors like breast cancer, cervical cancer or lung cancer (Garcia-Tunon, 2004; Kasprzak, 2007; Mindiola, 2008). Furthermore, IL-2R and/or IL-2 expression is found in human and murine tumor cell lines including a murine mastocytoma cell line (Alileche, 1993; Garcia de Galdeano, 1996; Hassuneh, 1997; Lin, 1995). IL-2R and IL-2 expression in carcinoma cell lines have been associated with increased cell proliferation (Reichert, 2000; Reichert, 1998). The most striking correlation of the IL-2R and cancer, however, is a soluble form of CD25 (CD25s) since high serum levels of CD25s are related to carcinogenesis and occasionally to prognosis in human cancer (Bien, 2008; Goto, 2005; Kallio, 2001; Kuhn, 2005; Ottaiano, 2006).

1.3 Aims and Hypotheses

The prognostic assessment and therapeutical strategies of canine MCT depend highly on the biological behavior of these tumors which is mainly correlated with histological grade. However, despite the presence of *c-KIT* mutations little is known about underlying molecular mechanisms for MCT development and malignant progression that could be responsible for the diverse malignant behavior of these tumors. Recently, the IL-2R α-subunit CD25 has been introduced as a marker to distinguish neoplastic from non-neoplastic human mast cells in SM patients which leads to the assumption that the IL-2R may have an impact on the development and tumor growth of neoplastic mast cell diseases. The aims of this project were therefore to evaluate CD25 as a possible tumor marker for canine MCT, to investigate the expression of the remaining subunits and the ligand of the IL-2R and to assess the usefulness of the IL-2R as a malignancy marker for canine MCT. Accordingly, the following hypotheses were tested in this work:

First Hypothesis

The IL-2R-α-subunit CD25 is expressed by canine cutaneous MCT and differentiates neoplastic from non-neoplastic canine cutaneous mast cells.

Second Hypothesis

The complete IL-2R and the ligand IL-2 are expressed by canine cutaneous MCT. The IL-2R is increased in higher grade tumors and thus contributes to mast cell tumor proliferation.

2 Research Publications in Journals with Peer-Review

2.1 CD25 is Expressed by Canine Cutaneous Mast Cell Tumors But Not by Cutaneous Connective Tissue Mast Cells

"CD25 is Expressed by Canine Cutaneous Mast Cell Tumors But Not by Cutaneous Connective Tissue Mast Cells"

Journal of Veterinary Pathology 2012 Nov; 49(6): 988-97. doi: 10.1177/0300985812439215 Epub 2012 Mar 23, PMID: 22446323

http://dx.doi.org/10.1177/0300985812439215

2.2 All Subunits of the Interleukin-2 Receptor are Expressed by Canine Cutaneous Mast Cell Tumours

"All Subunits of the Interleukin-2 Receptor are Expressed by Canine Cutaneous Mast Cell Tumours"

Journal of Comparative Pathology 2012 Dec; doi: 10.1016/j.jcpa.2012.11.232

[Epub ahead of print]

http://dx.doi.org/10.1016/j.jcpa.2012.11.232

3 Declaration of Own Portions of Work in the Research Publications

3.1 CD25 is Expressed by Canine Cutaneous Mast Cell Tumors But Not by Cutaneous Connective Tissue Mast Cells

Authors: Meyer A, Gruber AD, Klopfleisch R

Year: 2012

Journal: *Journal of Veterinary Pathology* 49(6): 988-97

Contributions by A. Meyer: Design, preparation, completion and evaluation of the experiments, subsequent preparation of the entire manuscript

Contributions of other authors: Assistance in design and evaluation of the experiments, review of the subsequent manuscript

3.2 All Subunits of the Interleukin-2 Receptor are Expressed by Canine Cutaneous Mast Cell Tumours

Authors: Meyer A, Gruber AD, Klopfleisch R

Year: 2012

Journal: *Journal of Comparative Pathology* doi: 10.1016/j.jcpa.2012.11.232.
[Epub ahead of print]

Contributions by A. Meyer: Design, preparation, completion and evaluation of the experiments, subsequent preparation of the entire manuscript

Contributions of other authors: Assistance in design and evaluation of the experiments, review of the subsequent manuscript

4 Concluding Discussion

The link between IL-2R expression and neoplastic mast cell diseases is a comparatively new approach in human medicine and has not been investigated in veterinary medicine so far. This is paralleled by the fact that molecular mechanisms contributing to MCT development and malignancy in dogs are widely unknown as well as reasons for the heterogeneous biological behavior of canine MCT. A link between cytokine receptor activity and mast cell tumor proliferation appears probable since cytokines like IL-3, IL-4, IL-9 and IL-10 mediate the proliferation and differentiation of non-neoplastic mast cells (Renauld, 1995; Shelburne, 2001).

To this end the present work investigated the potential influence of the cytokine receptor IL-2R in canine MCT with regard to its potential use as a diagnostic marker and its possible role in MCT carcinogenesis. Accordingly, the following two hypotheses were tested here.

4.1 First Hypothesis

The IL-2R-α-subunit CD25 is expressed by canine cutaneous MCT and differentiates neoplastic from non-neoplastic canine cutaneous mast cells.

To test the first hypothesis CD25 mRNA and protein expression was investigated in canine MCT of different histological grade according to the commonly used Patnaik grading system and the new 2-tier Kiupel grading system (Kiupel, 2010; Patnaik, 1984). CD25 protein expression was further analyzed in non-neoplastic resting connective tissue mast cells of normal canine skin and in activated connective tissue mast cells from canine allergic dermatitis specimens.

On the mRNA level, CD25 was significantly increased in grade III MCT and high grade MCT compared to grade I MCT and low grade MCT, respectively (Supplement 1). This increased expression in higher grade tumors was in accordance with our assumption that CD25 and thus the IL-2R may have an impact on MCT proliferation that is known to be increased in higher grade and thus more malignant MCT (Abadie, 1999; Bostock, 1989; Romansik, 2007).

The antibody used for the protein analysis was a monoclonal mouse-anti-human antibody (clone 4C9) since neither a canine-specific antibody nor an antibody with cross-reactivity in dogs was available at the time of the study. To ensure a specific CD25 protein expression in the canine specimens, the cross-reactivity of the antibody was tested before. Canine CD25 transfected human HEK 293 cells showed a distinct CD25 membrane staining compared with

lacking signals in the corresponding negative controls which clearly demonstrated the cross-reactivity of the antibody. Subsequently, CD25 expression was evaluated by immunohistochemistry in 90 canine cutaneous MCT with detection of CD25 protein in 81 of 90 MCT (90 %), a prevalence comparable to that in human SM patients (Baumgartner, 2008; Krokowski, 2005; Sotlar, 2004). Due to the fact that CD25 is normally expressed by T cells, the number of tumor infiltrating lymphocytes was assessed by a CD3 immunolabeling and was present at up to 15 % in higher grade tumors. However, a CD25/CD3 double immunolabeling confirmed that virtually all CD25 positive cells did not co-express CD3 and therefore only a few CD25 positive T cells contribute to CD25 expression in canine MCT.

The number of CD25 positive tumors was negatively correlated with tumor grade and thereby contradictory to the mRNA results. To further specify this, a semiquantitative analysis of the percentage of CD25 positive cells per tumor was assessed and revealed also a significant decrease of CD25 positive cells with increasing tumor grade (Supplement 1). The fact that not all neoplastic mast cells expressed CD25 is also described in SM patients and most likely reflects the heterogeneity of neoplastic cells within a tumor (Hollmann, 2008). The fact that the expression was negatively correlated with tumor grade, however, is less obvious to explain. It may be due to the advanced independence of tumor cells with increasing malignancy but certainly challenge the potential influence on MCT proliferation. It may also suggest a role in initial MCT development rather than in malignant progression.

Based on this, the expression of CD25 in normal resting and reactive cutaneous mast cells was investigated by immunofluorescence double labeling of CD25 and KIT. In contrast to the consistent co-expression of CD25 and KIT in MCT cells, none of the KIT-positive non-neoplastic mast cells in healthy skin specimens expressed the CD25 protein. This was in accordance with recent findings in human medicine, where CD25 expression is used to distinguish neoplastic from non-neoplastic resting or reactive mast cells in SM patients (Sotlar, 2004; Valent, 2001). In dogs, however, few reactive mast cells of allergic dermatitis specimens expressed CD25 as well. This finding points toward a link between CD25 expression and mast cell activation as it is known for T cells in the course of immune response (Boyman, 2012). Alternatively, CD25 expression in activated mast cells and MCT may support the idea of a progression from activated mast cells to neoplastic MCT cells. This hypothetical link between inflammation and the development of cancer is well known for several other tumor types but is mostly due to growth factors in the inflammatory microenvironment and not a malignant progression of the inflammatory cell itself (Coussens, 2002). In any case, CD25 expression of few reactive cutaneous mast cells diminishes the applicability of CD25 as a reliable neoplastic mast cell marker in dogs.

Apart from its potential pro-proliferative function, CD25 expression by canine MCT could also be a mechanism to escape from anti-tumor immune response. In this scenario neoplastic mast cells compete with tumor infiltrating T cells for IL-2 and capturing of IL-2 by MCT cells would suppress the proliferation and activation of tumor infiltrating T cells. This hypothesis is supported by the findings of this study where lower grade canine MCT with high CD25 expression contained less tumor infiltrating T-cells than higher grade tumors with low CD25 expression. However, higher numbers of tumor infiltrating T cells in higher grade canine MCT could also be a general feature of malignant tumors instead of a direct effect of CD25 expression. It finally also does not explain the biological significance of decreasing CD25 expression with increasing degree of malignancy in canine MCT. In any case, CD25 alone is not sufficient for IL-2R dependent signaling but the complete receptor assembly of all three subunits is required for functional activity of the IL-2R.

4.2 Second Hypothesis

The complete IL-2R and the ligand IL-2 are expressed by canine cutaneous MCT. The IL-2R is increased in higher grade tumors and thus contributes to mast cell tumor proliferation.

Based on the results of the first hypothesis the mRNA and protein expression levels of the remaining IL-2R subunits CD122 (IL-2Rβ) and CD132 (IL-2Rγ) and the ligand IL-2 were analyzed in canine MCT of different grade and *c-KIT* mutation status. Additionally, the binding capacity of IL-2 to MCT cells was tested in an immunohistochemistry based IL-2 linking assay.

The mRNA of CD122 and CD132 was increased in higher grade tumors compared to lower grade tumors and therefore alike the mRNA expression of the α-subunit CD25. The mRNA expression of the ligand IL-2, however, was consistently decreased with increasing tumor grade. In contrast, the *c-KIT* mutation status did not have a significant influence on the mRNA expression level of the IL-2R and IL-2 (Supplement 1).

Immunohistochemistry revealed that the CD122 and CD132 proteins as well as the IL-2 protein were expressed by canine neoplastic mast cells and hence confirmed the first part of the second hypothesis. As for CD25, the number of CD122 and CD132 positive tumors as well as the percentage of positive tumor cells clearly decreased with increasing tumor grade (Supplement 1). Therefore part two of the second hypothesis has to be rejected and hence the impact on MCT proliferation and carcinogenesis has to be reconsidered. A similar down-regulation of activating receptors with increasing malignancy has been shown for the

5-HT1AR in canine MCT and for growth factor receptors in canine mammary carcinomas (Froberg, 2009; Klopfleisch, 2010; Klopfleisch, 2011). This may reflect the independence of malignant and thus more dedifferentiated tumors from external proliferation stimuli but a definitive explanation of this phenomenon has not been found so far. Nevertheless, high IL-2R expression in lower grade tumors may imply relevance for early MCT development and well differentiated tumors but less importance at later stages of malignancy. IL-2R expression is therefore not sufficient to be a malignancy marker of canine MCT but may be used as a diagnostic marker for well differentiated tumors.

CD122 and CD132 proteins were detected in 90 % and 96 % of MCT, respectively, a prevalence similar to that for CD25. Altogether, 77 % of canine MCT expressed all three subunits and hence the complete IL-2R. This is noteworthy since the trimeric receptor assembly is necessary for IL-2R signaling and consequently only tumors that express all three subunits may have the property of IL-2R dependent receptor functions. Since both subunits are also components of the IL-15 or the IL-4 receptor it can be speculated that canine MCT cells may also express other cytokine receptors than the IL-2R (Giri, 1994; Kondo, 1993). The expression of the IL-15 or the IL-4 receptor, however, has not been analyzed in canine MCT so far.

The IL-2 protein was expressed in only 64 % of canine MCT, a lower prevalence compared to the IL-2R, which was mainly due to the fact that many grade III and high grade MCT completely lacked IL-2 expression. However, the generally decreased protein expression with higher tumor grade was the same as for the IL-2R. The fact that canine neoplastic mast cells express both the IL-2R and the ligand IL-2 leads to the assumption of a possible autocrine IL-2R signaling in canine MCT. An autocrine growth regulation of the IL-2R is well known for T cells and has been recently described for cervical cancer cells (Malek, 2010; Rangel-Corona, 2010). As for *c-KIT* mutated MCT, this may be an efficient mechanism of independent tumor cell proliferation but in this case without need of an activating mutation. Referring to this assumption it had to be evaluated whether IL-2R expressing mast cells are capable of IL-2 binding. A therefore established IL-2 linking assay revealed an immunohistochemically enhanced IL-2 signal after incubation with an IL-2 peptide. This indicates that IL-2R expressing neoplastic mast cells are able to bind IL-2 and thus IL-2R signaling may be relevant for canine MCT.

The growth factor receptor KIT is related to the IL-2R/IL-2 system since the KIT ligand SCF augments IL-2 mediated proliferation of NK cells (Fehniger, 1997). A link between KIT and the IL-2R in canine MCT has been shown here insofar as CD122 and CD132 and in particular the IL-2 protein expression was consistently decreased in *c-KIT* mutated tumors in this study. Concerning a biological significance this could be both an effect and a cause.

Hypothetically *c-KIT* mutated tumors might not need other external proliferation stimuli because of the permanently activated growth factor receptor KIT. On the other hand it has been shown that activated *c-KIT* is able to phosphorylate and activate the IL-2R in the absence of IL-2 (Rocha-Zavaleta, 2004). That means that in case of a mutated and thus permanently activated *c-KIT* the ligand IL-2 might not be needed to induce IL-2R signaling.

4.3 Therapeutic Relevance of IL-2R Expression in Canine MCT

IL-2R expression in canine MCT enables potential treatment strategies besides the commonly used radiotherapy and tyrosine kinase inhibitors in case of insufficient surgery. These may include drugs like rapamycin or ciclosporin that interfere with the IL-2R pathway in lymphocytes or the novel group of recombinant immunotoxins which are fusion proteins composed of a truncated toxin and a targeting ligand (FitzGerald, 2011; Kreitman, 2000; Reichert, 1999). For the IL-2R/IL-2 system two immunotoxins are currently being tested or approved for treatment of hematological malignancies. Denileukin diftitox (DAB389-IL2) is an immunotoxin composed of IL-2 and truncated diphtheria toxin that targets the IL-2R, while LMB-2 (anti-Tac(Fv)-PE38) is composed of a CD25 antibody fused to truncated Pseudomonas exotoxin and therefore specifically targets CD25 (FitzGerald, 2011; Kreitman, 2000). Another aspect of therapeutic approaches in this context is the administration of IL-2 as antitumor treatment in human and veterinary medicine (Finocchiaro, 2012; Grande, 2006; Stewart, 2006).

Assuming that IL-2R expressing neoplastic mast cells could be stimulated by therapeutically administered IL-2 would consequently exclude canine MCT from this therapy form. In striking contrast a recently published study revealed that intratumoral IL-2 application can induce regression of non-resectable MCT in dogs (Ziekman, 2013). Possible explanations may be that exogenous IL-2 does not influence IL-2R signaling in canine MCT cells, especially because the proposed mechanism of local IL-2 application is a vascular leakage and diminished circulation in the tumor rather than an immunostimulatory effect. Another important fact is that only non-resectable and thus presumably higher grade tumors were included in that study whereas higher grade tumors in the present study had a weak IL-2R expression. Therefore it should be considered to exclude at least lower grade canine MCT with high IL-2R expression from this form of therapy or to conduct a study of therapeutical IL-2 effects on canine MCT with different histological grades.

4.4 Conclusion

The aim of this thesis was to investigate additional molecular mechanisms besides the proto-oncogene *c-KIT* that may contribute to MCT development and carcinogenesis in dogs. In that context the so-called tumor microevolution seems to be important, for instance the acquirement of molecules that are normally restricted to other cell types but display advantages for tumor survival and tumor growth. The IL-2R and particularly the α-subunit CD25 seemed to be a promising target since the receptor promotes cell activation and proliferation in immune cells and expression of CD25 was correlated with a neoplastic mast cell phenotype in humans. Therefore two hypotheses were tested here, namely that the IL-2R α-subunit CD25 is expressed by canine cutaneous MCT and differentiates neoplastic from non-neoplastic mast cells and that the remaining subunits and the ligand IL-2 are expressed as well and an increased IL-2R expression in higher grade tumors contributes to mast cell tumor proliferation.

The findings of the study supported the first hypothesis since CD25 was expressed by canine cutaneous MCT and was able to differentiate neoplastic from non-neoplastic resting mast cells but was also slightly expressed in non-neoplastic activated mast cells. The latter, however, unfortunately diminishes the applicability of CD25 as a reliable tumor marker for neoplastic mast cells in dogs. The second hypothesis was partially confirmed since the majority of canine cutaneous MCT expressed both the IL-2R and the ligand IL-2 but the protein expression was consistently decreased with increasing tumor grade. Thus IL-2R expression in canine MCT seems not to significantly contribute to MCT proliferation, at least not in malignant tumors.

Taken together this implies that the IL-2R may be more relevant for early MCT development and lower grade tumors but less important at later stages of malignancy. This should also be taken into consideration when dealing with new treatment options such as intratumoral IL-2 application because well differentiated tumors with high IL-2R expression may not be appropriate for this form of therapy.

4.5 Outlook

Results of the present study proposed that IL-2R signaling in canine MCT may contribute to tumor development and tumor cell proliferation as it was shown for IL-2R expressing carcinoma cell lines so far (Rangel-Corona, 2010; Reichert, 1998; Rocha-Zavaleta, 2004). Vital requirements for that are the expression of the trimeric receptor, the capability of the receptor to bind the ligand IL-2 and the ability to trigger corresponding downstream signaling

pathways. Here the expression of the complete IL-2R in canine MCT was demonstrated and an IL-2 linking assay gave a first hint towards an IL-2 binding capacity of IL-2R expressing MCT cells. To verify this, in vitro studies for relevant downstream signaling molecules may be useful. Furthermore, expression of the IL-2R/IL-2 system and responding to exogenous IL-2 in canine MCT cell lines could be used to assess a pro-proliferative effect in vitro. In that context also a potential interaction of IL-2 and SCF, as shown for NK cells, could be analyzed in cell culture based proliferation assays (Fehniger, 1997).

Another attempt of the study was to identify CD25 as a potential tumor marker or malignancy marker for canine MCT. However, this could not be achieved because few non-neoplastic activated mast cells expressed CD25. Unfortunately, due to technical difficulties, it was not possible to assess the expression of the remaining subunits and IL-2 in non-neoplastic mast cells. It may be possible that some of these proteins clearly differentiate neoplastic from non-neoplastic mast cells as shown for CD25 in humans (Krokowski, 2005; Sotlar, 2004). However, the more useful tool would be to establish a malignancy marker rather than a tumor marker. Unfortunately, the IL-2R and IL-2 expression was negatively correlated with tumor grade and therefore not applicable as an MCT malignancy marker. Despite the histological grade is the most consistent prognostic factor, however, a clinical follow up would be helpful to determine a correlation of IL-2R expression and a malignant phenotype besides from histological grade.

The expression of a molecule that is normally restricted to other cell types leads to the assumption of causative mutations in the corresponding gene. Based on the results of this study a molecular gene analysis of the IL-2R and IL-2 may identify underlying genomic alterations to explain their expression in the majority of canine MCT.

5 Summary

Interleukin-2 Receptor Expression in Canine Cutaneous Mast Cell Tumors

Anja Meyer

Canine cutaneous mast cell tumors (MCT) are among the most frequently observed tumors in small animals and are the most common skin tumors in dogs. Many attempts have been made to predict their biological behavior because this tumor entity occurs from relatively benign tumors with a good long-term prognosis to highly malignant tumors with short survival times. So far, the histological grade is the most independent predictive prognostic factor; however, underlying molecular mechanisms that contribute to the variable malignancy of these tumors are widely unknown so far. One important aspect is the identification of mutations in the proto-oncogene *c-KIT* which has been associated with higher histological tumor grade and also with a worse prognosis. However, only up to 17 % of canine MCT exhibit relevant *c-KIT* mutations and thus additional factors must contribute to MCT carcinogenesis.

In human mast cell diseases CD25, the α-subunit of the interleukin-2 receptor (IL-2R), has recently been defined as a minor diagnostic criterion for systemic mastocytosis (SM). The IL-2R is normally restricted to lymphoid immune cells but has also been described in several human tumors so far. Therefore the aim of the present study was to investigate the expression of the IL-2R and the ligand interleukin-2 (IL-2) in canine cutaneous MCT with regard to a potential influence on MCT development, MCT proliferation and the manifestation of a malignant phenotype. To this end the mRNA and protein expression of the three subunits of the IL-2R and the ligand IL-2 were analyzed in 90 canine cutaneous MCT and correlated with the histological grade and the *c-KIT* mutation status. Furthermore co-expression of CD25 and KIT was investigated in non-neoplastic resting and activated mast cells compared with canine MCT. Finally an immunohistochemistry based IL-2 linking assay was established to reveal a potential IL-2 binding capacity of IL-2R expressing neoplastic mast cells.

Canine neoplastic mast cells expressed both the IL-2R and IL-2 but the expression was negatively correlated with tumor grade and *c-KIT* mutation status. In contrast, non-neoplastic resting mast cells lacked CD25 expression while few activated mast cells exhibited the CD25 protein. Additionally, IL-2R expressing tumor cells were shown to be able to bind the ligand IL-2. In conclusion the IL-2R/IL-2 system seems to have an impact on canine cutaneous MCT since the complete receptor and the ligand are expressed by canine neoplastic mast cells. The decreased expression in dedifferentiated tumors, however, diminishes their potential as a malignancy marker but raises questions as to a potential influence in early

MCT development, especially since CD25 was not present in non-neoplastic resting mast cells. The ability of neoplastic mast cells to bind the ligand IL-2 suggests a pro-proliferative IL-2R function, although the negative correlation between IL-2R expression and tumor grade questions a major role in MCT proliferation. In any case, the IL-2R expression of canine MCT cells should be taken into consideration when dealing with new forms of antitumor therapies. Especially the recently proposed intratumoral IL-2 application for canine MCT should be carefully applied and monitored, at least for lower grade tumors as shown by this study.

6 Zusammenfassung

Interleukin-2-Rezeptor Expression in kaninen kutanen Mastzelltumoren

Anja Meyer

Kanine kutane Mastzelltumoren (MZT) gehören zu den häufigsten Tumoren in der Kleintiermedizin und stellen den häufigsten Hauttumor des Hundes dar. Das biologische Verhalten von MZT ist sehr variabel und reicht von relativ gutartigen Tumoren mit guter Langzeitprognose bis hin zu bösartigen Tumoren mit sehr kurzen Überlebenszeiten. Um das biologische Verhalten dieser Tumoren möglichst präzise vorhersagen, ist der histologische Tumorgrad bisher der zuverlässigste unabhängige Prognosefaktor. Die zugrunde liegenden molekularen Mechanismen, welche zu der unterschiedlich ausgeprägten Malignität dieser Tumoren beitragen, sind bisher jedoch weitestgehend unbekannt. Lediglich die Identifikation von Mutationen im Proto-Onkogen *c-KIT* stellt einen relevanten Aspekt in der Karzinogenese von kaninen MZT dar, da *c-KIT*-Mutationen mit einem hohen Tumorgrad sowie mit einer schlechteren Prognose assoziiert wurden. Einschränkend muss jedoch beachtet werden, dass nur bis zu 17 % der MZT relevante *c-KIT*-Mutationen aufweisen und somit zusätzliche Mechanismen für die Karzinogenese von MZT eine Rolle spielen müssen.

In der Humanmedizin wurde kürzlich die Expression von CD25, der α-Untereinheit des Interleukin-2-Rezeptors (IL-2R), als diagnostisches Kriterium für die systemische Mastozytose (SM) beim Menschen definiert. Eine Expression des IL-2R ist normalerweise auf lymphoide Zellen, insbesondere T-Zellen, beschränkt, wurde aber auch für einige Tumoren beim Menschen beschrieben. Ziel dieser Studie war es daher, die Expression des IL-2R und des Liganden Interleukin-2 (IL-2) in kaninen MZT zu untersuchen und deren Einfluss hinsichtlich der Tumorentstehung, der Tumorproliferation und der Ausbildung eines bösartigen Tumorphänotyps zu bewerten. Dafür wurde die mRNA- und Proteinexpression der drei IL-2R-Untereinheiten sowie des Liganden IL-2 in 90 kaninen kutanen MZT untersucht und mit dem histologischen Tumorgrad sowie dem *c-KIT*-Mutationsstatus korreliert. Weiterhin wurde die Co-Expression des KIT-Rezeptors und des CD25-Proteins sowohl in kaninen MZT als auch in nicht-neoplastischen, ruhenden und aktivierten Mastzellen der Haut analysiert. Mittels eines immunhistologischen IL-2-Bindungsasseys sollte anschließend die Fähigkeit von MZT-Zellen untersucht werden, den Liganden IL-2 zu binden.

Kanine MZT-Zellen exprimierten sowohl den IL-2R als auch IL-2, welche jedoch auf Proteinebene negativ mit dem Tumorgrad und dem Vorhandensein einer *c-KIT*-Mutation korreliert waren. Demgegenüber exprimierten nicht-neoplastische, ruhende Mastzellen der Haut kein CD25, während wenige aktivierte Mastzellen das CD25-Protein aufwiesen.

Weiterhin konnte gezeigt werden, dass IL-2R-exprimierende Tumorzellen die Fähigkeit besitzen, den Liganden IL-2 zu binden. Zusammenfassend kann somit geschlussfolgert werden, dass der IL-2R und IL-2 relevant für kanine MZT sind, da sowohl der komplette Rezeptor als auch der Ligand von neoplastischen Mastzellen exprimiert werden. Die geringere Expression in entdifferenzierten Tumoren schließt die IL-2R-Expression als Malignitätsmarker jedoch aus. Dennoch erscheint ein Einfluss des IL-2R in der Initiation von MZT durchaus möglich, vor allem da CD25 zwar in MZT aber nicht in nicht-neoplastischen, ruhenden Mastzellen exprimiert wurde. Die Fähigkeit neoplastischer Mastzellen, den Liganden IL-2 zu binden, weist weiterhin auf eine Funktion des IL-2R in der Proliferation von MZT hin, wobei die negative Expression mit ansteigendem Tumorgrad einen relevanten Einfluss zumindest für bösartige Tumoren in Frage stellt. In jedem Fall sollte die Expression des IL-2R in kaninen Mastzelltumoren bezüglich neuer IL-2-abhängiger Therapieformen beachtet werden. Vor allem die kürzlich vorgeschlagene intratumorale IL-2-Applikation sollte für kanine MZT sorgfältig überdacht werden, laut der vorliegenden Studie zumindest für gut differenzierte MZT.

7 References

Abadie, J. J., Amardeilh, M. A. and Delverdier, M. E. (1999). Immunohistochemical detection of proliferating cell nuclear antigen and Ki-67 in mast cell tumors from dogs. *J Am Vet Med Assoc,* **215,** 1629-1634.

Alileche, A., Plaisance, S., Han, D. S., Rubinstein, E., Mingari, C., Bellomo, R., Jasmin, C. and Azzarone, B. (1993). Human melanoma cell line M14 secretes a functional interleukin 2. *Oncogene,* **8,** 1791-1796.

Andersen, C. L., Kristensen, T. K., Severinsen, M. T., Moller, M. B., Vestergaard, H., Bergmann, O. J., Hasselbalch, H. C. and Bjerrum, O. W. (2012). Systemic mastocytosis--a systematic review. *Dan Med J,* **59,** A4397.

Azmitia, E. C. (2001). Modern views on an ancient chemical: serotonin effects on cell proliferation, maturation, and apoptosis. *Brain Res Bull,* **56,** 413-424.

Baker-Gabb, M., Hunt, G. B. and France, M. P. (2003). Soft tissue sarcomas and mast cell tumours in dogs; clinical behaviour and response to surgery. *Aust Vet J,* **81,** 732-738.

Baumgartner, C., Sonneck, K., Krauth, M. T., Kneidinger, M., Fodinger, M., Hauswirth, A. W., Mullauer, L. and Valent, P. (2008). Immunohistochemical assessment of CD25 is equally sensitive and diagnostic in mastocytosis compared to flow cytometry. *Eur J Clin Invest,* **38,** 326-335.

Bien, E. and Balcerska, A. (2008). Serum soluble interleukin 2 receptor alpha in human cancer of adults and children: a review. *Biomarkers,* **13,** 1-26.

Bookbinder, P. F., Butt, M. T. and Harvey, H. J. (1992). Determination of the number of mast cells in lymph node, bone marrow, and buffy coat cytologic specimens from dogs. *J Am Vet Med Assoc,* **200,** 1648-1650.

Bostock, D. E. (1973). The prognosis following surgical removal of mastocytomas in dogs. *J Small Anim Pract,* **14,** 27-41.

Bostock, D. E. (1986). Neoplasms of the skin and subcutaneous tissues in dogs and cats. *Br Vet J,* **142,** 1-19.

Bostock, D. E., Crocker, J., Harris, K. and Smith, P. (1989). Nucleolar organiser regions as indicators of post-surgical prognosis in canine spontaneous mast cell tumours. *Br J Cancer,* **59,** 915-918.

Boston, S. E. (2011). Canine Mast Cell Tumors. *2011 ACVS Veterinary Symposium*, Chicago, USA, 347-352.

Boyman, O. and Sprent, J. (2012). The role of interleukin-2 during homeostasis and activation of the immune system. *Nat Rev Immunol,* **12,** 180-190.

Brodey, R. S. (1970). Canine and feline neoplasia. *Adv Vet Sci Comp Med,* **14,** 309-354.

Bronden, L. B., Nielsen, S. S., Toft, N. and Kristensen, A. T. (2010). Data from the Danish veterinary cancer registry on the occurrence and distribution of neoplasms in dogs in Denmark. *Vet Rec,* **166,** 586-590.

Cohen, D., Reif, J. S., Brodey, R. S. and Keiser, H. (1974). Epidemiological analysis of the most prevalent sites and types of canine neoplasia observed in a veterinary hospital. *Cancer Res,* **34,** 2859-2868.

Corrigall, V. M., Arastu, M., Khan, S., Shah, C., Fife, M., Smeets, T., Tak, P. P. and Panayi, G. S. (2001). Functional IL-2 receptor beta (CD122) and gamma (CD132) chains are expressed by fibroblast-like synoviocytes: activation by IL-2 stimulates monocyte chemoattractant protein-1 production. *J Immunol,* **166,** 4141-4147.

Coussens, L. M. and Werb, Z. (2002). Inflammation and cancer. *Nature,* **420,** 860-867.

Downing, S., Chien, M. B., Kass, P. H., Moore, P. E. and London, C. A. (2002). Prevalence and importance of internal tandem duplications in exons 11 and 12 of c-kit in mast cell tumors of dogs. *Am J Vet Res,* **63,** 1718-1723.

Endicott, M. M., Charney, S. C., McKnight, J. A., Loar, A. S., Barger, A. M. and Bergman, P. J. (2007). Clinicopathological findings and results of bone marrow aspiration in dogs with cutaneous mast cell tumours: 157 cases (1999-2002). *Vet Comp Oncol,* **5,** 31-37.

Escribano, L., Diaz-Agustin, B., Bellas, C., Navalon, R., Nunez, R., Sperr, W. R., Schernthaner, G. H., Valent, P. and Orfao, A. (2001). Utility of flow cytometric analysis of mast cells in the diagnosis and classification of adult mastocytosis. *Leuk Res,* **25,** 563-570.

Escribano, L., Orfao, A., Diaz-Agustin, B., Villarrubia, J., Cervero, C., Lopez, A., Marcos, M. A., Bellas, C., Fernandez-Canadas, S., Cuevas, M., Sanchez, A., Velasco, J. L., Navarro, J. L. and Miguel, J. F. (1998). Indolent systemic mast cell disease in adults:

immunophenotypic characterization of bone marrow mast cells and its diagnostic implications. *Blood,* **91,** 2731-2736.

Fehniger, T. A., Carson, W. E., Mrozek, E. and Caligiuri, M. A. (1997). Stem cell factor enhances interleukin-2-mediated expansion of murine natural killer cells in vivo. *Blood,* **90,** 3647-3653.

Finocchiaro, L. M. and Glikin, G. C. (2012). Cytokine-enhanced vaccine and suicide gene therapy as surgery adjuvant treatments for spontaneous canine melanoma: 9 years of follow-up. *Cancer Gene Ther,* **19,** 852-861.

FitzGerald, D. J., Wayne, A. S., Kreitman, R. J. and Pastan, I. (2011). Treatment of hematologic malignancies with immunotoxins and antibody-drug conjugates. *Cancer Res,* **71,** 6300-6309.

Fox, L. E., Rosenthal, R. C., Twedt, D. C., Dubielzig, R. R., MacEwen, E. G. and Grauer, G. F. (1990). Plasma histamine and gastrin concentrations in 17 dogs with mast cell tumors. *J Vet Intern Med,* **4,** 242-246.

Froberg, G. K., Lindberg, R., Ritter, M. and Nordlind, K. (2009). Expression of serotonin and its 5-HT1A receptor in canine cutaneous mast cell tumours. *J Comp Pathol,* **141,** 89-97.

Garcia-Tunon, I., Ricote, M., Ruiz, A., Fraile, B., Paniagua, R. and Royuela, M. (2004). Interleukin-2 and its receptor complex (alpha, beta and gamma chains) in in situ and infiltrative human breast cancer: an immunohistochemical comparative study. *Breast Cancer Res,* **6,** R1-R7.

Garcia de Galdeano, A., Boyano, M. D., Smith-Zubiaga, I. and Canavate, M. L. (1996). B16F10 murine melanoma cells express interleukin-2 and a functional interleukin-2 receptor. *Tumour Biol,* **17,** 155-167.

Giantin, M., Vascellari, M., Morello, E. M., Capello, K., Vercelli, A., Granato, A., Lopparelli, R. M., Nassuato, C., Carminato, A., Martano, M., Mutinelli, F. and Dacasto, M. (2012). c-KIT messenger RNA and protein expression and mutations in canine cutaneous mast cell tumors: correlations with post-surgical prognosis. *J Vet Diagn Invest,* **24,** 116-126.

Gil da Costa, R. M., Matos, E., Rema, A., Lopes, C., Pires, M. A. and Gartner, F. (2007). CD117 immunoexpression in canine mast cell tumours: correlations with pathological variables and proliferation markers. *BMC Vet Res,* **3,** 19.

Giri, J. G., Ahdieh, M., Eisenman, J., Shanebeck, K., Grabstein, K., Kumaki, S., Namen, A., Park, L. S., Cosman, D. and Anderson, D. (1994). Utilization of the beta and gamma chains of the IL-2 receptor by the novel cytokine IL-15. *EMBO J,* **13,** 2822-2830.

Goto, H., Tsurumi, H., Takemura, M., Ino-Shimomura, Y., Kasahara, S., Sawada, M., Yamada, T., Hara, T., Fukuno, K., Goto, N., Okuno, M., Takami, T., Seishima, M. and Moriwaki, H. (2005). Serum-soluble interleukin-2 receptor (sIL-2R) level determines clinical outcome in patients with aggressive non-Hodgkin's lymphoma: in combination with the International Prognostic Index. *J Cancer Res Clin Oncol,* **131,** 73-79.

Grande, C., Firvida, J. L., Navas, V. and Casal, J. (2006). Interleukin-2 for the treatment of solid tumors other than melanoma and renal cell carcinoma. *Anticancer Drugs,* **17,** 1-12.

Hahn, H. P. and Hornick, J. L. (2007). Immunoreactivity for CD25 in gastrointestinal mucosal mast cells is specific for systemic mastocytosis. *Am J Surg Pathol,* **31,** 1669-1676.

Hassuneh, M. R., Nagarkatti, P. S. and Nagarkatti, M. (1997). Evidence for the participation of interleukin-2 (IL-2) and IL-4 in the regulation of autonomous growth and tumorigenesis of transformed cells of lymphoid origin. *Blood,* **89,** 610-620.

Hollmann, T. J., Brenn, T. and Hornick, J. L. (2008). CD25 expression on cutaneous mast cells from adult patients presenting with urticaria pigmentosa is predictive of systemic mastocytosis. *Am J Surg Pathol,* **32,** 139-145.

Horny, H. P. (2009). Mastocytosis: an unusual clonal disorder of bone marrow-derived hematopoietic progenitor cells. *Am J Clin Pathol,* **132,** 438-447.

Horny, H. P., Metcalfe and D. D., Bennett J. (2008). Mastocytosis. In: *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues*, Swerdlow, S. H., Campo, E., Harris, N. L., Jaffe, E. S., Pileri, S. A., Stein, H., Thiele, J. and Vardiman, J. W., Lyon, France, 54-63.

Hottendorf, G. H. and Nielsen, S. W. (1968). Pathologic report of 29 necropsies on dogs with mastocytoma. *Pathol Vet,* **5,** 102-121.

Howard, E. B., Sawa, T. R., Nielsen, S. W. and Kenyon, A. J. (1969). Mastocytoma and gastroduodenal ulceration. Gastric and duodenal ulcers in dogs with mastocytoma. *Pathol Vet,* **6,** 146-158.

Iwata, N., Ochiai, K., Kadosawa, T., Takiguchi, M. and Umemura, T. (2000). Canine extracutaneous mast-cell tumours consisting of connective tissue mast cells. *J Comp Pathol,* **123,** 306-310.

Kaldrymidou, H., Leontides, L., Koutinas, A. F., Saridomichelakis, M. N. and Karayannopoulou, M. (2002). Prevalence, distribution and factors associated with the presence and the potential for malignancy of cutaneous neoplasms in 174 dogs admitted to a clinic in northern Greece. *J Vet Med A Physiol Pathol Clin Med,* **49,** 87-91.

Kallio, J. P., Tammela, T. L., Marttinen, A. T. and Kellokumpu-Lehtinen, P. L. (2001). Soluble immunological parameters and early prognosis of renal cell cancer patients. *J Exp Clin Cancer Res,* **20,** 523-528.

Kasprzak, A., Olejniczak, K., Przybyszewska, W. and Zabel, M. (2007). Cellular expression of interleukin 2 (IL-2) and its receptor (IL-2R, CD25) in lung tumours. *Folia Morphol (Warsz),* **66,** 159-166.

Kim, H. P., Imbert, J. and Leonard, W. J. (2006). Both integrated and differential regulation of components of the IL-2/IL-2 receptor system. *Cytokine Growth Factor Rev,* **17,** 349-366.

Kiupel, M., Webster, J. D., Bailey, K. L., Best, S., DeLay, J., Detrisac, C. J., Fitzgerald, S. D., Gamble, D., Ginn, P. E., Goldschmidt, M. H., Hendrick, M. J., Howerth, E. W., Janovitz, E. B., Langohr, I., Lenz, S. D., Lipscomb, T. P., Miller, M. A., Misdorp, W., Moroff, S., Mullaney, T. P., Neyens, I., O'Toole, D., Ramos-Vara, J., Scase, T. J., Schulman, F. Y., Sledge, D., Smedley, R. C., Smith, K., P, W. S., Southorn, E., Stedman, N. L., Steficek, B. A., Stromberg, P. C., Valli, V. E., Weisbrode, S. E., Yager, J., Heller, J. and Miller, R. (2010). Proposal of a 2-tier histologic grading system for canine cutaneous mast cell tumors to more accurately predict biological behavior. *Vet Pathol,* **48,** 147-155.

Klopfleisch, R., Hvid, H., Klose, P., da Costa, A. and Gruber, A. D. (2010). Insulin receptor is expressed in normal canine mammary gland and benign adenomas but decreased in

metastatic canine mammary carcinomas similar to human breast cancer. *Vet Comp Oncol,* **8,** 293-301.

Klopfleisch, R., Lenze, D., Hummel, M. and Gruber, A. D. (2011). The metastatic cascade is reflected in the transcriptome of metastatic canine mammary carcinomas. *Vet J,* **190,** 236-243.

Kondo, M., Takeshita, T., Ishii, N., Nakamura, M., Watanabe, S., Arai, K. and Sugamura, K. (1993). Sharing of the interleukin-2 (IL-2) receptor gamma chain between receptors for IL-2 and IL-4. *Science,* **262,** 1874-1877.

Kravis, L. D., Vail, D. M., Kisseberth, W. C., Ogilvie, G. K. and Volk, L. M. (1996). Frequency of argyrophilic nucleolar organizer regions in fine-needle aspirates and biopsy specimens from mast cell tumors in dogs. *J Am Vet Med Assoc,* **209,** 1418-1420.

Kreitman, R. J., Wilson, W. H., White, J. D., Stetler-Stevenson, M., Jaffe, E. S., Giardina, S., Waldmann, T. A. and Pastan, I. (2000). Phase I trial of recombinant immunotoxin anti-Tac(Fv)-PE38 (LMB-2) in patients with hematologic malignancies. *J Clin Oncol,* **18,** 1622-1636.

Krick, E. L., Billings, A. P., Shofer, F. S., Watanabe, S. and Sorenmo, K. U. (2009). Cytological lymph node evaluation in dogs with mast cell tumours: association with grade and survival. *Vet Comp Oncol,* **7,** 130-138.

Krokowski, M., Sotlar, K., Krauth, M. T., Fodinger, M., Valent, P. and Horny, H. P. (2005). Delineation of patterns of bone marrow mast cell infiltration in systemic mastocytosis: value of CD25, correlation with subvariants of the disease, and separation from mast cell hyperplasia. *Am J Clin Pathol,* **124,** 560-568.

Kuhn, D. J. and Dou, Q. P. (2005). The role of interleukin-2 receptor alpha in cancer. *Front Biosci,* **10,** 1462-1474.

Kuntz, C. A., Dernell, W. S., Powers, B. E., Devitt, C., Straw, R. C. and Withrow, S. J. (1997). Prognostic factors for surgical treatment of soft-tissue sarcomas in dogs: 75 cases (1986-1996). *J Am Vet Med Assoc,* **211,** 1147-1151.

Laprie, C., Abadie, J., Amardeilh, M. F., Net, J. L., Lagadic, M. and Delverdier, M. (2001). MIB-1 immunoreactivity correlates with biologic behaviour in canine cutaneous melanoma. *Vet Dermatol,* **12,** 139-147.

Leonard, W. J., Depper, J. M., Crabtree, G. R., Rudikoff, S., Pumphrey, J., Robb, R. J., Kronke, M., Svetlik, P. B., Peffer, N. J., Waldmann, T. A. et al. (1984). Molecular cloning and expression of cDNAs for the human interleukin-2 receptor. *Nature,* **311,** 626-631.

Letard, S., Yang, Y., Hanssens, K., Palmerini, F., Leventhal, P. S., Guery, S., Moussy, A., Kinet, J. P., Hermine, O. and Dubreuil, P. (2008). Gain-of-function mutations in the extracellular domain of KIT are common in canine mast cell tumors. *Mol Cancer Res,* **6,** 1137-1145.

Letourneau, S., Krieg, C., Pantaleo, G. and Boyman, O. (2009). IL-2- and CD25-dependent immunoregulatory mechanisms in the homeostasis of T-cell subsets. *J Allergy Clin Immunol,* **123,** 758-762.

Lin, W. C., Yasumura, S., Suminami, Y., Sung, M. W., Nagashima, S., Stanson, J. and Whiteside, T. L. (1995). Constitutive production of IL-2 by human carcinoma cells, expression of IL-2 receptor, and tumor cell growth. *J Immunol,* **155,** 4805-4816.

London, C. A., Galli, S. J., Yuuki, T., Hu, Z. Q., Helfand, S. C. and Geissler, E. N. (1999). Spontaneous canine mast cell tumors express tandem duplications in the proto-oncogene c-kit. *Exp Hematol,* **27,** 689-697.

London, C. A., Kisseberth, W. C., Galli, S. J., Geissler, E. N. and Helfand, S. C. (1996). Expression of stem cell factor receptor (c-kit) by the malignant mast cells from spontaneous canine mast cell tumours. *J Comp Pathol,* **115,** 399-414.

London, C. A. and Seguin, B. (2003). Mast cell tumors in the dog. *Vet Clin North Am Small Anim Pract,* **33,** 473-489, v.

Longley, B. J., Jr., Metcalfe, D. D., Tharp, M., Wang, X., Tyrrell, L., Lu, S. Z., Heitjan, D. and Ma, Y. (1999). Activating and dominant inactivating c-KIT catalytic domain mutations in distinct clinical forms of human mastocytosis. *Proc Natl Acad Sci U S A,* **96,** 1609-1614.

Ma, Y., Longley, B. J., Wang, X., Blount, J. L., Langley, K. and Caughey, G. H. (1999). Clustering of activating mutations in c-KIT's juxtamembrane coding region in canine mast cell neoplasms. *J Invest Dermatol,* **112,** 165-170.

Malek, T. R. (2008). The biology of interleukin-2. *Annu Rev Immunol,* **26,** 453-479.

Malek, T. R. and Castro, I. (2010). Interleukin-2 receptor signaling: at the interface between tolerance and immunity. *Immunity,* **33,** 153-165.

Miller, D. M. (1995). The occurrence of mast cell tumors in young Shar-Peis. *J Vet Diagn Invest,* **7,** 360-363.

Mindiola, R., Caulejas, D., Nunez-Troconis, J., Araujo, M., Delgado, M. and Mosquera, J. (2008). Increased number of IL-2, IL-2 receptor and IL-10 positive cells in premalignant lesions of the cervix. *Invest Clin,* **49,** 533-545.

Mullins, M. N., Dernell, W. S., Withrow, S. J., Ehrhart, E. J., Thamm, D. H. and Lana, S. E. (2006). Evaluation of prognostic factors associated with outcome in dogs with multiple cutaneous mast cell tumors treated with surgery with and without adjuvant treatment: 54 cases (1998-2004). *J Am Vet Med Assoc,* **228,** 91-95.

Murphy, S., Sparkes, A. H., Blunden, A. S., Brearley, M. J. and Smith, K. C. (2006). Effects of stage and number of tumours on prognosis of dogs with cutaneous mast cell tumours. *Vet Rec,* **158,** 287-291.

Murphy, S., Sparkes, A. H., Smith, K. C., Blunden, A. S. and Brearley, M. J. (2004). Relationships between the histological grade of cutaneous mast cell tumours in dogs, their survival and the efficacy of surgical resection. *Vet Rec,* **154,** 743-746.

Nakamura, Y., Russell, S. M., Mess, S. A., Friedmann, M., Erdos, M., Francois, C., Jacques, Y., Adelstein, S. and Leonard, W. J. (1994). Heterodimerization of the IL-2 receptor beta- and gamma-chain cytoplasmic domains is required for signalling. *Nature,* **369,** 330-333.

O'Keefe, D. A., Couto, C. G., Burke-Schwartz, C. and Jacobs, R. M. (1987). Systemic mastocytosis in 16 dogs. *J Vet Intern Med,* **1,** 75-80.

Ottaiano, A., Leonardi, E., Simeone, E., Ascierto, P. A., Scala, S., Calemma, R., Bryce, J., Caraco, C., Satriano, R. A., Gianfranco, N., Franco, R., Botti, G. and Castello, G. (2006). Soluble interleukin-2 receptor in stage I-III melanoma. *Cytokine,* **33,** 150-155.

Owen, L. N., Brodey, R. S., Gillette, E. L., Milouchine, V. N., Misdorp, W., Parodi, A. L., Syrkin, A. B. and Theilen, G. H. (1980). WHO-TNM Classification of Tumours in Domestic Animals. In: *WHO-Classification*, Owen, L.N., Geneva, Switzerland, 14-15.

Ozaki, K., Yamagami, T., Nomura, K. and Narama, I. (2002). Mast cell tumors of the gastrointestinal tract in 39 dogs. *Vet Pathol,* **39,** 557-564.

Patnaik, A. K., Ehler, W. J. and MacEwen, E. G. (1984). Canine cutaneous mast cell tumor: morphologic grading and survival time in 83 dogs. *Vet Pathol,* **21,** 469-474.

Patnaik, A. K., MacEwen, E. G., Black, A. P. and Luckow, S. (1982). Extracutaneous mast-cell tumor in the dog. *Vet Pathol,* **19,** 608-615.

Pena, L. L., Nieto, A. I., Perez-Alenza, D., Cuesta, P. and Castano, M. (1998). Immunohistochemical detection of Ki-67 and PCNA in canine mammary tumors: relationship to clinical and pathologic variables. *J Vet Diagn Invest,* **10,** 237-246.

Preziosi, R., Sarli, G. and Paltrinieri, M. (2004). Prognostic value of intratumoral vessel density in cutaneous mast cell tumors of the dog. *J Comp Pathol,* **130,** 143-151.

Rangel-Corona, R., Corona-Ortega, T., Soto-Cruz, I., Lopez-Labra, A., Pablo-Arcos, T., Torres-Guarneros, C. F. and Weiss-Steider, B. (2010). Evidence that cervical cancer cells secrete IL-2, which becomes an autocrine growth factor. *Cytokine,* **50,** 273-277.

Reguera, M. J., Rabanal, R. M., Puigdemont, A. and Ferrer, L. (2000). Canine mast cell tumors express stem cell factor receptor. *Am J Dermatopathol,* **22,** 49-54.

Reichert, T. E., Kashii, Y., Stanson, J., Zeevi, A. and Whiteside, T. L. (1999). The role of endogenous interleukin-2 in proliferation of human carcinoma cell lines. *Br J Cancer,* **81,** 822-831.

Reichert, T. E., Nagashima, S., Kashii, Y., Stanson, J., Gao, G., Dou, Q. P. and Whiteside, T. L. (2000). Interleukin-2 expression in human carcinoma cell lines and its role in cell cycle progression. *Oncogene,* **19,** 514-525.

Reichert, T. E., Watkins, S., Stanson, J., Johnson, J. T. and Whiteside, T. L. (1998). Endogenous IL-2 in cancer cells: a marker of cellular proliferation. *J Histochem Cytochem,* **46,** 603-611.

Renauld, J. C., Kermouni, A., Vink, A., Louahed, J. and Van Snick, J. (1995). Interleukin-9 and its receptor: involvement in mast cell differentiation and T cell oncogenesis. *J Leukoc Biol,* **57,** 353-360.

Ritter, M., El-Nour, H., Hedblad, M. A., Butterfield, J. H., Beck, O., Stephanson, N., Holst, M., Giscombe, R., Azmitia, E. C. and Nordlind, K. (2012). Serotonin and its 5-HT1 receptor in human mastocytosis. *Immunopharmacol Immunotoxicol,* **34,** 679-685.

Rocha-Zavaleta, L., Huitron, C., Caceres-Cortes, J. R., Alvarado-Moreno, J. A., Valle-Mendiola, A., Soto-Cruz, I., Weiss-Steider, B. and Rangel-Corona, R. (2004). Interleukin-2 (IL-2) receptor-betagamma signalling is activated by c-Kit in the absence of IL-2, or by exogenous IL-2 via JAK3/STAT5 in human papillomavirus-associated cervical cancer. *Cell Signal,* **16,** 1239-1247.

Romansik, E. M., Reilly, C. M., Kass, P. H., Moore, P. F. and London, C. A. (2007). Mitotic index is predictive for survival for canine cutaneous mast cell tumors. *Vet Pathol,* **44,** 335-341.

Roskoski, R., Jr. (2005). Structure and regulation of Kit protein-tyrosine kinase--the stem cell factor receptor. *Biochem Biophys Res Commun,* **338,** 1307-1315.

Rothwell, T. L., Howlett, C. R., Middleton, D. J., Griffiths, D. A. and Duff, B. C. (1987). Skin neoplasms of dogs in Sydney. *Aust Vet J,* **64,** 161-164.

Sanchez, J., Ramirez, G. A., Buendia, A. J., Vilafranca, M., Martinez, C. M., Altimira, J. and Navarro, J. A. (2007). Immunohistochemical characterization and evaluation of prognostic factors in canine oral melanomas with osteocartilaginous differentiation. *Vet Pathol,* **44,** 676-682.

Sarli, G., Preziosi, R., Benazzi, C., Castellani, G. and Marcato, P. S. (2002). Prognostic value of histologic stage and proliferative activity in canine malignant mammary tumors. *J Vet Diagn Invest,* **14,** 25-34.

Scase, T. J., Edwards, D., Miller, J., Henley, W., Smith, K., Blunden, A. and Murphy, S. (2006). Canine mast cell tumors: correlation of apoptosis and proliferation markers with prognosis. *J Vet Intern Med,* **20,** 151-158.

Seguin, B., Besancon, M. F., McCallan, J. L., Dewe, L. L., Tenwolde, M. C., Wong, E. K. and Kent, M. S. (2006). Recurrence rate, clinical outcome, and cellular proliferation indices as prognostic indicators after incomplete surgical excision of cutaneous grade II mast cell tumors: 28 dogs (1994-2002). *J Vet Intern Med,* **20,** 933-940.

Sfiligoi, G., Rassnick, K. M., Scarlett, J. M., Northrup, N. C. and Gieger, T. L. (2005). Outcome of dogs with mast cell tumors in the inguinal or perineal region versus other cutaneous locations: 124 cases (1990-2001). *J Am Vet Med Assoc,* **226,** 1368-1374.

Sharon, M., Klausner, R. D., Cullen, B. R., Chizzonite, R. and Leonard, W. J. (1986). Novel interleukin-2 receptor subunit detected by cross-linking under high-affinity conditions. *Science,* **234,** 859-863.

Shelburne, C. P. and Ryan, J. J. (2001). The role of Th2 cytokines in mast cell homeostasis. *Immunol Rev,* **179,** 82-93.

Siddiqui, E. J., Thompson, C. S., Mikhailidis, D. P. and Mumtaz, F. H. (2005). The role of serotonin in tumour growth (review). *Oncol Rep,* **14,** 1593-1597.

Simoes, J. P., Schoning, P. and Butine, M. (1994). Prognosis of canine mast cell tumors: a comparison of three methods. *Vet Pathol,* **31,** 637-647.

Sotlar, K., Horny, H. P., Simonitsch, I., Krokowski, M., Aichberger, K. J., Mayerhofer, M., Printz, D., Fritsch, G. and Valent, P. (2004). CD25 indicates the neoplastic phenotype of mast cells: a novel immunohistochemical marker for the diagnosis of systemic mastocytosis (SM) in routinely processed bone marrow biopsy specimens. *Am J Surg Pathol,* **28,** 1319-1325.

Stefanello, D., Valenti, P., Faverzani, S., Bronzo, V., Fiorbianco, V., Pinto da Cunha, N., Romussi, S., Cantatore, M. and Caniatti, M. (2009). Ultrasound-guided cytology of spleen and liver: a prognostic tool in canine cutaneous mast cell tumor. *J Vet Intern Med,* **23,** 1051-1057.

Stewart, R. J., Hill, F. W., Masztalerz, A., Jacobs, J. J., Koten, J. W. and Den Otter, W. (2006). Treatment of ocular squamous cell carcinomas in cattle with interleukin-2. *Vet Rec,* **159,** 668-672.

Stricker, T. P. and Kumar, V. (2010). Neoplasia. In: *Robbins and Cotran Pathologic Basis of Disease*, Kumar, V., Abbas, A. K., Fausto, N. and Aster, J. C., Philadelphia, USA, 259-330.

Takahashi, T., Kadosawa, T., Nagase, M., Matsunaga, S., Mochizuki, M., Nishimura, R. and Sasaki, N. (2000). Visceral mast cell tumors in dogs: 10 cases (1982-1997). *J Am Vet Med Assoc,* **216,** 222-226.

Takeshita, T., Asao, H., Ohtani, K., Ishii, N., Kumaki, S., Tanaka, N., Munakata, H., Nakamura, M. and Sugamura, K. (1992). Cloning of the gamma chain of the human IL-2 receptor. *Science,* **257,** 379-382.

Thamm, D. H., Mauldin, E. A. and Vail, D. M. (1999). Prednisone and vinblastine chemotherapy for canine mast cell tumor--41 cases (1992-1997). *J Vet Intern Med,* **13,** 491-497.

Thamm, D. H., Turek, M. M. and Vail, D. M. (2006). Outcome and prognostic factors following adjuvant prednisone/vinblastine chemotherapy for high-risk canine mast cell tumour: 61 cases. *J Vet Med Sci,* **68,** 581-587.

Thamm, D. H. and Vail, D. M. (2007). Mast Cell Tumors. In: *Small Animal Clinical Oncology*, Withrow, S. J. and Vail, D. M., St. Louis, USA, 402-424.

Turrel, J. M., Kitchell, B. E., Miller, L. M. and Theon, A. (1988). Prognostic factors for radiation treatment of mast cell tumor in 85 dogs. J Am Vet Med Assoc, **193,** 936-940.

Valent, P., Akin, C., Escribano, L., Fodinger, M., Hartmann, K., Brockow, K., Castells, M., Sperr, W. R., Kluin-Nelemans, H. C., Hamdy, N. A., Lortholary, O., Robyn, J., van Doormaal, J., Sotlar, K., Hauswirth, A. W., Arock, M., Hermine, O., Hellmann, A., Triggiani, M., Niedoszytko, M., Schwartz, L. B., Orfao, A., Horny, H. P. and Metcalfe, D. D. (2007). Standards and standardization in mastocytosis: consensus statements on diagnostics, treatment recommendations and response criteria. *Eur J Clin Invest,* **37,** 435-453.

Valent, P., Horny, H. P., Escribano, L., Longley, B. J., Li, C. Y., Schwartz, L. B., Marone, G., Nunez, R., Akin, C., Sotlar, K., Sperr, W. R., Wolff, K., Brunning, R. D., Parwaresch, R. M., Austen, K. F., Lennert, K., Metcalfe, D. D., Vardiman, J. W. and Bennett, J. M. (2001). Diagnostic criteria and classification of mastocytosis: a consensus proposal. *Leuk Res,* **25,** 603-625.

Valli, V. E., Jacobs, R. M., Parodi, A. L., Vernau, W. and Moore, P. F. (2002). *WHO International Histological Classification of Tumors of Domestic Animals*, Armed Forces Institute of Pathology, Washington, USA.

van Daele, P. L., Beukenkamp, B. S., Geertsma-Kleinekoort, W. M., Valk, P. J., van Laar, J. A., van Hagen, P. M. and van der Velden, V. H. (2009). Immunophenotyping of mast

cells: a sensitive and specific diagnostic tool for systemic mastocytosis. *Neth J Med,* **67,** 142-146.

Wang, J., Wicker, L. S. and Santamaria, P. (2009). IL-2 and its high-affinity receptor: genetic control of immunoregulation and autoimmunity. *Semin Immunol,* **21,** 363-371.

Wang, X., Rickert, M. and Garcia, K. C. (2005). Structure of the quaternary complex of interleukin-2 with its alpha, beta, and gammac receptors. *Science,* **310,** 1159-1163.

Webster, J. D., Kiupel, M., Kaneene, J. B., Miller, R. and Yuzbasiyan-Gurkan, V. (2004). The use of KIT and tryptase expression patterns as prognostic tools for canine cutaneous mast cell tumors. *Vet Pathol,* **41,** 371-377.

Webster, J. D., Yuzbasiyan-Gurkan, V., Kaneene, J. B., Miller, R., Resau, J. H. and Kiupel, M. (2006). The role of c-KIT in tumorigenesis: evaluation in canine cutaneous mast cell tumors. *Neoplasia,* **8,** 104-111.

Webster, J. D., Yuzbasiyan-Gurkan, V., Miller, R. A., Kaneene, J. B. and Kiupel, M. (2007). Cellular proliferation in canine cutaneous mast cell tumors: associations with c-KIT and its role in prognostication. *Vet Pathol,* **44,** 298-308.

Webster, J. D., Yuzbasiyan-Gurkan, V., Thamm, D. H., Hamilton, E. and Kiupel, M. (2008). Evaluation of prognostic markers for canine mast cell tumors treated with vinblastine and prednisone. *BMC Vet Res,* **4,** 32.

Zeiser, R. and Negrin, R. S. (2008). Interleukin-2 receptor downstream events in regulatory T cells: implications for the choice of immunosuppressive drug therapy. *Cell Cycle,* **7,** 458-462.

Zemke, D., Yamini, B. and Yuzbasiyan-Gurkan, V. (2002). Mutations in the juxtamembrane domain of c-KIT are associated with higher grade mast cell tumors in dogs. *Vet Pathol,* **39,** 529-535.

Ziekman, P. G., Otter, W. D., Tan, J. F., Teske, E., Kirpensteijn, J., Koten, J. W. and Jacobs, J. J. (2013). Intratumoural interleukin-2 therapy can induce regression of non-resectable mastocytoma in dogs. *Anticancer Res,* **33,** 161-165.

8 Supplemental Material

Supplement 1 mRNA and Protein Expression Levels and Significant Differences of the Interleukin-2 Receptor Subunits and the Ligand Interleukin-2

mRNA	**CD25**	**CD122**	**CD132**	**IL-2**
GI vs. GIII	↑ [p=0.047] *	↑ [p=0.310]	↑ [p=0.054]	↓ [p=0.160]
LG vs. HG	↑ [p=0.023] *	↑ [p=0.188]	↑ [p=0.104]	↓ [p=0.257]
WT vs. M	n.d.	↓ [p=0.118]	↑ [p=0.299]	↑ [p=0.953]
Protein	**CD25**	**CD122**	**CD132**	**IL-2**
G I vs. G II	↓ [p=0.012] *	↓ [p=0.162]	↓ [p=0.000] *	↓ [p=0.228]
G I vs. G III	↓ [p=0.000] *	↓ [p=0.153]	↓ [p=0.000] *	↓ [p=0.000] *
G II vs. G III	↓ [p=0.000] *	↓ [p=0.781]	↓ [p=0.043] *	↓ [p=0.000] *
LG vs. HG	↓ [p=0.000] *	↓ [p=0.207]	↓ [p=0.000] *	↓ [p=0.000] *
WT vs. M	n.d.	↓ [p=0.480]	↓ [p=0.098]	↓ [p=0.000] *

P values [ ] and significant differences * (P <0.05) were analyzed between grade I (GI), grade II (GII) and grade III (GIII) MCT, between low-grade (LG) and high-grade (HG) MCT and between c-*KIT* wild-type MCT (WT) and c-*KIT* mutated MCT (M)

↑ Higher expression in the compared group

↓ Lower expression in the compared group

vs.: versus; n.d.: not done;

9 Publications

Oral Presentations:

"The Interleukin-2 Receptor Is Expressed By Canine Mast Cell Tumors"

A. Meyer, A.D. Gruber, R. Klopfleisch

29th Meeting of the European Society of Veterinary Pathology and the European College of Veterinary Pathologists, Uppsala, Sweden (07.-10.09.2011)

"Die IL-2-Rezeptor-Untereinheit CD25 als Mastzelltumormarker – Expressionsstudien in kaninen Mastzelltumoren"

A. Meyer, A.D. Gruber, R. Klopfleisch

Post graduate research symposium at the Freie Universität Berlin, Berlin, Germany (01.07.2011)

"Die IL-2-Rezeptor-Untereinheit CD25 als Mastzelltumormarker – Expressionsstudien in kaninen Mastzelltumoren"

A. Meyer, A.D. Gruber, R. Klopfleisch

54. Annual Conference of the German Veterinary Medical Society, Section Veterinary Pathology, Fulda, Germany (12.-13.03.2011)

Poster Presentations:

"Interleukin-2-Rezeptor-Expression in kaninen Mastzelltumoren"

A. Meyer, A.D. Gruber, R. Klopfleisch

Post graduate research symposium at the Freie Universität Berlin, Berlin, Germany (13.07.2012)

"Expressionsanalyse von Stammzellmarkern und Differenzierungsgenen in kaninen Mastzelltumoren"

A. Meyer, A.D. Gruber, R. Klopfleisch

Post graduate research symposium at the Freie Universität Berlin, Berlin, Germany (02.07.2010)

10 Acknowledgments / Danksagung

Als erstes gilt mein Dank Herrn Prof. Dr. Achim D. Gruber, Ph.D., Leiter des Institutes für Tierpathologie, der mir durch die Aufnahme in sein Institut und durch die Vergabe des Themas die Anfertigung dieser Dissertation ermöglichte. Ich danke ihm in diesem Rahmen auch für die stetige Unterstützung sowie hilfreichen Anregungen im Rahmen meines Ph.D.-Betreuungsteams.

Herrn Prof. Dr. Robert Klopfleisch danke ich für die persönliche Betreuung und stetige Hilfsbereitschaft in den letzten 3 Jahren sowie vor allem für seinen Optimismus und seine Motivierung besonders in schwierigeren Phasen dieser Arbeit. Außerdem danke ich ihm sowie Frau Prof. Dr. Barbara Kohn für ihre Unterstützung und Hilfestellung im Rahmen meines Ph.D.-Betreuungsteams.

Frau Michaela Dauer und Frau Monika Schaerig gilt mein ausdrücklicher Dank für die Einarbeitung in diverse Labortechniken sowie die stete Unterstützung und Hilfestellung in diesem Bereich.

Den Mitarbeitern des Institutes für Tierpathologie danke ich für die überaus schöne und entspannte Arbeitsatmosphäre, die mich jeden Tag sehr gerne hat hierher kommen lassen. Kristina Dietert möchte ich ganz besonders für ihre uneingeschränkte Hilfsbereitschaft und Anleitung im Labor sowie für ein immer offenes Ohr danken. Olivia Kershaw danke ich für ihre stetige Hilfsbereitschaft in allen großen und kleinen Krisensituationen und für ihre aufmunternden Worte in den richtigen Momenten.

Meiner lieben Kollegin und Zimmerpartnerin Patricia Schlieben danke ich für alles - für die liebevolle Atmosphäre in unserem kleinen Zimmer, für ihre Hilfsbereitschaft in allen Lebenslagen und für ihre Ruhe, ihre Geduld und ihren Optimismus in Situationen, in denen ich sie nicht hatte.

Meiner Familie möchte ich von Herzen danken, dass sie immer an mich glauben und in jeder Lebenslage hilfsbereit an meiner Seite stehen. Meinen lieben Freunden Vivian, Claudia, Josefine, Vanessa und Elisa danke ich dafür, dass sie immer für mich da sind. Malte danke ich für seine Liebe, seine Geduld und vor allem für seine positive Einstellung und die Fähigkeit, mich auch in schwierigen Situationen zum Lachen zu bringen.

Selbständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbstständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Berlin, den 11.02.2013 Anja Meyer