CONSTRUCTION AND CHARACHTERISATION OF A STABLY TRANSFECTED BHK CELL LINE PERMANENTLY SECRETING THE CANINE INTERLEUKIN 12 AS A SOURCE FOR ADOPTIVE CANCER IMMUNOTHERAPY IN DOGS

Inaugural Dissertation
submitted to the
Faculty of Veterinary Medicine
in partial fulfillment of the requirements
for the PhD-Degree
of the Faculties of Veterinary Medicine and Medicine
of the Justus Liebig University Giessen

by
Kocoski, Vladimir

of
Struga,
Republic of Macedonia

Giessen 2008

From the Institute of Veterinary Pathology
Director: Prof. Dr. Manfred Reinacher
of the Faculty of Veterinary Medicine of the Justus Liebig University Giessen

First Supervisor and Committee Member:
Prof. Dr. Eberhard Burkhardt

Second Supervisor and Committee Member:
Prof. Dr. Lothar Stitz

Committee Members:
Prof. Dr. Dr. Hans Michael Piper
Prof. Dr. Holger Hackstein

Date of Doctoral Defence:
31.03.2008

I declare that I have completed this dissertation single-handedly without the unauthorized help of a second party and only with the assistance acknowledged therein. I have appropriately acknowledged and referenced all text passages that are derived literally from or are based on the content of published or unpublished work of others, and all information that relates to verbal communications. I have abided by the principles of good scientific conduct laid down in the charter of the Justus Liebig University of Giessen in carrying out the investigations described in the dissertation.

TO MY PARENTS

1	**INTRODUCTION**	**1**
2	**LITERATURE OVERVIEW**	**3**
2.1	DNA Cloning	3
2.2	Protein expression in mammalian cells	4
2.2.1	General principles	4
2.2.2	The Tet System for inducible mammalian expression	5
2.3	Biology of IL-12 in human and mice	6
2.4	Biology of IL-12 in the dog	10
2.5	IL-12 as a single chain	10
2.6	Investigation of the IL-12 effects on PBMC	11
2.6.1	PBMC Isolation	11
2.6.2	Cytotoxicity assay	12
2.6.3	Cell proliferation	13
3	**MATERIALS AND METHODS**	**14**
3.1	Cloning	14
3.1.1	Polymerase Chain Reaction	14
3.1.1.1	KOD Hot Start DNA Polymerase	14
3.1.1.2	Taq Polymerase	15
3.1.1.3	A-overhang addition and TA cloning	15
3.1.2	Gel electrophoresis	16
3.1.3	Restriction digestion	16
3.1.4	Ligation	17
3.1.5	Transformation of E.coli	17
3.1.6	Plasmid DNA extraction	18
3.2	Construction of the recombinant canine single chain IL-12 (rcscIL-12)	19
3.2.1	Completion of the cDNA coding for the IL-12 p35 sequence	19
3.2.2	Correction of the IL-12 p40 cDNA sequence (DpnI site-directed mutagenesis)	20
3.2.3	Construction of the rcscIL-12	21
3.2.4	Construction of the rcscIL-12/IREs/Luciferase/pTRUE plasmid	22
3.3	Establishing of a stably transfected BHK Tet-On cell line	22
3.3.1	Stable transfection	22

3.3.2	Cloning of the transfected cells	23
3.3.3	Luciferase assay	24
3.3.4	Immunofluorescence	24
3.3.5	SDS-PAGE	25
3.3.6	Western blot	26
3.4	Detection of the rcscIL-12 bioactivity	27
3.4.1	PBMC isolation	27
3.4.2	PBMC pre-stimulation	27
3.4.3	IFN-γ ELISA	28
3.4.4	BrdU ELISA	29
3.4.5	Cytotoxicity assay (Rose Bengal Assay)	30
4	**RESULTS**	**32**
4.1	Construction of the rcscIL-12	32
4.1.1	Repair of the cDNA coding for the IL-12 p35 sequence	32
4.1.2	Correction of the cDNA coding for the IL-12 p40 sequence	33
4.1.3	Construction of the rcscIL-12	34
4.1.4	Construction of the rcscIL-12/IRES/Luciferase/pTRUE plasmid	34
4.2	Establishing of rcscIL-12 containing BHK Tet-On cell line	36
4.2.1	Transfection of the BHK Tet-On cell line	36
4.2.2	Detection of the rcscIL-12 in the established cell line	41
4.2.2.1	Detection of the rcscIL-12 protein	41
4.2.3	Confirmation of bioactivity of the IL-12-	42
4.3	Investigation of the biological effects of rcscIL-12 on canine PBMC	45
4.3.1	Resting canine PBMC do not produce IFN-γ after stimulation with IL-12	45
4.3.2	IL-12 requires presence of IL-2 to induce IFN-γ production in IL-2 lymphoblasts	46
4.3.3	Proliferation of canine IL-2 lymphoblasts upon IL-12 stimulation	47
4.3.4	Evaluation of the cytotoxic activity of canine IL-2 lymphoblasts upon stimulation with IL-12	48
5	**DISCUSSION**	**50**
6	**SUMMARY**	**55**
7	**ZUSAMMENFASSUNG**	**57**

8 BIBLIOGRAPHY 59
9 ATTACHMENT I-VII

Abbreviations

A	Adenine
AAV	Adeno-associated viruses
AP	Alkaline phosphatase
APC	Antigen presenting cells
APS	Ammonium persulfate
BCIP	(5-Bromo-4-Chloro-3'-Indolyphosphate p-Toluidine Salt)
BHK	Baby hamster kidney
Blasts	Cells stimulated to divide
bp	Basepair
BrdU	5-bromo-2-deoxyuridine
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CMV	Cytomegalovirus
CRA	Chromium release assay
CTAC	Canine thyreo adenocarcinoma
C	Cytosine
Cy-5	Carbocyanine 5
DC	Dendritic cells
ddH_2O	Double-destillated water
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleo triphosphates
EDTA	Ethylenediamine tetraacetic acid
FL	Fas ligand
G	Guanine
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulfuric acid
IFN-γ	Interferon gamma
cIFN-γ	Canine Interferon gamma
IL-2	Interleukin 2
IL-12	Interleukin 12
IL-12R	Interleukin 12 receptor

IRES	Internal ribosomal entry site
kb	Kilobasis
KOD	*Kodakaraensis, Thermococcus*
LAK cell	Lymphokine activated killer cell
LPS	Lipopolysacharide
mA	Miliampers
µg	Microgram
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulfate
min.	Minutes
µl	Microliter
ml	Mililiter
mM	Milimole
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)
mRNA	Messenger ribonucleic acid
NaCl	Sodium (Natrium) cloride
NBT	(Nitro-Blue Tetrazolium Chloride)
ng	Nanogram
NK	Natural killer
nm	Nanometers
O.D.	Optical density
PAGE	Polyacrylamid gel electrophoresis
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PHA	phytohemaglutinine
pM	Picomole
POD	Peroxydase
RNA	Ribonucleic acid
RBA	Rose bengal assay
rcIL-12	Recombinant canine Interleukin-12
rcscIL-12	Recombinant canine single-chain Interleukin-12
rpm	Revolution per minute

rtTA	Reversed tetracycline-controlled transactivator
sec.	Second(s)
SDS	Sodium dodecyl sulfate
Taq	*Thermococcus aquaticus*
TBE	Tris-borate-EDTA buffer
Tet	Tetracycline
Th cell	T helper cell
T	Thymidine
TRE	Tetracycline responsive element
tTA	Tetracycline-controlled transactivator
U	Unit
UV	Ultraviolet
V	Volts
v/v	Volume per volume
VP16	Herpes Simplex virion partical protein 16
w/v	weight per volume
XTT	Na-3,3`-[1-(phenylamino)carbonyl-3,4-tetrazolium]-bis

1 Introduction

The pursuit to improve specificity of tumor therapy has driven tumor research over the last 20 years. As a consequence, the tumor immunotherapy has now become one of the most extensively researched areas on the field of tumor therapy. Due to the great progress in basic research, numerous approaches based on cancer immunotherapy have evolved over the last decade. Generally, they can be classified into two different strategies: those that employ the constituents of humoral immunity, mostly antibodies, and those that make use of the cellular immunity. Among the latter, two main directions are eminent one exploiting the antigen-specific cytotoxic T cells, whereas the other relies on natural killer (NK) cells. The advantage of NK cells in contrast to cytotoxic T cells is that they do not require prior contact with the antigen in order to eliminate it. Additionally, the cytotoxicity of NK cells can be enhanced by various stimuli, including several cytokines. In this context, previous results from our lab performed in dogs, as well as numerous studies in human and mice have shown an increase in anti-tumor activity of lymphokine-activated killer (LAK) cells (cytotoxic $CD8^{+}$ T cells and/or NK cells) following stimulation with interleukin (IL) -2. Another cytokine known to stimulate T cells and especially NK cells is IL-12. Enhancement of the NK cell cytotoxic and other anti-tumor activities by IL-12 have been well documented in human and mice, but detailed investigations in dogs have not been carried out so far. Thus, detailed investigations of the in-vitro effects of IL-12 on canine lymphocytes are of importance, before investigating its effects in vivo. However, under in-vivo conditions, cells are exposed to almost constant concentrations of IL-12, which differ from in-vitro experiments where the cytokine is supplied in defined intervals. In order to provide in vitro conditions, which would resemble the conditions found in vivo we sought to construct a cell line that could secrete IL-12 into its supernatant upon induction with Doxycycline. For this purpose, the cDNA sequence of the canine IL-12 genes was first cloned in a Tet-On vector carrying tetracycline inducible promoter, and was stably transfected into a baby hamster kidney (BHK) Tet-On cell line. This cell line contains already stably transfected protein known as reversed tetracycline-dependent transcriptional transactivator (rtTA). Its function is to bind to the tetracycline inducible promoter in the presence of tetracycline and hence to initiate the expression of the cloned IL-12 gene. Instead of tetracycline, another drug of the same group, doxycycline, is commonly used for induction of gene expression. Consequently, this new cell-line can be used to

investigate the potential of constant and permanent IL-12 stimulation to enhance anti-tumor activities of lymphokine-activated cells. Due to certain characteristics of the IL-12 biology mentioned below, the IL-12 coding cDNA was constructed to code for a single-chain protein. Besides using the constructed BHK Tet-On cell line for in vitro stimulation of lymphocytes, the constructed vector carrying the IL-12 could also be applied in tumor immunotherapy, by means of gene delivery into the tumor itself.

2 Literature Overview

2.1 DNA Cloning

The amplification of given DNA fragments can be achieved in two different ways, either by polymerase chain reaction (PCR) or by a cell-based approach. In case of PCR (SAIKI et al., 1985), the DNA fragment is amplified in vitro, using a purified enzyme (DNA-dependent DNA polymerase). In the cell-based approach, the DNA fragment is introduced into a cell, mostly in bacteria, due to their simple manipulation. Among bacteria, Escherichia coli is most widely used. For introduction of the DNA fragment into the cell various DNA sequences capable of self-renewal (vectors) are used. Vectors are either modified sequences of a viral genome, or plasmids. The plasmids are naturally occurring double-stranded circular DNA sequences found in bacteria, which multiply independently of the bacterial genome. Vector plasmids are derived from naturally occurring plasmids, by removing the unnecessary sequences and adding additional sequences needed for cloning. These necessary sequences are: one origin of replication, one selection marker and a multiple cloning site (SAMBROOK AND RUSSEL, 2001). The origin of replication (defined sequence of base pairs) allows plasmid replication in the host cell, whereas the selection marker is a gene coding for a protein which renders the host cell resistant to a given drug. The gene coding for ß-lactamase (ABRACHAM et al., 1940), an enzyme which inactivates ampiciline by cleaving, is the mostly used selection marker. Genes coding for enzymes that inactivate other antibiotics such as neomycin, kanamycin and puromycin are also in use. The multiple cloning site (MCS) is a nucleotide sequence that contains restriction sites for different restriction endonucleases. The restriction endonucleases (restriction enzymes) were discovered in bacteria where they serve as defense mechanism against bacteriophages (viruses infecting bacteria) by cleaving its genome. Restriction enzymes work by binding to defined stretches of nucleotide bases (restriction sites), following which they cleave them. Each restriction site is specific for one or several enzymes, and hence it can be cleaved only by it/them. With help of the restriction enzymes the plasmid, as well as the sequence of interest are cut and afterwards ligated using another enzyme called DNA

ligase. In this way, the gene of interest is integrated into the plasmid sequence. The plasmid vector containing the sequence of interest is then introduced (transformed) into bacteria which can be easily propagated. However, the process of DNA uptake is not a constitutive characteristic of bacteria, and hence chemical or physical methods are used to render them transiently passable for the plasmid vector. The most routinely used method is the $CaCl_2$ method, where bacteria are incubated on ice in presence of $CaCl_2$, following which they are exposed to 42°C for short time (30-120 seconds). Since not all bacteria exposed to transformation acquire the plasmid, those that are transformed have to be selected from the non-transformed bacteria. This is achieved with help of the selection marker carried by the plasmid. Thus, when the bacteria are incubated in medium containing the appropriate drug (antibiotic), only the transformed bacteria survive. Subsequently, the transformed bacteria are grown in sufficient quantities and using chemical approaches the plasmid is isolated, after which it can be used in downstream applications.

The vector plasmids used only for DNA amplification are also known as cloning vectors. When the vector is provided with a bacterial promoter, the protein coding sequence inserted downstream of it will be transcribed by the bacterial DNA-dependent RNA polymerase and consequently expressed. These vectors are named bacterial expression vectors and are used when the protein needs to be purified and further structurally or functionally investigated.

2.2 Protein expression in mammalian cells

2.2.1 General principles

The need for correct protein folding and posttranslational modifications prevents the expression of many mammalian proteins in bacteria e.g. *Escherichia coli* (E. coli). It is known that some of the posttranslational modifications are unique to mammalian cells, so expression in bacteria does not lead to formation of a functional protein. For these reasons, such proteins must be expressed in mammalian cells by use of mammalian expression vectors. These vectors possess an appropriate mammalian promoter as well as a polyadenylation signal, which provides correct posttranscriptional processing of the mRNA molecule. Another important consideration when expressing a protein in mammalian cells is the mode of integration of its coding sequence into the recipient cell. In this regard, there are principally

two types of vectors used for mammalian expression. The first are viral-based vectors, predominately derived from adeno-associated viruses (AAV), which insert the sequence at specific sites on the chromosomal DNA. The second type can be any plasmid-based vector, whose characteristic is to enter the cell nucleus and integrate into chromosomes at random and with much lower efficiency than a viral-based vector. There are different opinions among scientists on how to improve chromosomal integration of non-viral vectors, but there is no single one that is widely accepted. Nevertheless, one method to improve chromosomal integration is the linearization of plasmid sequence, thus providing the wanted stable transfection.

2.2.2 The Tet System for inducible mammalian expression

When inserting an expression vector into the desired cell, the mode of regulation of gene expression is an important experimental issue. There are numerous vectors for expression in mammalian cells, offering various modes of transcriptional regulation. Thus, some of them utilize promoters providing strong and constitutive protein expression (e.g. CMV), whereas others are inducible. Among vectors carrying inducible promoters, tetracycline responsive element (TRE) - based vector is widely used. This vector was initially developed as a system that negatively regulates protein expression and hence called Tet-Off system (GOSSEN et al., 1992). The Tet-Off system was adapted from elements controlling the expression of tetracycline resistance gene in bacteria. Here, regulatory nucleotide sequences (operator/ promoter) located upstream of the gene and a regulatory protein (tetracycline repressor) binding these sequences regulate its expression. Thus, in bacteria in absence of tetracycline, a repressor protein binds to the operator sequence and thereby prevents RNA polymerase from binding the promoter and activating transcription. Conversely, in presence of tetracycline, the repressor undergoes conformational changes and releases the operator, rendering the promoter binding site free for the polymerase to transcribe downstream genes. In the Tet-Off vector, seven copies of the tetracycline operator sequence followed by a minimal cytomegalovirus (CMV) promoter are placed 5’ to the sequence to be inserted. This order of sequences is called tetracycline responsive element (TRE). Protein expression from the TRE is controlled by a regulatory protein called tetracycline-controlled transactivator (tTA). tTA is a fusion protein of the first 207 amino acids of the tetracycline repressor and the C-terminal 127 amino

acids of the activation domain of VP16, which is herpes simplex virus encoded transcriptional activator. The addition of this activation domain to the Tet-repressor, together with the presence of the minimal CMV promoter (s.a.) in the TRE allows for start of transcription upon binding of the tTA to the TRE sequence. Consequently, in the Tet-Off system, in absence of tetracycline the tTA is bound to TRE and hence transcription of the downstream gene is provided. In contrast, upon tetracycline addition the tTA binds it, releasing the TRE, which consequently stops the transcription. To achieve convenient manipulation of protein expression, GOSSEN et al. (1995) modified the binding affinities of the tTA, so that in presence of tetracycline or its analogues, such as doxycycline, it binds to the operator (GOSSEN et al. 1995). This change was achieved by exchanging four amino acids in the tetracycline repressor and due to its “reverse” behavior the modified tTA was named reversed transcriptional transactivator (rtTA).

This transactivator protein is stably transfected with help of an additional (helper) plasmid in a prior step, using neomycin resistance gene as selection marker. Expression of rtTA in the helper plasmid is controlled by the strong constitutive promoter pCMV, allowing constant expression of the rtTA.

The Tet system offers more advantages compared to other inducible mammalian expression vectors, helping to improve the regulation of protein expression. Thus, YIN et al. (1996) reported that the Tet system provides very high expression levels, which are comparable to the levels obtained by strong constitutive promoters such as CMV. Another advantage of the Tet system is its high specificity, owing to the fact that the DNA regulatory elements controlling gene expression are found only in bacteria and hence they can not be influenced by the present mammalian regulatory proteins.

2.3 Biology of IL-12 in human and mice

As one of the most important cytokines in the T helper cells 1 (Th_1) immune response, IL-12 has been extensively investigated (GERMANN et al., 1993; STRAUSS-AYALI et al., 2005). Many studies have focused on the pro-inflammatory and anti-tumor effects of IL-12 (KOBAYASHI et al., 1989; BRUNDA et al., 1993, GATELY et al., 1993). IL-12 is a heterodimeric cytokine of 70 kDa that is secreted predominantly by antigen-presenting cells (APC), especially dendritic cells (DC) (KOBAYASHI et al., 1989, GUBLER et al., 1991).

Various stimuli such as whole bacteria (*Mycobacterium tuberculosis, Listeria spp., Neiseria meningitides* etc.), IFN-γ plus LPS, lipoteichoic preparations of Gram-positive bacteria, and CpG motifs of bacterial DNA etc., can induce IL-12 secretion by the APC in-vitro as well as *in vivo* (D'ANDREA et al., 1992). IL-12 is composed of two subunits p40 and p35, named after their molecular weights (in kDa), and they are located on different chromosomes. The p35 subunit can be detected in non-stimulated cells, whereas p40 is expressed only in stimulated cells and significantly in excess to p35 (TRINCHIERI, 2003). In order for a functional IL-12 protein to be produced, both peptides must be expressed simultaneously in one cell (GUBLER et al., 1991). IL-12 exerts its actions by binding to its heterodimeric receptor, which consists of two subunits named IL-12 receptor beta1 (IL-12Rß1) and IL-12 receptor beta2 (IL-12Rß2). IL-12 induced functions are mediated by its major downstream molecule, the transcription factor signal transducer and activator of transcription 4 (STAT4) (THIERFELDER et al., 1996). Regarding the biological activities of IL-12, it has been shown that: (1) it is the major cytokine driving the bias towards Th_1 phenotype at the initial phase of the immune response by stimulating IFN-γ production (GERMANN et al., 1993); (2) it has a stimulatory role in the cytotoxic effector function of the NK cells and $CD8^+$ T cytotoxic cells (CHEHIMI et al., 1992); and (3) it shows growth factor characteristics for T and NK cells (GATELY et al., 1991). Some of these effects, especially the cytotoxicity, are mediated by a number of molecules being up-regulated by target cells upon binding of IL-12 to its receptor. Among these molecules the most important are: IFN-γ, perforin (HASHIMOTO et al., 1999), Fas ligand FL (EBERT, 2004) and different adhesion molecules such as CD69, CD71, CD54, CD2 (RABINOWICH et al., 1993).

IFN-γ. This signatory molecule of IL-12 stimulation is secreted by the two main IL-12 responsive lymphocyte populations: T cells (Th1 and $CD8^+$ cytotoxic T cells) and NK cells (CHAN et al., 1991). HODGE et al. (2002) have shown that in addition to regulation of IFN-γ on transcriptional level, IL-12 also confers post-transcriptional regulation of IFN-γ by stabilizing the nuclear fraction of its mRNA. Despite being one of the major Th_1 cytokines and thus playing a predominant role against intracellular pathogens, IFN-γ has additional roles, for example in the immune response against cancer. This can be observed either through the direct action of IFN-γ on the cancer cells (DUNN et al., 2004) or indirectly through its anti-angiopoietic effects.

Stimulation of cytotoxicity. In addition to the stimulation of IFN-γ production from NK cells, IL-12 was originally discovered for its stimulatory effects on the MHC-unrestricted cytotoxic activity of peripheral blood leukocytes (PBL) (KOBAYASHI et al., 1989). In the same study, it was shown that the $CD3^-$ $CD5^-$ $CD56^+$ NK cell fraction represented the population of unfractionated PBL responsible for increased lymphokine activated killer (LAK) activity upon IL-12 stimulation. Following this discovery, a large amount of research was conducted in order to clarify the exact molecular action of the IL-12 induced cytotoxic activity of the lymphocytes. Hence, different molecules participating in the effector phase of the cytotoxic process, such as perforin (HASHIMOTO et al., 1999), granzymes (BONNEMA et al., 1994; DEBLAKER-HOHE et al., 1995) and TNF-α, were found to be up-regulated by IL-12.

Lymphocyte proliferation. Another effect of IL-12 is its ability to stimulate proliferation of peripheral blood mononuclear cells (PBMC). A large amount of data exists on this topic, reporting on the effects of IL-12 on proliferation of the whole PBMC population, as well as of distinct lymphocyte fractions. Thus, IL-12 causes proliferation of PHA-stimulated lymphoblasts as well as proliferation of NK cells and of $CD4^+$ and $CD8^+$ T lymphocytes of such mitogen-stimulated PBMC (GATELY et al., 1991). Conversly, experiments investigating the proliferation of purified NK cells upon IL-12 stimulation showed that freshly isolated NK cells did not proliferate in response to IL-12 stimulation. Additionally it was also shown that IL-12 inhibited the IL-2 induced proliferation of pre-activated NK cells by 80-90%. (ROBERTSON et al., 1992).

IL-12 forms. All of the effects of IL-12 mentioned above are mediated through its heterodimeric IL-12 p70 form, but existence of other forms has also been reported among different species (GATELY et al., 1996; HEINZEL et al., 1997). The presence of additional forms of IL-12 is due to the fact that IL-12 p40 is expressed in excess compared to IL-12 p35 (D'ANDERA et al., 1992) subunit, hence allowing formation of p40 homodimers and monomers. Nevertheless, the excess of the IL-12 p40 subunit shows different patterns of molecular interactions depending on the species. Additionally, different patterns of molecular interactions were also observed, depending on whether primary cells or transfected culture cells were investigated. Thus, immunoprecipitation of IL-12 p40 from human primary dendritic cells (DC) stimulated to produce IL-12, showed that p40 co-precipitated with the p35 chain only and not with another p40 chain. This confirmed that IL-12 p40 in human is expressed only as a monomer (CARRA et al., 2000). In contrast to primary cells, cells

transfected with plasmid carrying the human IL-12 cDNA showed presence of p40 homodimers. This homodimeric form exhibited significantly lower binding affinity for the IL-12Rß1 than the IL-12 heterodimer (CARRA et al., 2000). Nevertheless, due to over-expression of the p40 subunit, p40 homodimers compete with the p70 heterodimeric form for the IL-12 receptor and hence act as an antagonist. This was shown by MATTNER et al. (1993) and LING et al. (1995) who demonstrated inhibition of IL-12 p70 induced lymphocyte proliferation by the p40 homodimeric form. In contrast to human primary cells, murine IL-12 producing primary cells revealed the existence of IL-12 p40 homodimers, which are an effective antagonist of the biological action of murine IL-12 p70 heterodimeric form (HEINZEL et al., 1997). The absence of any biological, agonistic or antagonistic activity was demonstrated for the porcine IL-12 p40 homodimer (FOSS, 1999).

IL-12 in tumor therapy. The anti-tumor activity of IL-12 has been confirmed by in vivo experiments in mice (BRUNDA et al., 1993). This has made IL-12 an attractive tool for anti-cancer therapy following delivery to tumor patients. Among the studies performed, different ways of application (local, systemic), as well as different forms of IL-12 (cDNA, protein) have been investigated in their potential to reduce tumor growth or even to eradicate established tumors. Thus, some of these studies showed that intra-tumoral injection of adenoviral vector encoding the IL-12 cDNA induced complete regression of several different tumors experimentally established in mice. These tumors include subcutaneous murine adenocarcinomas and fibrosarcomas (GAMBOTTO et al., 1999), breast (BRAMSON et al., 1996) and colorectal carcinoma (MAZZOLINI et al., 1999), orthotopically implanted murine tumors including medullary thyroid carcinoma (NASU et al., 1999) and hepatocellular carcinoma (BARAJAS et al., 2001). Additionally, some of these studies (GAMBOTTO et al., 1999) also reported rejection of subsequent rechallenge with the same tumors, indicating a persistence of a long lasting anti-tumor immunity. In contrast to gene delivery studies, there are no studies investigating the potential of in vitro IL-12 stimulated, adoptively transferred lymphokine activated killer (LAK) cells, despite the well investigated IL-12 stimulative effect on T cell mediated and especially on NK cell mediated cytotoxic activity. Hence, studies investigating the potential of using adoptively transferred LAK cells for cancer immunotherapy are needed.

2.4 Biology of IL-12 in dog

Canine IL-12 was cloned independently by two groups (OKANO et al., 1997; BÜTTNER et al., 1998) and it was shown that both subunits have more than 80% identity with IL-12 of several other species (human, mouse, bovine), as determined by computer-aided sequence comparison (BÜTTNER et al., 1998). In accordance with the structural similarity, the biological activities of the canine IL-12 proved to be similar to those described for the respective human and murine counterparts. Because of this, the biological activities of canine IL-12 were investigated mostly in clinical studies in dogs infected with Leishmania species (STRAUSS-AYALI et al., 2005). Apart from this study there are only a few other studies that have investigated the biological activities of IL-12 in dogs. Thus, PHILLIPS et al. (1999) have demonstrated an increased proliferation of canine phytohemaglutinine (PHA)-lymphoblasts as well as an increased cytotoxic activity of freshly isolated canine PBMC after 3 days stimulation, but using human recombinant IL-12. Although this group has demonstrated biological effects of human IL-12 on canine lymphocytes, its use in cancer immunotherapy is connected with the risk of initiating antibody response in dog. Another group working with canine IL-12 (SALDARRIAGA et al., 2006), investigated the increase of the IFN-γ mRNA level upon stimulation with canine IL-12 fused heterodimeric cDNA (single cDNA coding for the both IL-12 subunits). They also showed that in vivo application of the fused heterodimeric IL-12 cDNA had the same effects regarding the increase in the IFN-γ mRNA level. However, other effects like proliferation or cytotoxic activity of the canine PBMCs, or of their subpopulations have not been investigated.

2.5 IL-12 as a single chain

Considering the facts about the IL-12 biology mentioned above and the interest of introducing its nucleotide sequence into the tumor site as an effective tumor therapy, the construction of a single plasmid carrying the both IL-12 subunits has emerged as convenient solution. This plasmid offers a solution to overcome the disadvantages that accompany gene therapy where both subunits are transfected on separate plasmids. Exemplarily, one of the drawbacks in applying the IL-12 sequence to a patient is the fact that functional IL-12 requires the presence of both chains in the same cell (GUBLER et al., 1991).

Since transfection is a very random process, a double transfection with two different plasmids lowers the chances for a successful outcome. Additionally, gene therapy is accompanied by certain risks, because the heterogenic genetic material is introduced into the patient. This is specially to be regarded when delivering the sequence with help of viral vectors, some of which potentially confer their own threat for the patient. The first attempt to overcome these problems resulted in construction of a IL-12 cDNA sequence inserted into a retroviral construct, where an internal ribosomal entry site (IRES) element from the encephalomyocarditis virus was used for separate translation of each of the subunits (ZITVOGEL et al., 1994). Although the vector was successfully transfected, this approach showed a drawback in the progressive reduction of the expression rate downstream of each IRES. In this case the difference in the expression rate of the two subunits makes the formation and thus the activity of the IL-12 heterodimer less predictable and less reproducible between experiments. As a solution, a single nucleotide sequence encoding both p40 and p35 subunits connected by a linker sequence, was constructed by ANDERSON et al. (1997). The linker sequence encodes a decapeptide chain containing two elastin motifs. It adopts its structure in such a way that it does not intervene with the formation of intra- and interpeptide structures of both IL-12 subunits, thus allowing to form the natural conformation.

2.6 Investigation of the IL-12 effects on PBMC

2.6.1 PBMC Isolation

The most widely used technique for routine separation of peripheral blood cells is density gradient centrifugation, based on the different specific densities of cells. Numerous media suitable for this purpose have been described, with Ficoll® being the first choice due to its simple handling and pre-adjusted osmolarity. Another often used density centrifugation medium is the colloidal silica solution, Percoll® (Pharmacia, Sweden), with particles of a diameter of 15-30 nm and slightly hypoosmotic in comparison to the blood. The hypoosmolarity of Percoll® can be easily corrected by adding 1 part of 1.5 N NaCl solution to 9 parts of the Percoll® stock solution. Because of the difference in densities among lymphocyte populations in different species precise determination of the conditions for isolation of PMBC from the blood must be performed for every species. Thus, an optimal

medium for isolation of canine PBMC is Percoll®, which showed better results in comparison to Ficoll® (GONDOLF, 1994; GONDOLF et al., 1996). The most appropriate concentrations for isolation of different sub-fractions of the canine PBMC using Percoll® were determined in our lab (GONDOLF, 1994; GONDOLF et al., 1996). According to these results 58.5 % Percoll® working solution, allows isolation of PBMC fraction enriched in NK cells.

2.6.2 Cytotoxicity assay

The Chromium-Release Assay (CRA) has been widely used as a gold standard for investigating the cytotoxic activity. This assay analyses the release of radioactive ^{51}Cr from labeled target cells into the supernatant. The subsequently measured radioactivity in the supernatant is analogous to the cytolysis of target cells caused by a given agent. However, the need for special precautions when working with the CRA and its high price are significant disadvantages. As an alternative to the CRA, a new non-radioactive colorimetric method for determining the cytotoxic activity against adherent target cells using Rose-Bengal color was established in our department (GONDOLF 1994, GONDOLF et al., 1996). The Rose-Bengal Assay (RBA) is based on the loss of adherence of the killed (dead) target cells. To determine this, target cells are plated in a 96-well plate and incubated until they form a monolayer, after which effector cells are added at various effector to target (E:T) ratios. The effector and target cells are incubated in a co-culture allowing direct contact between them. As negative controls monolayers without effector cells are used. Following incubation, the supernatant together with the effector cells and the non-adherent dead target cells are removed from the wells. The remaining target cells are incubated with the Rose-Bengal color allowing them to absorb it. Following incubation, the color is released into the supernatant by an ethanol-containing solution. The intensity of the color in the supernatant is determined by measuring its optical density (O.D.) in a spectrophotometer at 570 nm, using the 630 nm for a background correction. Among the adherent target cells, canine thyroid adenocarcinoma (CTAC), as NK-sensitive and VERO as NK-resistant cell lines were investigated by GONDOLF (1994, 1996). Experiments showed that the formation of a target cell monolayer is best obtained after 26 hours, if plated at a density of $1x10^4$/well. Furthermore, the investigation of different incubation times of effector and target cells showed that 14 hours are optimal for obtaining reliable results.

Important advantage of RBA over the CRA is the fact that CRA allows measuring of cytotoxic processes due to cell membrane disruption only. Since the apoptotic processes are lacking cell membrane disruption, the detection capacity of CRA is limited only to necrotic processes, excluding detection of apoptotic events. In contrast, both necrotic and apoptotic events lead to loss of cell adherence, making RBA more sensitive then the CRA (GONDOLF 1994, GONDOLF et al., 1996).

2.6.3 Cell proliferation

Cell proliferation is one of the criteria widely used to determine cell activity, response to stimulation and its physiological state. The various assays used for measuring cell proliferation can be divided into two groups: (1) assays such as MTT, XTT and Alamar Blue which indirectly analyze proliferation by measuring the metabolic activity of cells; (2) assays that directly determine the newly synthesized DNA, using radioactive labeled nucleotides, as in [^{3}H]-thymidine incorporation assay (TdR), or non-radioactive labeled nucleotides, as in Bromodeoxyuridine (BrdU) incorporation assay. Another possibility to directly confirm the mitotic activity is the measurement of total DNA increase using different nucleophilic fluorescent dyes, which can be quantified using flow cytometry. Because of the simplicity in handling, the use of non-radioactive materials and lower cost, the ELISA based BrdU method (WAGNER 1998, WAGNER et al., 1999) was used to investigate the proliferation of canine PBMC. In this approach, cells are incubated with BrdU in presence of the appropriate stimulus for the required time. During the incubation time the cells entering mitosis incorporate the BrdU, whereas the resting cells do not. Afterwards the cells are centrifuged, fixed and permeabilized. In this way the incorporated BrdU is ready for detection by a specific peroxidase (POD) labeled anti-BrdU antibody. In the following step H_2O_2 based peroxidase substrate is added to the wells and the reaction mix is incubated for up to 30 minutes. The peroxidase activity is stopped by adding H_2SO_4 after which the O.D. of each well is determined in a spectrophotometer at 450 nm wavelength, using the wavelength of 630 nm for background correction.

3 Materials and Methods

3.1 Cloning

3.1.1 Polymerase chain reaction (PCR)

3.1.1.1 KOD Hot Start DNA Polymerase

When a reliable amplification of the sequences of interest was required, KOD Hot Start DNA Polymerase (Novagen®) with 3'→5' "proof-reading" activity was used. PCR with this polymerase was performed according to the manufacturer's recommendations. Shortly, the PCR was performed in a 50 µl reaction volume in presence of 1x buffer for KOD Hot start DNA Polymerase, 0.2 mM dNTPs (final concentration each), 1mM $MgSO_4$, 10 pM of each primer (MWG) and 1U KOD Hot start DNA Polymerase. Depending on the aim of a given PCR reaction, different amounts of DNA were used as a template. For site-directed mutagenesis 5-15 ng template plasmid DNA was used, while 30 ng were used where genomic DNA served as a template. The PCR program was set as follows:

1. Initial denaturation at 94°C for 2 min.
2. Cyclic denaturation at 95°C for 15 sec.
3. Annealing step 30 sec.:
   - for site-directed mutagenesis 60°C was used
   - for every other purpose the optimal temperature was determined by a gradient PCR
4. Extension 1min/kb at 68°C
5. Final extension 5min at 68°C

After each PCR performed, the product of interest was analyzed by gel electrophoresis and further processed according to the requirements of the subsequent experiment.

3.1.1.2 Taq Polymerase

PCR reactions that did not require the “proof-reading” activity of the polymerase were performed with Taq Polymerase (PeqLab), according to the manufacturer’s instructions. These PCR reactions were performed in 25 µl reaction volume, 1X buffer for the Taq-Polymerase (1.5mM $MgCl_2$), 0.2 mM dNTPs (final concentration each), 10 pM of each primer and 2,5 U Taq-Polymerase. Depending on the source of the DNA, the appropriate amount of template was used. The PCR program used with the Taq Polymerase was as follows:

1. Initial denaturation at 94°C for 2:30 min.
2. Cyclic denaturation at 92°C for 30 sec.
3. Annealing step 30 seconds:
   - the optimal temperature was determined by a gradient PCR for each primer pair
4. Extension1 min/kb at 72°C
5. Final extension 5min at 72°C

3.1.1.3 A-overhang addition and TA cloning

The A-addition kit (Qiagen) was used for adding the adenine nucleotide on the 3’ end of the DNA fragments with blunt ends, obtained after amplification with KOD Hot Start Polymerase. For this purpose, 5 µl of the PCR reaction volume were taken immediately after the amplification and incubated with 2µl of the 5X A-addition master mix (Qiagen) and 3µl ddH_2O in a total volume of 10µl, at 37°C for 30 min. Following incubation, an aliquot of this reaction was inserted into the pGEM-T® cloning vector (Promega), as recommended by the manufacturer. Shortly, 5 µl of the 2X rapid ligation buffer (pGEM-T® Vector System I kit, Promega), 1µl of the pGEM-T® vector, 1µl of the T4 DNA Ligase (3 Weiss units/µl, pGEM-T® Vector System I kit, Promega), and 3 µl of the fragment taken directly from the A-addition reaction volume were incubated at 4°C overnight, identically as described for the other ligation reactions (3.1.4). Following ligation, the ligation production was transformed into E. coli (HB101) as described (3.1.5).

3.1.2 Gel electrophoresis

After each PCR, the size of the amplicons was confirmed on polyagarose electrophoresis. Depending whether the amplicon had to be isolated, or only confirmed on gel, different agarose (SeaKem LE Agarose, Lonza) concentrations were used. Thus, 0.8% agarose was used for isolation of the amplicons, since most of them were larger then 1kb. In one case where the product was 200 bp, a 2% agarose gel was used. For detection purposes 1-2% agarose was used, depending on the expected product size. For preparing of the gel, which was cast in a small (6x10 cm) chamber, the agarose was dispersed in 1xTBE buffer and heated in a microwave oven until completely dissolved. Before casting the gel, ethidium bromide was added to the agarose. Five parts of each amplification reaction were mixed with one part of a 6X loading dye (6X loading dye solution, Fermentas) and loaded into gel slots. The electrophoresis was run using power supply system (Consort E425) at 80 V/cm/h in 1x TBE buffer. The PCR products resolved in the gel were visualized under exposure at 254 nm UV light (UV-lamp, Bachofer) and documented using the EDAS 120 system (KODAK).

3.1.3 Restriction digestion

Different restriction enzymes were used to digest the different sequences needed to create rcscIL-12. As a general rule, the restriction digestion was carried out in 60 µl volume, using between 3-6 µg of the DNA sequence to be digested, but always in equal molar ratios. Following the general principles of the restriction digestion, 1x of the corresponding buffer was used, whereas the restriction enzyme was applied at concentration of 1U pro 1µg of target DNA. All restriction digestions were performed at 37° C for 1 hour, except for the BsmBI enzyme (NEB), when 55°C was used. Where double digestion was not feasible due to the incompatibility between some buffers and enzymes, the DNA was consecutively digested. Thus, following the digestion with the first enzyme the DNA was “cleaned-up” using commercially available DNA binding columns (Macherey Nagel) as recommended by the manufacturer. For this purpose, the reaction volume after the first digestion was applied on

DNA binding column, which was centrifuged for 2 min at 7000 rpm in a microcentrifuge, and the flow-through discarded. The bound DNA was eluted from the column with 40 µl ddH2O and was used for the second digestion, again in a volume of 60 µl. The digested fragments were gel electrophoresis purified and isolated using a commercially available gel removal column (Nucleo Trap® Extraction kit, Macherey Nagel). For this purpose, the reaction volume of the digested DNA was loaded onto a 0,8% gel, the electrophoresis run at 80 V in 1x TBE buffer for 1h, the gel was visualized under 254nm UV light (UV-Lamp, Bachofer) and documented using EDAS 120 system (KODAK). The fragment of interest was cut out and transferred onto the appropriate column, which was centrifuged at 7000 rpm for 2 min. The eluate containing the DNA fragment of interest was further used in the corresponding ligation reaction.

3.1.4 Ligation

For the purpose of ligation, the fragments of interest were incubated together in an equal molar ratio in the presence of 1U of E.coli DNA Ligase at 4°C overnight. The ligation product was either transformed into E. coli in the case of plasmids or was used as a PCR template when further manipulation was necessary.

3.1.5 Transformation of E.coli

When a further amplification of a desired plasmid was needed, the plasmid was transformed into E.coli cells (HB101, K12 strain). For this purpose, the bacterial cell suspension (50 µl) was thawed on ice for 8-10 minutes and afterwards the appropriate plasmid containing reaction volume was added, followed by an additional incubation of 20 minutes on ice. As a next step the cells were “heat-shocked” at 42°C for 2 min followed by an additional incubation on ice for 2 minutes. 100 µl LB (Roth) medium were added and the entire content was spread out onto a Petri-dish (82 mm diameter) containing LB (Roth) agar supplied with 50µg/ml ampicilin (Roth). Depending on the actual purpose of the cloning, the selection was done with help of the white-blue screening, when using plasmids carrying the gene for ß-galactosidase. The LB agar containing plates used for blue-white screening were supplied

with 40µg/ml X-Gal (Roth) and 50µg/ml IPTG (Roth). Otherwise, when using plasmids not carrying the ß-galactosidase gene, random picking of 10 colonies was performed. In both cases the selected clones were cultured overnight in LB medium containing ampicilin (50µg/ml)· The transformed colonies were identified by means of a colony PCR with a crude bacterial lysate serving as a template.

3.1.6 Plasmid DNA extraction

The colonies positive in colony PCR were used for plasmid extraction using column chromatography-based plasmid preparation kit (NucleoSpin® Plasmid, Macherey Nagel) according to the manufacturer's recommendations:

1. The bacterial cell suspension was grown in 10 ml LB medium at 37°C for 14-16 hours.
2. After the incubation time the bacterial cells were pelleted at 11.000 x g for 2 minutes, the supernatant removed and the bacterial cell pellet resuspended in 250 µl of the appropriate resuspension buffer (buffer A1). The resuspended cells were vortexed in order to resuspend any remaining clumps.
3. 250 µl of a sodium dodecyl sulfate (SDS) containing lysis buffer (A2) was added to the cell suspension and the tube was gently inverted to allow complete lysis.
4. 300µl of the A3 buffer were added to the lysate, allowing the proteins to further denaturate and precipitate. At the same time this buffer creates optimal conditions for DNA binding to the NucleoSpin column.
5. The lysate was centrifuged at 11.000 x g for 5-10 minutes and the DNA containing supernatant was transferred onto the column.
6. The column was incubated for 1-2 minutes to allow the DNA molecules to bind and then it was centrifuged in a microcentrifuge at 11.000 x g for 1 min.
7. After the flow-through was discarded, 600 µl of ethanol containing buffer (A4) were added to the column to remove the contaminants like salts, metabolites and soluble macromolecular cellular components. The column was centrifuged at 11.000 x g for 1 minute.

8. The flow-through was discarded again and the column was centrifuged additionally at 11.000 x g for 2 min. in order to dry the silica membrane completely.
9. After drying 50 µl of the elution buffer (AE) providing low ionic and slightly alkaline conditions (5mM Tris-Cl, pH 8.5) were added to the column and it was incubated at RT for 1 min.
10. The eluted plasmid DNA was collected by centrifugation at 11.000 x g for 1 min. and the eluate was transferred to a new tube.

The plasmid DNA concentration was determined after every isolation procedure using an UV spectrophotometer (UV-1202, Shimadzu) at 1:1000 dilution of the plasmid DNA solution with molecular grade H_2O.

3.2 Construction of the recombinant canine single chain IL-12 (rcscIL-12)

3.2.1 Completion of the cDNA coding for the IL-12 p35 sequence

The sequences coding for the canine IL-12 p35 and IL-12 p40 subunits (BÜTTNER et al., 1998) were kindly provided by Prof. Dr. Mattias Büttner ("*Landesamt für Gesundheit und Lebensmittelsicherheit Oberschleißheim"*, Germany). Because of the time spent at -20°C confirmation of the sequences was performed by sequencing at MWG Biotech (Ebersberg, Germany). The sequencing results showed that the sequence coding for p35 contained the in-frame sequence of the first six exons (bp 1-494), with exception of the last 20 nucleotides of exon six (bp 494-514). Additionally, the complete seventh exon (bp 515-669) was also absent and hence the addition of the bases 494-669 to the existing sequence of the first six exons was required in order to complete the p35 subunit. For this purpose, the missing exon 7 was amplified from genomic DNA, using the following primers: forward primer 5' *cgtctcagccctgaatttcaacagtgtgact* 3'; reverse primer 5'*acgcgtttaggaagaattcagataactcatcattc tatcgatggtcaccg* 3'. This PCR reaction was performed under conditions described for KOD Hot Start DNA Polymerase. The rest of the sequence, from exons 1 to 6, which existed in the plasmid provided by Prof. Dr. Büttner (s.a.) was also multiplied by a PCR reaction as described (1.1.1.), using the following primers: forward primer 5'*atgtgcccgccgcgcggcctcc* 3' and 5'*cgtctccgggcctgtaacagctcatcgatagctgtcaacatgttttgatccaga* 3' as a reverse primer. For the

purpose of connecting these two fragments, BsmBI restriction sites (*cgtctcxxxx*) were introduced in the reverse primer for the exon 1-6 sequence and in the forward primer for the exon 7 sequence. After the sequences had been confirmed by sequencing (MWG), they were reamplified, digested with the BsmBI enzyme and ligated together (3.1.4). The ligation product was used as PCR template in the next reaction in order to re-amplify the complete p35 sequence using the forward primer for exon 1-6 sequence and the reverse primer for the sequence of exon 7. This amplicon was isolated and ligated into a plasmid vector as described above. After the positive clone had been chosen, it was sent for sequencing to MWG and its sequence confirmed.

3.2.2 Correction of the p40 Sequence (DpnI site-directed mutagenesis)

The sequencing of the plasmid containing the cDNA coding for the p40 subunit revealed the sequence presented in fig. 1. Positions 375 and 826, shown as capital letters, did not match the published consensus sequence of p40 (PubMed Acc.Nr.:NM_001003292). Thus, at position 375 a surplus adenine (A) was detected rendering the nucleotide sequence out of frame and at position 826 an adenine was confirmed instead of guanine (G).

1 atgcatcctcagcagttggtcatctcctggttttccctcgttttgctggcgtctcccctc 60
61 atgaccatatgggaactggagaaagatgtttatgttgtagagttggactggcaccctgat 120
121 gcccccggagaaatggtggtcctcacctgccatacccctgaagaagatgacatcacttgg 180
181 acctcagcgcagagcagtgaagtcctaggttctggtaaaactctgaccatccaagtcaaa 240
241 gaatttggagatgctggccagtatacctgccataaaggaggcaaggttctgagccgctca 300
301 ctcctgttgattcacaaaaaagaagatggaatttggtccactgatatcttaaaggaacag 360
*361 aaagaatccaaaaa**A**taagatctttctgaaatgtgaggcaaagaattattctggacgttt 420*
421 cacatgctggtggctgacggcaatcagtactgatttgaaattcagtgtcaaaagtagcag 480
481 aggcttctctgacccccaaggggtgacatgtggagcagtgacactttcagcagagagggt 540
541 cagagtggacaacagggattataagaagtacacagtggagtgtcaggagggcagtgcctg 600
601 cccctctgccgaggagagcctacccatcgaggtcgtggtggatgctattcacaagctcaa 660
661 gtatgaaaactacaccagcagcttcttcatcagagacatcatcaaaccagacccacccac 720
721 aaacctgcagctgaagccattgaaaaattctcggcacgtggaggtcagctgggaataccc 780
*781 cgacacctggagcaccccacattcctacttctccctgacattttgc**A**tacaggcccaggg 840*
841 caagaacaatagagaaaagaaagatagactctgcgtggacaagacctcagccaaggtcgt 900
901 gtgccacaaggatgccaagatccgcgtgcaagcccgagaccgctactatagttcatcctg 960
961 gagcgactgggcatctgtgtcctgcagttaggttccacccccaggatgaatcttgg 1016

Fig 1: IL-12 p40 sequence as obtained

In order to correct these mismatched bases, these positions were consecutively replaced using DpnI mutagenesis approach. For this purpose the plasmid carrying the cDNA coding for p40, was completely amplified by PCR using KOD Hot start polymerase, (3.1.1.1) with the appropriate primers (position 375: forward primer 5` *ggaacagaaagaatccaaaaataagactttt ctgaaatgtg* 3`; reverse primer5`*cacatttcagaaagatcttatttttggattctttctgttcc* 3`; position 826 forward primer 5`*cttctccctgacattttgcgtacaggcccagggcaagaac* 3`, reverse primer 5`*gttcttgccctgggcctgtacgcaaaatgtcagggagaag* 3`). The PCR amplicon was digested with DpnI at 37°C for 1h. Following digestion the sample was used to transform E.coli as described (3.1.5) and the plasmid was subsequently isolated. The isolated plasmid was sent to sequencing in order to confirm that the change of interest has occurred.

3.2.3 Construction of the rcscIL-12

In order to bring both IL-12 subunits (p35 and p40) together, they were reamplified using the following primers: p40 forward primer 5' *ggtaccatgcatcctcagcagttggtc* 3'; p40 reverse primer 5' *cgtctcaggacaccgactcccgggacactgcaggacacagatgcccagtc 3';* p35 forward primer 5' *cgtctcggtcccaggtgtcggtatgtgcccgccgcgcggcctcc* 3'; p35 reverse primer 5' *acgcgtttaggaagaattcagataactcatcattctatcgatggtcaccg* 3'. After the PCR had been performed, the amplicons were double digested with the following enzymes: p40 was initially digested with KpnI at 37°C for one hour in one for all (OFA) buffer, followed by digestion with BsmBI at 55°C for one hour. Similarly, the p35 was digested with PstI at 37°C for one hour in the NEBuffer 3 and afterwards with BsmBI at 55°C for an additional hour. The resulted restriction fragments were separated using gel electrophoresis and the sequences were isolated from the agarose gel. The fragments were ligated using the E.coli DNA ligase at 4°C over night, and 1µl of the ligated product was used as a template for a PCR performed under conditions for KOD Hot Start Polymerase with the p40 forward and p35 reverse primers. The resulting PCR product was cloned into pGEM-T (Invitrogen) vector as described previously.

3.2.4 Construction of the sccIL-12-IRES/Lucifease-pTRUE plasmid

For the purpose of creating this construct the sequences needed were digested at concentration of 3µg/ml in the appropriate buffers. Thus, the pTRUE vector (derived from pTRE, Clontech®, personal communication: PD Dr. Tautz, University of Luebeck, Germany) was consecutively digested with SacII and PstI (NEB®) due to the incompatibility of the two enzymes for a simultaneous digestion. The digestion was first performed with SacII enzyme in the presence of NEBuffer 4 buffer for two hours followed by a step of buffer removing using a DNA binding column. Afterwards the SacII-linearised pTRUE vector was digested using the PstI enzyme in the H-Buffer (TaKaRa®) for an additional hour. After digesting the pTRUE vector, it was dephosphorylated in presence of 1µl of a calf intestinal alkaline phosphatase at 37°C for 5 min. Similarly, the sequence coding for the rcscIL-12 was also consecutively digested with SacII and MluI enzymes (NEB®) under the same conditions as for SacII (s. a.), followed by a step of a buffer removal. The linearized rcscIL-12 vector was digested with the MluI enzyme in the NEBuffer 3 for one hour. The IRES/Luciferase cassette was double digested with MluI and PstI in the NEBuffer 3 for one hour. These three restriction fragments were isolated from a preparative 0.8 % gel, and following isolation they were ligated at 4°C in a presence of 1 x ligase buffer and 1U of E.coli DNA ligase over night. The resulting ligation product was transformed into E.coli cells (s. a.).

3.3 Establishing of a stably transfected BHK-Tet-On cell line

3.3.1 Stable transfection

The stable transfection of the BHK-Tet-On cell line with the sccIL-12 carrying plasmid was performed using lipofection. The sccIL-12 construct (s. 1.3.1) was previously cloned in E. coli (s.1.3.3). After a column chromatography was applied for purification, its concentration was determined using UV-spectrophotometer. Total amount of 6 µg of the plasmid was linearized at 37°C in the presence of 1 x buffer and in a total volume of 60 µl for 1 hour. Thereafter, 3 µg of the linearized construct (10 µl of the 300 ng/µl plasmid solution) was added to 10 µl of the liposome solution (Lipofectamin®, Qiagen) and 70 µl medium without

serum and the mixture was incubated at room temperature for 10 minutes. Together with the linearized construct containing the IL-12 gene, another one, also linearized vector coding for Puromycine resistance (personal communication PD Dr. Tautz, University of Luebeck, Germany) was added to the mixture at a concentration 100 times lower (3ng/ml) than the concentration of the rscsIL-12 containing plasmid. This transfection mixture was added to the BHK Tet-On cells (previously grown to confluence in a 6-well plate overnight) and these were incubated for two hours. Following incubation, the transfection mixture was removed and RPMI (PAA, Austira) culture medium containing 10% serum was added to the cells in order to incubate them for an additional 24 hours.

3.3.2 Cloning of the transfected cells

After 24 hours of incubation, the stably transfected cells were completely trypsinized and transferred to a 10 cm Petri dish containing 10 ml of complete medium supplied with 1µg/ml Puromycine dihydrochloride (Alexis corporation) and 0.4 mM neomycin sulfate (G418, Amresco). From this Petri dish further 10-fold serial dilutions were made (from 1: 10 to 1: 10. 000) in the same medium, in final volumes of 10 ml. On the following days the development of, as good as possible from one another separated areas, or cell isles was monitored in every dish. From the dilution with most distinctly separated cell isles, ten were randomly selected, carefully trypsinized and transferred into separate wells of a 24-well plate. They were allowed to grow to confluence and those populations forming monolayers were brought to 10-cm Petri-dishes. After they had grown to confluence in Petri-dishes, they were further subcultured and two portions of 1 x 10^6 cells of each population were concomitantly used for luciferase assay. One of the portions was incubated with Doxcycline, while the other one was left without, serving as a negative control. After the luciferase assay was performed, the best two populations according to the absolute values of the luciferase assay and the induction index (the difference between the Doxycycline induced and non-induced cells), were further subcloned using limited dilution cloning. The resulting clones were again tested for luciferase activity as described (3.3.3). The two clones with the highest luciferase assay values in the first subcloning underwent a second subcloning procedure.

3.3.3 Luciferase assay

The luciferase assay was performed with duplicate portions of 1x10^6 cells were cultured at 37°C, 5%CO_2 in a 6-well plate overnight. On the next day they were lysed according to the following protocol:

1. The medium was flicked off.
2. The cells were washed with 1X PBS solution.
3. The cells were incubated with 400 µl/well of the 1X lysis buffer, and after the cell layer had been gently scraped, the resulting cell lysate was transferred into 1.5ml Eppendorf tubes. These were stored on ice until further use.

For measuring the luciferase activity with the Luciferase Assay System kit (Promega), 20 µl of every cell clone lysate were incubated with 100µ of the 1X luciferase substrate (Luciferase Assay System, Promega). The resulting light emission was detected in a luminometer and was expressed as absolute value. 20µl PBS were used as a blank, and these values were withdrawn from the values of the probes. All probes were measured in duplicate and their mean was calculated. The values of the Doxycycline induced cells and of cells incubated without Doxycycline were compared, with their ratio serving as a measure for the Luciferase inducibility by Doxycycline (induction index).

3.3.4 Immunofluorescence

The cells of both finally chosen clones were cultured in duplicates in 6-well plates at concentration of 1x10^6cells/ml overnight. One of the duplicates of each cell clone was cultured in presence of doxycycline, whereas the other one was cultured without it. On the next day the cells were processed as follows:

1. The medium was removed.
2. The cells were washed with 1ml PBS pro well.
3. The cells were fixed by incubation in 750 µl methanol-aceton solution (1:1 pre-mixed solution, cooled at -20°C) at - 20°C for 20 min.
4. The methanol-aceton solution was removed and the cells were air-dried.

5. The cells were re-hydrated with 750 µl/well of the PBS/Tween solution at room temperature for 1 min.
6. The cells were washed with 750 µl/well of the PBS solution.
7. Incubation with the primary rabbit anti-luciferase antibody (Abcam, 1:1000) at 37°C for one hour.
8. The cells were washed 3 times with 750 µl/well of the PBS solution.
9. Incubation with the secondary goat anti-rabbit Cy-5 labeled antibody (Chemicon 1:500) at 37°C for one hour.
10. The cells were washed 3 times with 750 µl/well o f the PBS solution.

The labeled cells were visualized under inverted fluorescent microscope Axiovert35 (Zeiss).

3.3.5 SDS-PAGE

The sodium dodecyl sulfate (SDS) – polyacrylamid gel electrophoresis (PAGE) was performed in tricine gel (Schaegger and Jagow, 1987), cast in chambers with dimensions of 8.5 x 6 cm. The electrophoresis was run in an electrophoresis chamber manufactured in the working factory of the MZI Giessen. Solution of 12% acryl amide in gel buffer was used for preparing the resolving gel. The used acryl amide (AppliChem, Darmstadt) was a 40 % solution mixture of acryl amide and N, N'-methylen-bis-acrylamide in 29:1 ratio. Polymerization of the resolving gel was initialized by adding 0.05% (w/v) Ammonium persulfate (APS) and 0.1 % (v/v) TEMED. The stacking gel was prepared as a 4% acryl amide solution in gel buffer, polymerized by addition of 0.08% (w/v) ammonium persulfate (APS) and 0.1 % (v/v) TEMED. The probes (cells and supernatant) were lysed in SDS lysis buffer supplied with 5% (v/v) ß-mercaptoethanol and incubated at 94°C for 10 minutes to achieve complete protein denaturation. The probes were run at 120 V for 90 min., together with a pre-stained molecular marker (Pre-stained Protein Marker Broad Range, NEB). Following electrophoresis, the gel was removed and the stacking gel was removed. The remaining resolving gel was consequently processed for Western blot.

3.3.6 Western blot

The transfer of the electrophoretically separated proteins (3.3.5) to the nitro-cellulose membrane Bio Trace NT (Pall Gelman Laboratory) was carried out in electric field using the "semi-dry" blotting technique. For this purpose the Trans-Blot® SD (BioRad) electro blotting system was used, as recommended by the manufacturer. The gel was soaked into the cathode buffer for 5 minutes, whereas the membrane in the anode buffer. Additionally, 6 filter papers (Whatman) with the size of the gel were soaked into the appropriate transfer buffers: 3 of them into the anode, and the other 3 into the cathode buffer. The three filter papers soaked into the anode buffer were layed directly on the anode plate, followed by the membrane and the gel. The other 3 filter papers soaked into the cathode buffer were placed on the top of the gel. This stacked formation was gently pressed with a glass pippet in order to remove the air from between the layers. Finally, the cathode was placed over the cathode-soaked filter papers and electrical current was applied (5mA/cm^2 and maximally 13 V) for 30 min. Following the protein transfer, the membrane was incubated in a 5% (w/v) low-fat milk solution in PBS-Tween (0.05% (v/v) Tween-20 in PBS) at room temperature for at least 1 hour, in order to block the free binding sites of the membrane. In addition, the membrane was incubated at 4°C overnight in order to reduce the non-specific binding of the antibodies even more. On the next day the membrane was rinsed with PBS-Tween and incubated with the polyclonal goat anti-canine IL-12 antibody (R&D Systems) at concentration of 0.15µg/ml (diluted in PBS/Tween) for 1 hour. Afterwards the membrane was washed for 10 minutes three times in PBS/Tween, followed by incubation with 0.5 mg/ml of the second rabbit anti-goat (H+L) alkaline phosphatase AP-conjugated antibody (Novus Biologicals) at room temperature for 1 hour. The membrane was washed three times with PBS/Tween for 10 min. Following the last washing step, the membrane was incubated in 15 ml of a pre-mixed substrate solution BCIP/NBT (Sigma). After the development of a visible reaction in form of a colored line, the reaction was stopped by adding ddH_2O to the substarte solution. The membrane was documented and stored at 4°C.

3.4 Detection of rcscIL-12 bioactivity

3.4.1 PBMC Isolation

The density gradient centrifugation was used for PBMC isolation according to a protocol previously established in our lab and adapted for canine PBMC (GONDOLF, 1994; GONDOLF et al., 1996). Male, healthy, vaccinated and routinely dehelmintized Beagle dogs between 1.5 - 4 years of age (routine blood donors from the small animal clinic, department of internal medicine and from the clinic of obstetrics, gynecology and andrology) were used as blood donors. The blood was sampled in the morning by venupuncture of the jugular vein under standard sterile conditions. After sampling, the heparanized blood was diluted at ratio of 1:3 with sterile RPMI (PAA) medium supplied with 1% peniciline/streptomycine (PAA) and without fetal calf serum. Five milliliters of the diluted blood were layed on top of 3 ml of the 58.5% Percoll (Amersham) medium in conical glass tubes. The layered blood was centrifuged at 800 x g for 25 minutes without using the brake. The resulting band (fig. 23, attachment) was removed by a pasteur pipette and lymphocyte bands from 3-4 glass tubes were pooled together in a new conical tube containing 5ml RPMI (1% peniciline/streptomycine) culture medium supplied with 10 % fetal calf serum. The pooled lymphocytes were pelleted by centrifugation at 400 x g for 5 min. using the brake, the supernatant was discarded and the pellets from 3 tubes were pooled again by resuspension in 2 ml RPMI complete medium. Such pooled lymphocytes were centrifuged and another three pellets were pooled together until only one tube left. This was resuspended in 3 ml complete medium and the cell number and viability was determined by Trypan blue exclusion assay.

3.4.2 PBMC pre-stimulation

The isolated PBMCs were always incubated at concentration of 5 x 10^6/ml. Depending on the experiment they were treated with 100 U/ml of the rhIL-2 (PeproTech) for different intervals (5 or 7 days). In each case after the initial three days of incubation the cells were re-supplied with the same amount of medium and rhIL-2 (100 U/ml).

3.4.3 IFN-γ ELISA

Canine IFN-γ Quantikine®, a sandwich ELISA based kit (R&D Systems) was used to quantify the amounts of canine IFN-γ produced by the canine PBMCs. The IFN-γ was determined in the supernatants of cultured lymphocytes. For this purpose, suspensions of the cultured cells were transferred into 1.5 ml conical tubes which were centrifuged in a microcentrifuge at full speed (14.000 rpm) for 2 minutes, in order to pellet the cells and the small amount of debris present in the suspension. After obtaining a cell-free supernatant, the protocol was performed according to the manufacturer's recommendations:

1. 50 µl of each probe, 50 µl PBS (negative control) or 50 µl of the standard IFN-γ solution (positive control) were transferred into wells of the ELISA plate pre-coated with mouse monoclonal anti canine IFN-γ antibody and incubated for 2 hours.
2. Afterwards the wells were washed 3 times with 300 µl /well of the washing buffer.
3. 100 µl/well of the biotynilated monoclonal anti cIFN-γ antibody was added and the wells incubated for one hour.
4. The biotynilated antibody was removed and the wells were washed 3 times with 300 µl/well of the washing buffer.
5. 100 µl/well of a streptavidine-attached horse reddish peroxidase (HRP) was added to the samples and the wells were incubated for 30 min.
6. The streptavidine-HRP was removed and the wells washed again 3 times with 300 µl/well of the washing buffer
7. 100 µl/well of a pre-mixed peroxidse substrate was added and incubated for 30 minutes in dark.
8. 100µl/well of the stop solution (1 M H_2SO_4) were added and the optical density was determined within the next 5 min. by a spectrophotometer (Titertek Multiscan Plus, Titertek) at 450 nm wavelength with 630 nm wavelength serving as a background correction.

The raw O.D. values were then plotted on a graph and the approximate amount of cIFN-γ produced was determined from the standard curve which was obtained in each experiment with known concentrations of IFN-γ (0-4000 pg/ml).

3.4.4 BrdU ELISA

The BrdU incorporation was used to investigate the proliferation of stimulated cells. For this purpose ELISA-based BrdU detection was applied (WAGNER et al., 1998, WAGNER, 1999) using the BrDU ELISA kit (Roche Diagnostics), according to the manufacturer's recommendations. Shortly, triplicates of each probe were incubated in 100 µl volume in 96-well multiplate for the given time with the appropriate stimuli and the appropriate controls. Then they were processed according to the following protocol:

1. 10 µl of the BrdU labeling solution were added per well.
2. The cells were incubated at 37°C for 16 h.
3. The lymphocyte suspensions were thoroughly mixed by pippeting up and down in order to disrupt eventual cell agglutinates.
4. The 96-well plate was centrifuged at 400 x g for 10 min.
5. The supernatant was removed by flicking off.
6. The cells were dried with a hair dryer for 10 min.
7. 200 µl of ethanol based fixation/permeabilisation solution was added per well and the plate was incubated for 30 min.
8. The fixative was removed by flicking off, and the plate was left to drain on a clean paper.
9. 100 µl of the peroxidase labeled anti-BrdU antibody working solution was added to each well and the plate was incubated for 90 min.
10. The antibody solution was removed by flicking off, and the wells were washed three times with 300 µl/well of the washing solution.
11. After removing the washing buffer a ready-to-use substrate solution was added to the wells, and they were incubated until a blue color developed.
12. 25 µl of 1 M H_2SO_4 were added to stop the substrate solution and afterwards the optical density of the wells was determined at 450 nm in less then 5 minutes. The wavelength of 630 nm served as a correction.

For evaluating the results of the BrdU ELISA the mean was calculated from the triplicates and the results were displayed as raw values of the optical density.

3.4.5 Cytotoxicity assay (Rose Bengal Assay)

For determining the cytotoxic activity of canine PBMC, a method established in our lab (GONDOLF, 1994; GONDOLF et al., 1996) was used. Two routinely utilized cell lines, namely CTAC as a NK-sensitive and VERO as a NK-non-sensitive cell line were used as target cells for the cytotoxic activity of the PBMC. According to the optimal conditions determined by GONDOLF et al. (1996), $1x10^4$ target cells were plated in a 96-well plate and incubated for 26 hours in order to achieve optimal monolayers. After this incubation time, effector PBMC were added in different effector to target (E:T) ratios (varying from 100: 1 to 25:1) and incubated at 37°C (5% CO_2) for additional 14 h. Following this incubation period the effector cells and the killed, no longer adherent target cells were removed using a multichannel pipet. Subsequently, 100 µl of 0,25% Rose Bengal (Sigma) working solution was added to stain the remaining, still adherent target cells for 3 min. The Rose Bengal color was removed from the supernatant by flicking off and the cells were washed three times with 300 µl/well of the PBS-NaCl washing solution (pH 7.4) to completely remove the extracellular color. The remaining target cells, which had absorbed the dye were lysed with 200 µl/well of ethanol-PBS (50:50 v/v) solution and incubated for 5 min. in order to allow releasing of the dye from the cells. The optical density of the resulting solution was determined in a spectrophotometer at 570 nm wavelength, with the wavelength of 630 nm serving as a background correction. The O.D. values of target cells incubated without effector cells were considered as 100 % and the cytotoxicity of the effector cells was determined using the following formula:

$$\text{Cytotoxic activity} = 100 - \left(\frac{\text{O.D. of the sample}}{\text{O.D. of the control}}\right) \text{ x } 100$$

It should be mentioned that negative values can result using this formula in some of the effector-target suspensions (sample) were effector cells did not exhibit cytotoxic activity. However, despite the lack of cytotoxic activity, adherence of effector to target cells occurred

leading to higher absorbance (higher O.D. values) in these wells than in the control wells (target cells only). These negative values were considered as zero cytotoxicity.

4 RESULTS

4.1 Construction of the rcscIL-12

4.1.1 Repair of the cDNA coding for the IL-12 p35 sequence

The cDNA coding for the IL-12 p35 sequence was completed as described (s. 3.2.1). The amplicon of the first six exons is presented in fig. 2 as amplified with the respective primers. All three bands represent amplicons of the same sequence amplified using different annealing temperatures by gradient PCR. All products exhibited the expected length of 514 bp and correct amplification was afterwards confirmed by sequencing (MWG Biotech, Germany).
Fig. 3 shows the amplicon obtained by amplification of exon 7 from genomic DNA using the appropriate primers (3.2.1). After isolating and cloning of this amplicon, its sequence was confirmed by sequencing (MWG Biotech, Germany).

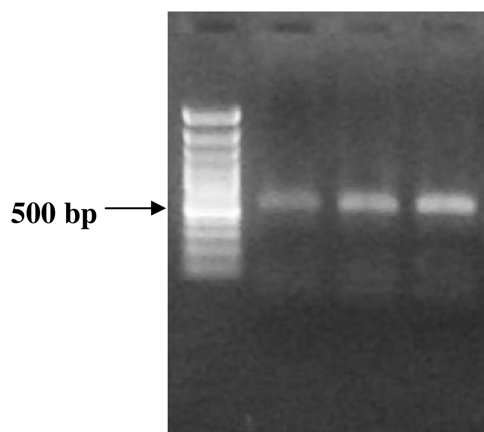

Fig 2: Amplicons (gradient PCR) of the sequence of exons1-6 of the p35 subunit; **all three bands** show the same length of 500 bp

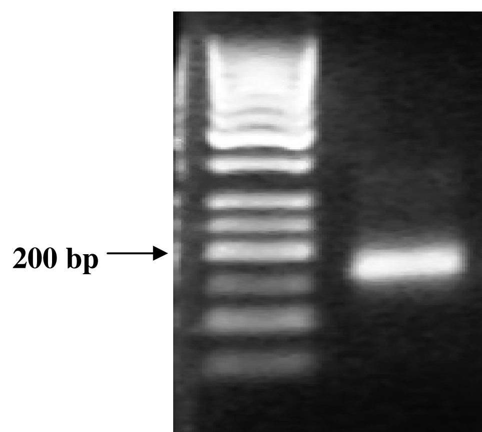

Fig 3: Amplicon of the sequence of exon**7** of the p35 subunit (**right lane**), with length of 170 bp

The amplicons of the sequences for exons 1-6 and for exon 7 were processed as described (3.2.1). Fig. 4 shows the amplicons of the ligation product of exon 1-6 with exon 7 using the corresponding primers (3.2.1). All bands represent the same amplicons obtained at different

annealing temperatures using gradient PCR. The resulting complete sequence of the p35 subunit was confirmed by sequencing at MWG.

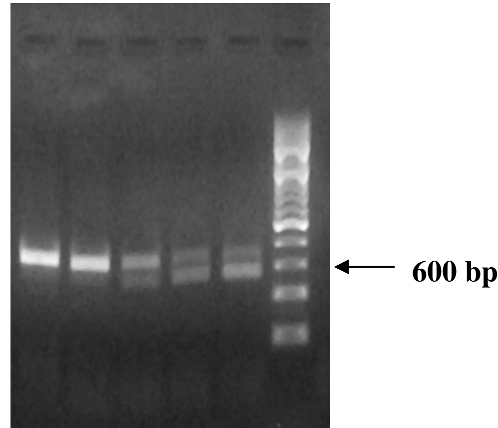

Fig 4: Amplicons of the complete sequence of the p35 subunit ; **all 5 lanes** show the same lenght of 669 bp

4.1.2 Correction of the cDNA coding for the IL-12 p40

The result of the corrections of the mismatched bases is the sequence presented in fig. 5. After the last site-directed mutagenesis had been performed, the resulting plasmid was isolated and sent for sequencing (MWG Biotech, Germany).

```
  1   atgcatcctcagcagttggtcatctcctggttttccctcgttttgctggcgtcacccctc 60
_ 61  atgaccatatgggaactggagaaagatgtttatgttgtagagttggactggcaccctgat 120
_ 121 gcccccggagaaatggtggtcctcacctgccatacccctgaagaagatgacatcacttgg 180
_ 181 acctcagcgcagagcagtgaagtcctaggttctggtaaaactctgaccatccaagtcaaa 240
_ 241 gaatttggagatgctggccagtatacctgccataaaggaggcaaggttctgagccgctca 300
_ 301 ctcctgttgattcacaaaaaagaagatggaatttggtccactgatatcttaaaggaacag 360
_ 361 aaagaatccaaaaataagatctttctgaaatgtgaggcaaagaattattctggacgttt 420
_ 421 cacatgctggtggctgacggcaatcagtactgatttgaaattcagtgtcaaaagtagcag 480
_ 481 aggcttctctgacccccaaggggtgacatgtggagcagtgacactttcagcagagagggt 540
_ 541 cagagtggacaacagggattataagaagtacacagtggagtgtcaggagggcagtgcctg 600
_ 601 cccctctgccgaggagagcctacccatcgaggtcgtggtggatgctattcacaagctcaa 660
_ 661 gtatgaaaactacaccagcagcttcttcatcagagacatcatcaaaccagacccacccac 720
_ 721 aaacctgcagctgaagccattgaaaaattctcggcacgtggaggtcagctgggaataccc 780
_ 781 cgacacctggagcaccccacattcctacttctccctgacattttgcGtacaggcccaggg 840
_ 841 caagaacaatagagaaaagaaagatagactctgcgtggacaagacctcagccaaggtcgt 900
_ 901 gtgccacaaggatgccaagatccgcgtgcaagcccgagaccgctactatagttcatcctg 960
_ 961 gagcgactgggcatctgtgtcctgcagttaggttccacccccaggatgaatcttgg 1016
```

Fig 5: IL-12 p40 sequence after the **mismatch corrections** (indicated in red colour, capital letters) performed by site-directed mutagenesis

4.1.3 Construction of the rcscIL-12

The ligation product of the cDNAs coding for the p35 and p40 subunits was cloned and processed as described (3.2.3). Fig. 6 shows the amplification product of this constructed sequence with the expected length of 1.6 kb.

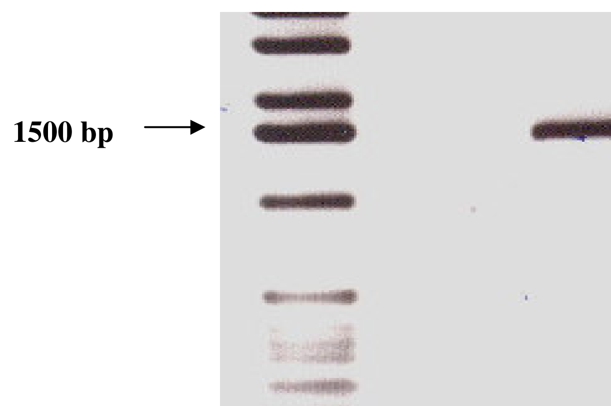

Fig 6: Amplicon of the sccIL-12 sequence (**right lane**)

4.1.4 Construction of the rcscIL-12/Luciferase/pTRUE vector

The successful construction of the rcscIL-12 enabled the completion of the plasmid required for expression of functional canine IL-12. Fig. 7 shows the amplicons of all sequences required to construct this plasmid: the rcscIL-12 (band 1, *1.6 kb*), the IRES/Luciferase cassette (band 2, *2.5 kb*) and the pTURE vector (band 3, *3 kb*). Following the cloning of this vector construct, restriction digestion was used as a first confirmation of the successful cloning. Fig. 8a shows the restriction fragments obtained after digestion with the following restriction enzymes: Avr II (lane 2), and Mlu I and Sph I (lane 3). Lane 1 shows the undigested plasmid with total length of 7.1 kb. A closer view of the restriction sites is presented in fig.8b. Thus, the blue lines represent the restriction sites for the Avr II, positioned at base pair 90 of the IRES/Luciferase cassette and at base pair 214 of the rcscIL-12 sequence. The resulting restriction fragments are 1.5 and 5.6 kb in length. Restriction digestion with Mlu I and Sph I, which cut at base pair 1 and base pair 1200 of the IRES/luciferase cassette, respectively, produces restriction fragments of 1.2 and 5.9 kb.

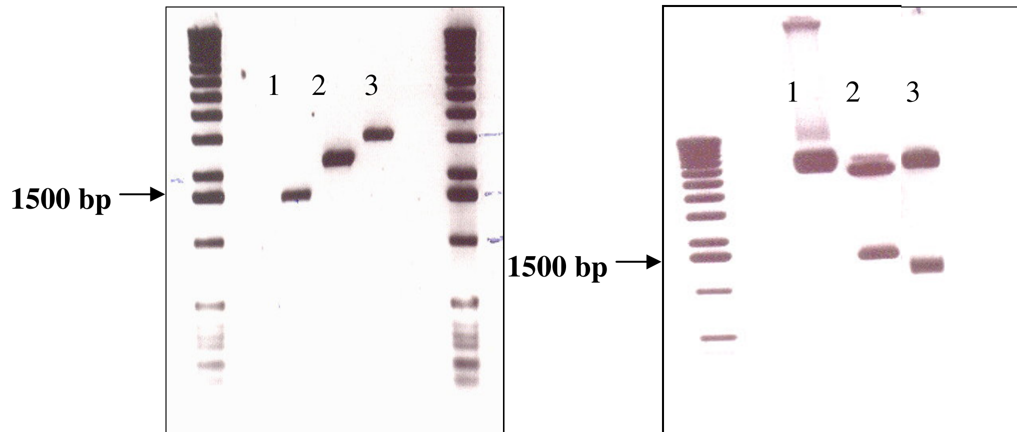

Fig 7: PCR control of the sequences to be ligated: **Lane1:** rcscIL-12; **lane 2:** IRES/luciferase cassette; **lane 3:** pTRUE vector

Figure 8a: Restriction digestion control after the ligation of the rcscIL12 and IRES/luciferase into pTRUE vector. **Lane 1:**undigested plasmid; **lane 2:** restriction fragments of the digestion with the AvrII; **lane 3:**restriction fragments of the digestion with the MluI and SphI

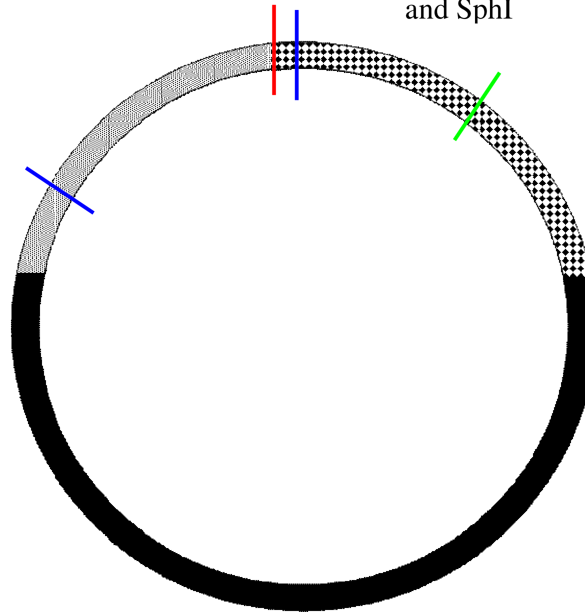

Fig 8b: The pTRUE↔rcscIL-12↔IRES/Luciferase plasmid. **Dotted line:** rcscIL-12 (1.6 kb); **spotted line:** IRES/Luciferase cassette (2.5 kb); **filled line:** pTURE plasmid (3 kb). **Blue lines:** restriction sites for the AvrII; **red line:** restriction site for the MluI; **green line:** restriction site for the the Sph I.

4.2 Establishing of rcscIL-12 containing BHK Tet-On cell line

4.2.1 Transfection of the BHK Tet-On cell line

Following successful cloning of the pTRUE vector with the cDNAs encoding rscsIL-12 and luciferase, the construct was stably transfected into the BHK Tet-On cell line. Transfected cells surviving the neomycine /puromycine selection pressure were selected as described (3.3.2). As demonstrated in the luciferase assay (fig. 9, table 1), only four from ten populations surviving the selection process reached confluence in a 10 cm Petri dish. Cells from these populations were incubated with Doxycycline overnight, and the luciferase activity was analyzed. The following values of absolute light units were recorded after the luminescence measurement: population 2 (6393.9), population 3 (16.315), population 5 (45618) and population 6 (44503). Aliquots of the same cell populations incubated without Doxycycline showed much more lower values of the absolute light units: population 2 (128.36); population 3 (4.682); population 5 (724.33) and population 6 (634.17). According to these values the induction index was calculated for each population as described (3.3.3), giving the following values: population 2 (49.8), population 3 (3.48), population 5 (62.97) and population 6 (70.17).

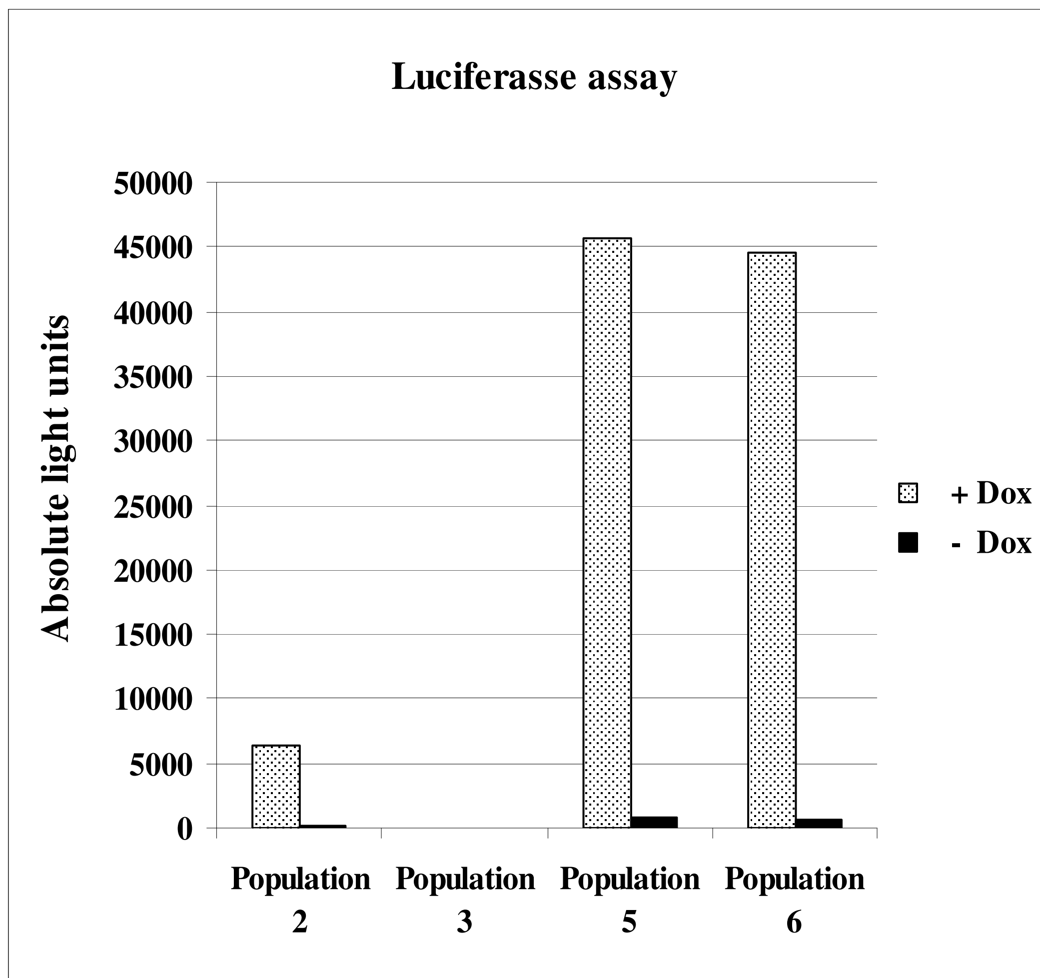

Fig 9: Luciferase assay values of the four populations from the initial cloning. *Dotted columns:* cells incubated **with** doxycycline; *closed columns:* cells incubated **without** Doxycycline

According to these criteria, the highest values of the luciferase activity were shown by populations 5 and 6. Additionally, these populations showed the best induction index, 62.9 and 70.1, respectively. In order to ensure the clonal nature of the future cell lines, cells of populations 5 and 6 were cloned using limited dilution cloning, as described (3.3.2). Following cloning, five clones from each population were grown to confluence in a 10 cm Petri dish and were tested on luciferase activity.

The results of the first subcloning procedure are presented in fig. 10. According to the luciferase assay values, the induction index (in brackets) was as follows: clone 5.1 (72.5) clone 5.2 (45.1), clone 5.3 (82.4), clone 5.5 (66.9), clone 6.1 (20.9), clone 6.2 (125.2), clone 6.3 (106), clone 6.5 (64.08). Due to the highest induction index, the clones 6.2 and 6.3 were subcloned a second time.

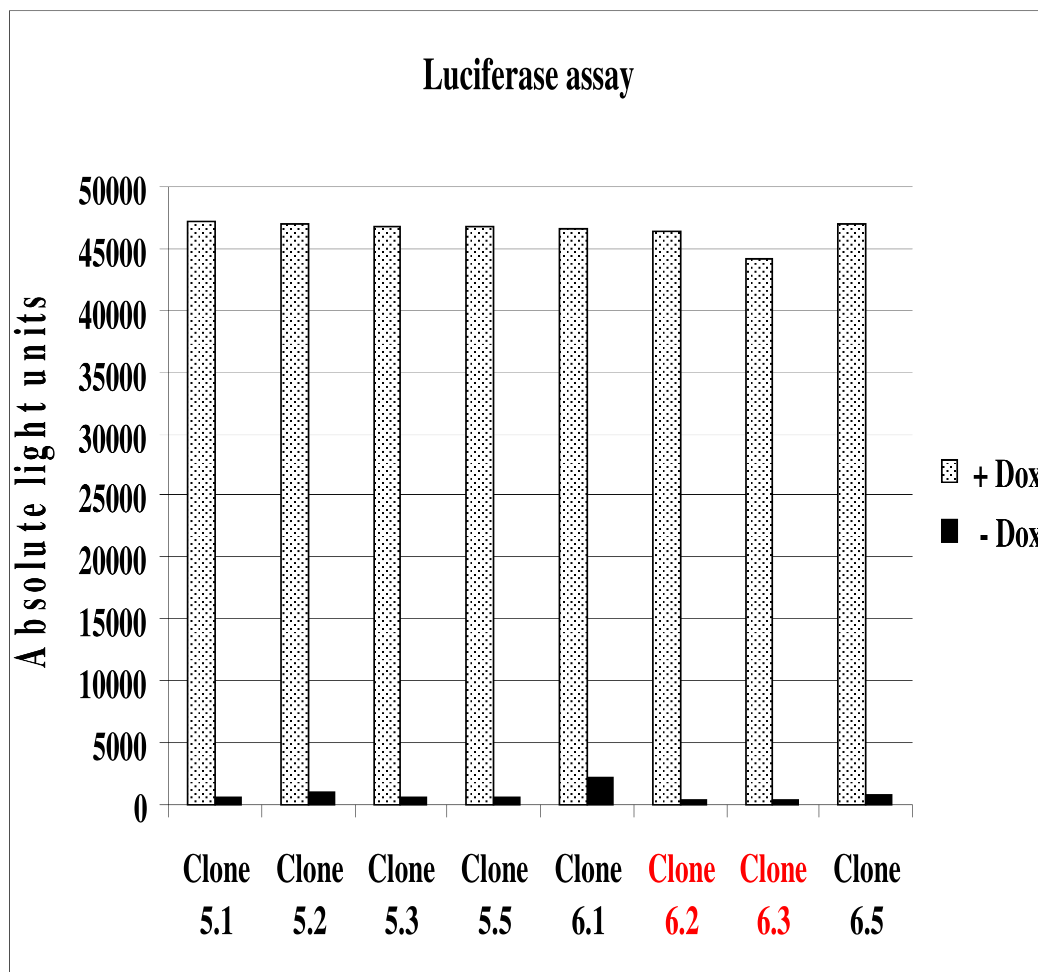

Fig 10: Luciferase assay values of the first subcloning. *Dotted columns:* cells incubated **with** doxycycline; *closed columns:* cells incubated **without** Doxycycline

The values of the luciferase assay of the second subcloning procedure are presented in fig. 11. The values of the induction index of the respective clones are: clone 6.2.1 (102), clone 6.2.2 (216), clone 6.2.3 (54), clone 6.2.4 (154), clone 6.2.5 (128), clone 6.3.1 (137), clone 6.3.2 (184), clone 6.3.3 (177), clone 6.3.4 (143), clone 6.3.5 (221). On the basis of these data, the clones 6.2.2 and 6.3.5 were finally chosen as established cell lines.

Fig 11: Luciferase assay values of the second subcloning. *Dotted columns:* cells incubated **with** doxycycline; *closed columns:* cells incubated **without** Doxycycline

A successful single-cell cloning assumes the presence of the transfected plasmid in every cell. Since the nature of the luciferase assay does not discriminate whether every cell contains the transfected plasmid, immunofluorescent staining of the transfected cells was additionally performed as described (3.3.4). As can be seen in figures 12a (clone 6.2.2) and 12b (clone 6.3.5), both clones showed 100 % of positive cells, indicating that every cell contained the rcscIL-12 expressing plasmid.

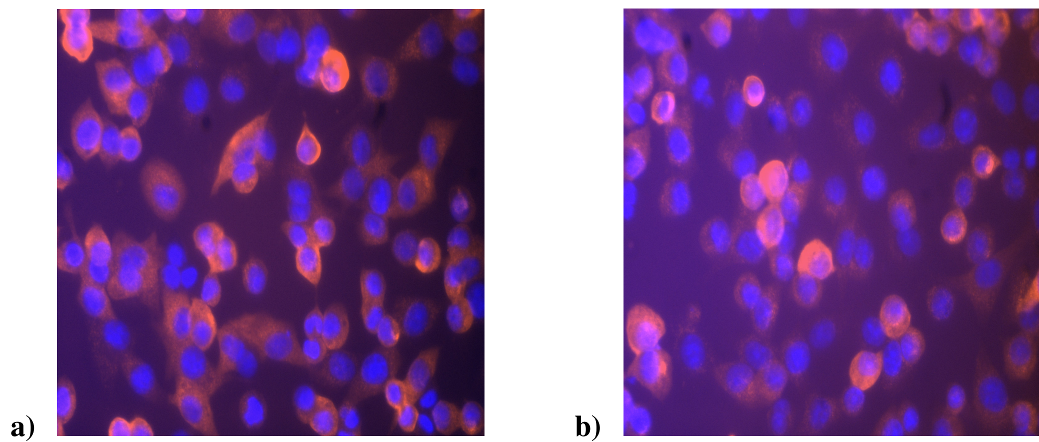

Fig 12: Demonstration of bright orange-red fluorescence of the antibody-labeled luciferase protein in the cytoplasm of all cells: **a)** clone 6.2.2; **b)** clone 6.3.5

4.2.2 Detection of the rcscIL-12 in the established cell line

4.2.2.1 Detection of the rcscIL-12 protein

Using Western blot analysis, the presence of the rscsIL-12 protein was confirmed in cell lysates as well as in the supernatant of the cell lines. Fig. 13 shows the Western blot performed with the lysate and the supernatant of the cell clone 6.2.2.

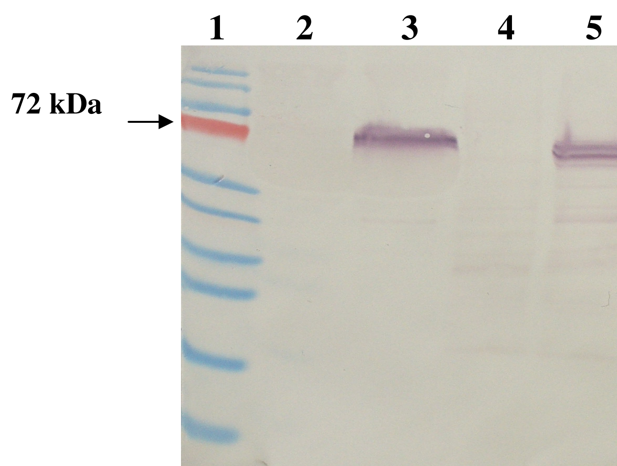

Fig 13: NBT/BCIP-based detection of the rcscIL-12 protein (~70 kDa) by Western blot analysis. *Lane 1:* marker; *Lane 2:* supernatant of **Doxycycline non-induced** cells; *Lane 3:* supernatant of **Doxycycline induced cells**; *Lane 4:* lysate of **Doxycycline non-induced cells**; *Lane 5:* lysate of **Doxycycline induced cells**. The positive reacting bands are indicated by brown color

The red band in the molecular weight marker (lane 1) resembles molecular weight of 72 kDa, indicating that the protein detected with a specific goat anti-cIL-12 polyclonal antibody on the membrane (lanes 3 and 5) is of similar molecular weight. In contrast, in the supernatant of non-induced cells (lane 2) as well as in lysates of non-induced cells (lane 4) we could not detect the protein of interest.

4.2.3 Confirmation of bioactivity of the rcscIL-12

Initially we investigated which assay is the optimal assay for investigating IL-12 bioactivity. In our experiments IFN-γ production by IL-2 induced canine lymphoblasts (in the following text called IL-2 lymphoblasts) proved to be the assay of choice over proliferation and cytotoxicity assays. Recombinant canine IL-12 (rcIL-12) (R&D Systems) was used as a standard in these establishing experiments.

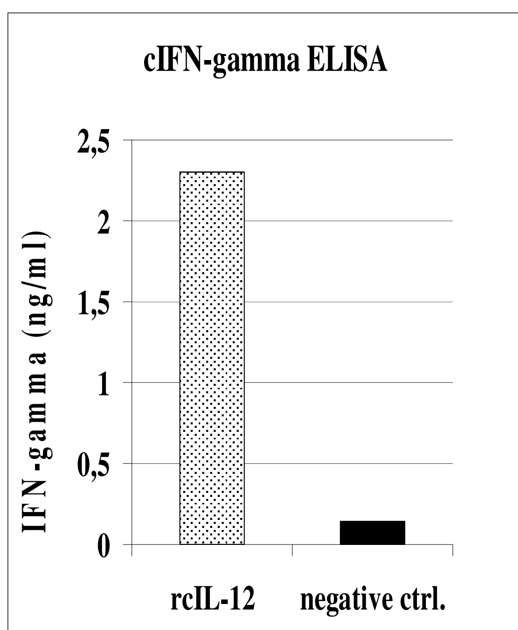

Fig 14: IFN-γ concentration in supernatants of canine IL-2 lymphoblasts after **48 h** incubation. *Dotted column:* incubated with **IL-12**; *closed column:* incubated with **medium only.**

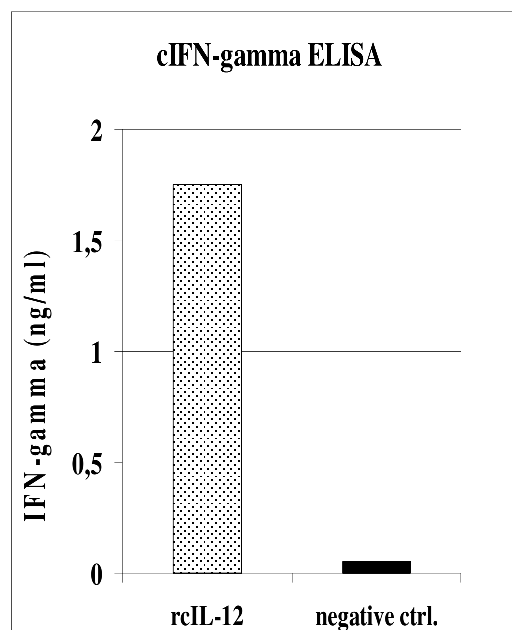

Fig 15: IFN-γ concentration in supernatants of canine IL-2 lymphoblasts after **72 h** incubation. *Dotted column:* incubated with **IL-12** ; *closed column:* incubated with **medium only**.

Thus, following incubation of IL-2 lymphoblasts with rcIL-12 for 48 h (fig.14) or 72 h (fig.15), a clear increase in the IFN-γ production was observed. Thus, IL-2 induced canine lymphoblasts incubated with medium only produced 0.08 and 0.05 ng/ml IFN-γ, whereas IL-2 lymphoblasts incubated with recombinant canine IL-12 (R&D Systems) produced 2.3 and 1.75 ng/ml IFN-γ after 48 and 72 hours, respectively.

As a next step the biological activity of the supernatant from the established BHK Tet-On cell line was analyzed. Fig.16 shows a representative experiment in which IFN-γ production by IL-2 lymphoblasts was measured after incubation with supernatant from clone 6.2.2. These experiments demonstrated that supernatants of both Doxycycline induced and Doxycyline non-induced cells of clone 6.2.2, stimulated IFN-γ production by the IL-2 lymphoblasts to the same extent.

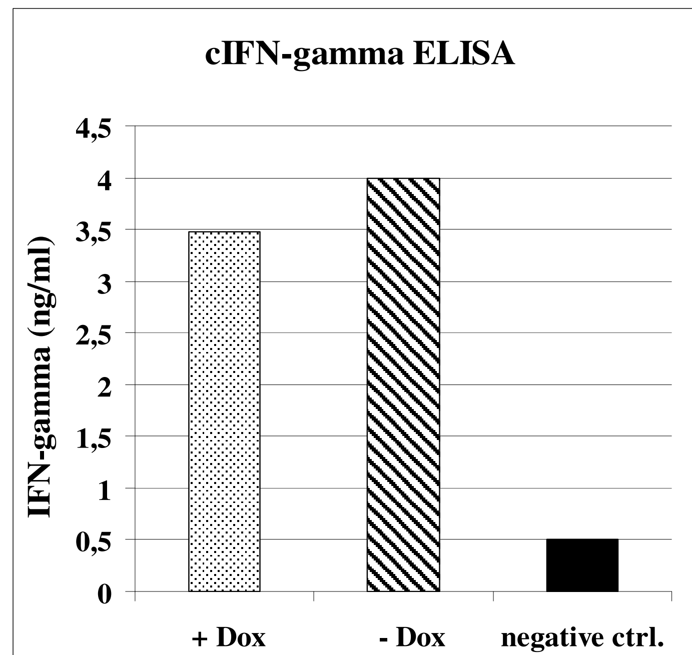

Fig 16: IFN-γ concentration in supernatants of canine IL-2 lymphoblasts incubated with the supernatants of cells of clone 6.2.2; *Dotted column:* supernatant from Doxycycline **induced cells**; *slashed column:* supernatant from Doxycycline **non-induced cells**; *closed column:* **medium only**.

In order to verify that the secretion of IFN-γ was due to the presence of rcscIL-12 in the supernatant of the transfected cell line (clone 6.2.2) a neutralization assay was performed. Therefore, a neutralizing anti-canine IL-12 antibody was incubated with the supernatant of clone 6.2.2 before adding it to the IL-2 pre-stimulated lymphoblasts, and IFN-γ production was measured. Fig. 17 shows the results of three representative neutralization experiments.

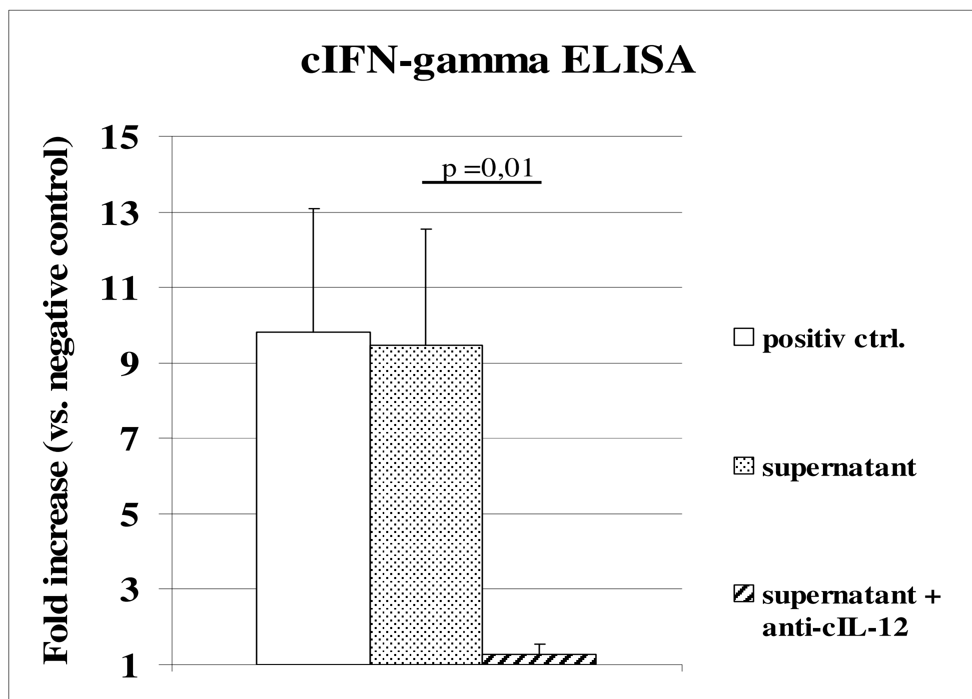

Fig 17: Fold increase of IFN-γ concentration in supernatants of canine IL-2 lymphoblasts incubated for 24 h; *open column:* with **rcIL-12** as a positive control; *dotted column:* with **IL-12-containing supernatant** of the 6.2.2 cell line; *slashed column:* with **IL-12 containing supernatant + anti-cIL-12 antibody**.
The mean value of the negative control (IL-2 lymphoblasts incubated with medium only) was taken as 1

IL-2 lymphoblasts incubated with the rcIL-12 as positive control, produced 8.76 fold more IFN-γ than IL-2 lymphoblasts incubated with medium only (negative control). Similar amounts of IFN-γ (8.44 fold more than the negative control) were produced by IL-2 lymphoblasts when incubated with the supernatant from clone 6.2.2. In contrast, IL-2 lymphoblasts incubated with the clone 6.2.2 supernatant in presence of the neutralizing anti canine IL-12 antibody demonstrated only insignificant increase of IFN-γ compared to the negative control.

4.3 Investigation of the biological effects of rcscIL-12 on canine PBMC

4.3.1 Resting PBMC do not produce IFN-γ after stimulation with IL-12

In order to determine the optimal conditions for IL-12 mediated stimulation of the anti-tumor activities in canine PBMC, we investigated if their pre-stimulation is needed, using IFN-γ production as read-out. Fig.18 demonstrates the IFN-γ production by freshly isolated resting PBMC after incubation with rcscIL-12 for 18 h. Freshly isolated PBMC did not produce significant amounts of IFN-γ in response to rcscIL-12 stimulation. In contrast, in the experiments presented previously (fig.14 – fig.17) showing significant increase in IFN-γ production, the canine PBMC were stimulated with IL-2 prior to the IL-12 addition.

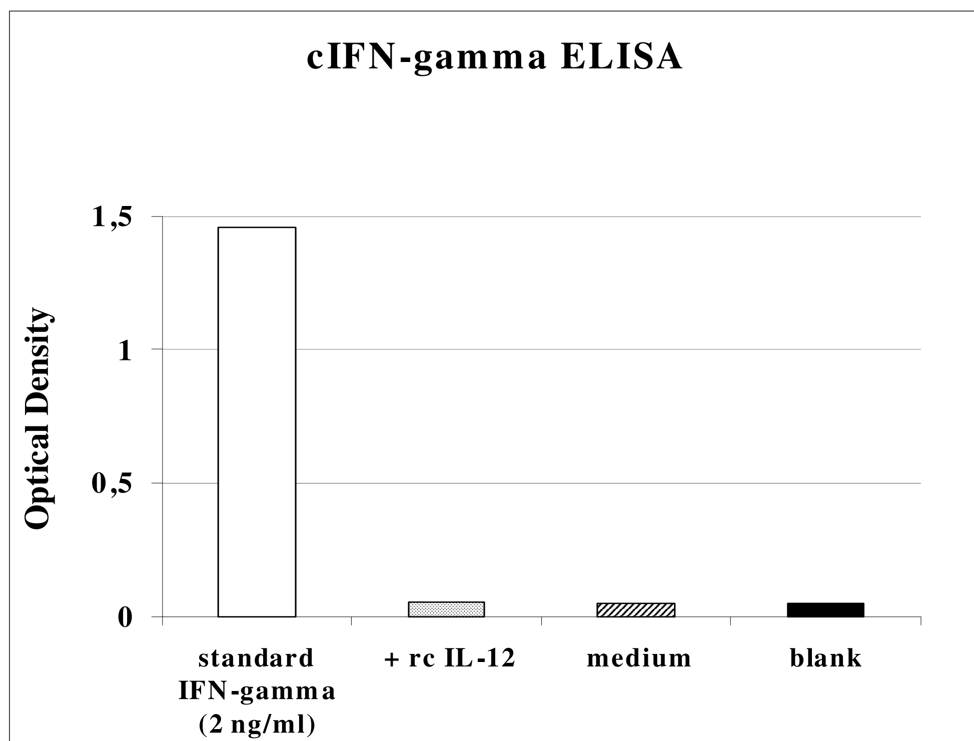

Fig 18: O.D. of IFN-γ ELISA; resting PBMC incubated for 18 h; *Dotted column:* with **rcIL-12**; *slashed column:* with **medium**; *closed column:* blank; *open column:* IFN-γ standard.

4.3.2 IL-12 requires presence of IL-2 to induce IFN-γ production in IL-2 lymphoblasts

In the IL-12 stimulation experiments presented, IL-2 pre-stimulated lymphoblasts were centrifuged only once before adding the IL-12. This provided that small amounts of IL-2 were present during the incubation period with IL-12. However, upon complete removal of IL-2 from the suspension (by three consecutive centrifugations), the IL-2 lymphoblasts did not produce detectable levels of IFN-γ following incubation with IL-12 (fig.19).

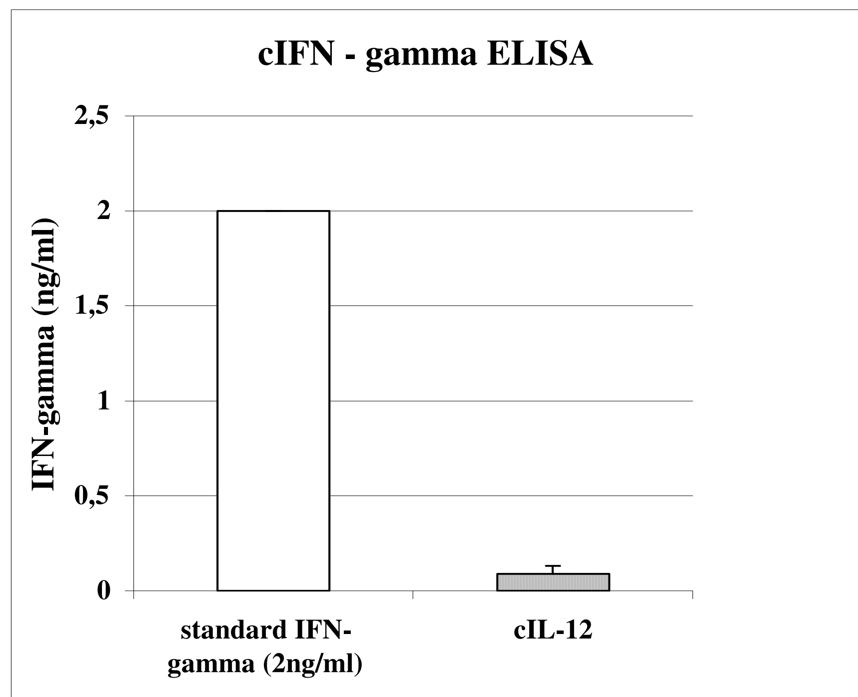

Fig 19: IFN-γ production upon IL-12 stimulation of IL-2 lymphoblasts after complete removal of the IL-2 by three consecutive centrifugations; *open column:* **standard IFN-γ**; *dotted column:* IL-lymphoblasts stimulated with **rcscIL-12**

These experiments clearly show (fig. 18, fig. 19) that IL-12 alone is not sufficient to induce detectable levels of IFN-γ and that IL-2 pre-stimulation of canine lymphocytes is a prerequisite for efficient induction of IFN-γ by IL-12 in vitro.

4.3.3 Proliferation of canine IL-2 lymphoblasts upon IL-12 stimulation

After optimizing the conditions for IL-12 stimulation of canine PBMC, their proliferation was analyzed, using BrdU incorporation based ELISA. In contrast to commonly used protocols, we used IL-2 as a pre-stimulus instead of PHA-P. As described (3.4.2) cells were pre-incubated with IL-2 and then stimulated with 50 µl of the supernatant of clone 6.2.2. The results of a representative experiment are presented in fig. 20, showing that there is not significant difference between IL-2 lymphoblasts incubated with and IL-2 lymphoblasts incubated without IL-12. In order to demonstrate that the cells were properly pre-activated with IL-2, a portion of the canine PBMC were pre-incubated with medium only instead with IL-2. These cells demonstrated significantly lower O.D. values than IL-2 lymphoblasts, indicating that IL-2 itself has indeed pre-stimulated the canine PBMC.

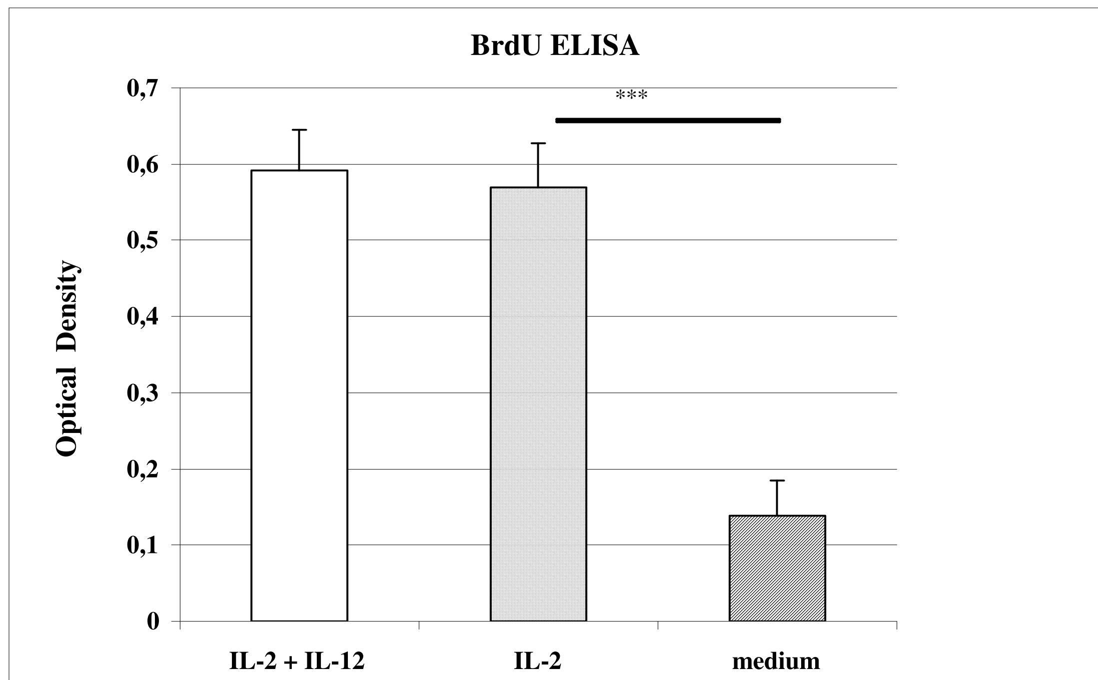

Fig 20: Cell proliferation measured by BrdU ELISA after 48 hours incubation of IL-2 lymphoblasts. *Empty column:* with **IL-2 + IL-12**; *dotted column:* **IL-2** only; *slashed column:* **medium only**.

4.3.4 Evaluation of the cytotoxic activity of canine IL-2 lymphoblasts upon stimulation with IL-12

To investigate the anti-tumor effect of IL-12, the cytotoxic activity of IL-2 lymphoblasts upon IL-12 stimulation was determined using the RBA, as described (3.4.5). The results of these experiments are presented in fig. 21. Highly variable increases in the cytotoxic activity of IL-12 stimulated IL-2 lymphoblasts compared to IL-2 lymphoblasts incubated without IL-12 were observed. Thus, in only three from eight dogs, IL-12 stimulated IL-2 lymphoblasts showed increase in cytotoxic activity (dogs 1, 6 and 8) of more than 30 % than IL-2 lymphoblasts incubated with medium only. IL-12-stimulated IL-2 lymphoblasts of three other dogs (dogs 2, 3 and 4), showed only a slight increase (less than 10%) in cytotoxic activity when compared to IL-2 lymphoblasts incubated with medium only. IL-12 stimulated IL-2 lymphoblasts of two other dogs (dogs 5 and 7) revealed 20-30 % increase in their cytotoxic activity compared to that of IL-2 lymphoblasts alone.

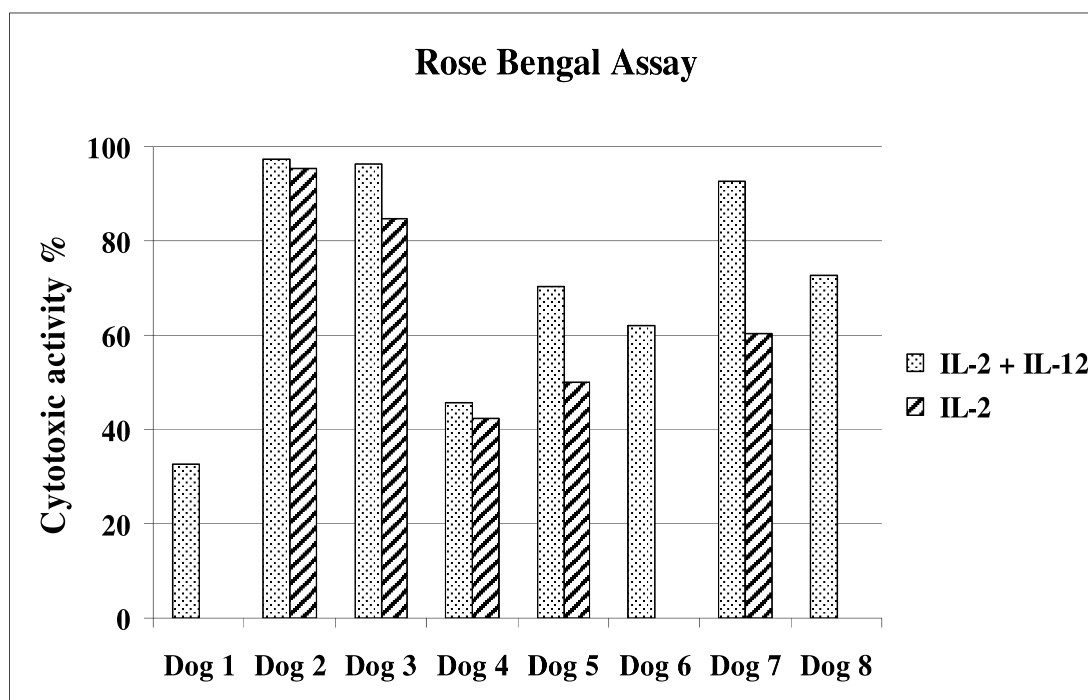

Figure 21. Cellular cytotoxicity RBA Assay. IL-2 blasts incubated for 14 hours with the CTAC target cells. *Spotted columns:* in presence of **rcscIL-12**; *slashed columns:* in presence **of medium only**.

Taken together, it can be stated that the presence of IL-12 induced cytolytic activity in canine lymphoblasts, which was not even present in cells incubated without IL-12, or in the majority of lymphocyte preparations more or less significantly increased the cytolytic activity.

5 Discussion

The goal of this project was (1) to provide a continuous source of canine IL-12 for use in tumor immunotherapy studies in dogs by establishing a stably transfected BHK Tet-On cell line, and (2) to investigate the biological activities of the recombinant IL-12 in order to find the optimal conditions for in vitro stimulation of the anti-tumoral activities of canine PBMC. For accomplishing the first goal the stably transfected cell line must demonstrate continuous IL-12 secretion over long time periods, ideally over several hundred passages. A stable and reliable IL-12 producing cell line is of great importance for the planned future studies of adoptive canine immunotherapy. Thus, the IL-12 producing cell line, together with another, IL-2 producing BHK Tet-On cell line previously established in our lab, can be used for stimulation of canine lymphocytes in a co-culture system (FALKENBERG et al., 1995; FALKENBERG, 1998). In this system, a bioreactor comprising of two compartments separated by a semi-permeable membrane with a cut off of 100 kDa, the IL-12 and IL-2 producing cell lines will be able to stimulate the canine lymphocytes, thus providing in vitro conditions very similar to conditions found in vivo. With help of the semi-permeable membrane it will be provided that only IL-12 (70 kDa), IL-2 (35 kDa) and some smaller molecules pass the barrier between the compartments, ensuring that there is no direct contact between the lymphocytes and the BHK cell line(s). In this way by continuous secretion of cytokines optimally stimulated canine lymphocytes will be available for adoptive immunotherapy of tumor-bearing dogs.

The main prerequisite for obtaining such a cell line is its clonal character. Our experimental data from the luciferase assay and especially from the immunofluorescent staining of the luciferase protein, certainly confirmed the clonal character of the established cell line. Additionally, the stability of the cell line was affirmed by Western blot, demonstrating the presence of IL-12 protein in the transfected BHK Tet-On cell lysate, as well as in the supernatant of cells of the 45-th passage.

One important issue concerning the expression of biologically active IL-12, regardless if it would be used for in vitro stimulation or for an intra-tumoral delivery, is to avoid production of any other IL-12 form than the IL-12 p70 form. As mentioned (2.3), depending on the species and the source of IL-12, other forms of IL-12, such as IL-12 p40 monomers and

homodimers are produced the latter exhibiting antagonistic effects on the IL-12 p70 activity (HEINZEL et al., 1997).

Since existence and function of these forms have not been investigated in dogs, their formation and biology are not predictable. In order to avoid any possible interference of these forms with the IL-12 p70, (HEINZEL et al., 1997) we chose to make a single-chain canine IL-12 construct. For this purpose, the cDNA sequences coding for p40 and p35 subunits were ligated together as described (3.2.3), resulting in IL-12 p70 monocystronic cDNA. This monocystronic cDNA assures transcription of both subunits from one promoter, avoiding the existence of separate mRNA transcripts of each of the subunits. Additionally, in order to ensure that the translated product of the monocystronic cDNA remains as an intact polypeptide chain during protein processing in the rough endoplasmic reticulum (ER), the leading sequence of the cDNA coding for the p35 subunit was omitted. Thus, we provide conditions which do not allow production of free p40 or p35 subunit by the transfected cell line. This was confirmed using western blot, demonstrating only one band of approximately 70 kDa in the cell lysates and supernatants of the established cell line, which corresponds to the molecular weight of the IL-12 p70 heterodimer (KOBAYASHI et al., 1989). Since the anti-canine IL-12 antibody used detects p40 regardless in which form it is present, we conclude that the only form present in the established cell line is indeed the IL-12 p70 heterodimeric form.

For investigating the expression rate of the rccsIL-12 by the transfected cell line, the luciferase assay was used as an indirect indicator. Since the luciferase expression can be induced upon Doxycycline addition, inducibility and expression rate of the luciferase reporter gene were investigated. These experiments revealed more than 200-fold increase in luciferase activity of lysates of Doxycycline induced cells in comparison to lysates of Doxycycline non-induced cells (s. 4.2.1., fig 11, table 3). The substrate excess in the luciferase assay provides conditions where one molecule of luciferase cleaves only one substrate molecule. This in turn means that one unit of luciferase activity is equal to one luciferase molecule. Due to the fact that luciferase and rcscIL-12 transcripts are located on monocystronic mRNA, the number of rcscIL-12 molecules is equal to the number of luciferase molecules. This linearity allows us to conclude that protein synthesis of rcscIL-12 increases by the same factor as the luciferase activity. These data are also important since only such approximate mode of quantification of IL-12 production was available, due to the lack of canine IL-12 ELISA at present time.

The second important point revealed by these experiments is the fact that supernatants from Doxycycline non-induced transfected BHK cells stimulated the same amount of IFN-γ by IL-2 induced lymphoblasts (IL-2 lymphoblasts) as the supernatants from Doxycycline induced transfected BHK cells. This indicates that rcscIL-12 basal transcription also exists in absence of Doxycycline and is sufficient to stimulate the same amount of IFN-γ as supernatants from Doxycycline induced cells. Although the absolute IL-12 concentration could not be determined, our experimental data comply with the general observations made in IL-12 studies in humans and mice. These observations demonstrate that IL-12 is a very potent cytokine being active in very low picomolar concentrations (CHAN et al., 1991).

Finally, the neutralization assay performed with the neutralizing anti-canine IL-12 antibody confirmed that the IFN-γ production by canine IL-2 lymphoblasts upon incubation with the supernatant is due to the presence of rcscIL-12 alone.

The second goal of this study was to determine the optimal conditions for IL-12 mediated anti-tumor effect of canine PBMC. Thus, the most important functions responsible for this effect such as IFN-γ production, lymphocyte proliferation, and the cytotoxic activity of canine PBMC toward tumor cell lines, were investigated in various experimental settings.

Concerning IFN-γ production we found that resting canine PBMC do not produce detectable amounts of IFN-γ upon incubation with IL-12, whereas IL-2 induced lymphoblasts produce significant amounts of IFN-γ after IL-12 addition. The finding that resting canine PBMC do not produce IFN-γ after IL-12 stimulation is in contrast to studies who showed IFN-γ production by freshly isolated human lymphocytes after IL-12 stimulation (CHAN et al., 1991). The same study also showed that IFN-γ can be equally induced in NK as well as in T cells. However, the reason for this difference between resting canine lymphocytes and canine lymphoblasts can only be hypothesized at current time. Moreover, a detailed analysis of the IFN-γ production by distinct canine lymphocytes is not feasible at this time due to lack of phenotypical characterization of canine NK cells. Nevertheless, since IFN-γ has important anti-tumor effects, finding the optimal conditions for its production by canine lymphocytes is an important issue. Thus, it would be important to determine which is the optimal IL-12 concentration stimulating the highest amount of IFN-γ. Additionally, by investigating the kinetic of IFN-γ production in the course of time after IL-12 stimulation, the time interval of the highest and the most constant IFN-γ production can be determined. In this context our experiments only indicated that at 48 hours after IL-12 incubation canine IL-2 lymphoblasts

produced a significant amount of IFN-γ (2,3 ng/ml), whereas a slight decrease (1,8 ng/ml) can be observed after 72 hours.

In contrast to other groups working with canine IL-12 who reported proliferation of canine PHA-P stimulated lymphoblasts (PHILLIPS et al., 1999), we did not find significant difference between IL-12 induced IL-2 lymphoblasts and IL-2 lymphoblasts incubated with medium only. However, the difference in the pre-stimulation protocol could be the explanation for this observed discrepancy. Thus, it is known from studies in human and mice that IL-2 preferentially induces proliferation of resting NK cells, whereas PHA-P stimulates the proliferation of $CD3^+$ cells (ROBERTSON et al., 1992). Clarifying this question in dogs is not feasible at present due to the already mentioned lack of phenotypical characterization of canine NK cells.

Finally, the cytotoxic function of canine IL-2 lymphoblasts upon incubation with rcscIL-12 was investigated in 8 dogs. The results of these experiments revealed increased cytotoxic activity of canine IL-2 lymphoblasts upon incubation with rcscIL-12 for 16 hours, in all investigated animals. However, the increase was very variable between animals. Thus, in three of 8 dogs, IL-2 lymphoblasts alone did not demonstrate cytotoxic activity, whereas IL-12 stimulated lymphoblasts showed between 35-75% cytotoxic activity (s. 4.3.4, fig 21, table 11). In three other dogs the increase in cytotoxic activity of IL-12 stimulated IL-2 lymphoblasts compared to IL-2 lymphoblasts without additional stimulation with IL-12 was between 20 and 30 %, whereas in two dogs this increase was less than 10% (s. 4.3.4, fig 21, table 11). These variations did not allow statistical analysis of the whole population investigated, although a tendency of increase in cytotoxic activity could be observed in IL-12 stimulated IL-2 lymphoblasts. The redundancy in regulation of proteins responsible for cytotoxic activity, such as perforin and granzymes, could be one reason for these individual differences. Thus, the expression level of perforin in human is regulated by various different cytokines, such as IL-2, IL-4, IL-6, IL-7, IL-12 etc (YU et al., 1999). Additionally to the redundancy, genetic differences in regulatory sequences, for example polymorphism in perforin promoters, also play a role in the regulation of the cytotoxic proteins expression. Significant individual differences were also observed in a previous study from our lab (FUNK et al., 2003; FUNK et al., 2005) investigating the cytolytic potential of canine PBMC from tumor-bearing dogs. To sum up, our experimental data from the cytotoxicity assay give rationale for an approach where lymphocytes of each tumor-bearing dog would be screened in

vitro for their cytotoxic potential prior to submitting the patient to an adoptive immunotherapy.

Additionally to the possibility of using the established cell line as a source for continuous lymphocyte stimulation with IL-12, the cDNA of the constructed rcscIL-12 could be used for gene delivery therapy in tumor patients. By introducing the IL-12 cDNA into the tumor, it would consequently secrete IL-12 and thus provide direct stimulation of tumor-infiltrating lymphocytes (BARAJAS et al., 2001; BRAMSON et al., 1996; FUJITA et al., 2007; GAMBOTTO et al., 1999). Some of the IL-12 gene delivery studies showed that the anti-tumor activity of IL-12 is additive to classical anti-tumor therapies, like radiotherapy (FUJITA et al., 2007). Another advantage of gene delivery as a therapeutical approach is demonstrated by a recent study, which provides evidence that application of IL-12 directly into the tumor reverts the phenotype of tumor-associated macrophages from a pro-tumor into a pro-inflammatory phenotype (WATKINS et al., 2007). This reversal of macrophage phenotype is characterized by significant reduction in tumor growth and progression. The interest of gene delivery therapy has grown also due to the latest results from a study demonstrating that introduction of foreign DNA into tumor cells does not have to be performed exclusively with viral vectors (SHI et al., 2002). Moreover, according to the same study, transfection of naked (without using any carriers) plasmid into tumor cells results in protein production by the tumor.

As a conclusion, recombinant canine single-chain IL-12 was constructed and its biological activities confirmed, making it a potential tool for further investigations in the area of immunology and especially tumor immunotherapy, as well for in vitro as for clinical investigations.

As a protein source, the stably transfected cell line can be used in an appropriate bioreactor (FALKENBERG et al., 1995; FALKENBERG, 1998) where it would stimulate lymphocytes, which can be thereupon adoptively transferred into tumor patients. Furthermore, the nucleotide sequence could also be used for clinical studies exploring the effect of IL-12 delivery directly into the tumor environment.

6 Summary

1. In the course of this project a new source for canine IL-12 was established in the stably transformed BHK Tet-On cell line carrying the cDNA coding for the canine single-chain interleukin-12 (IL-12). To create this construct, the cDNAs coding for the IL-12 subunits p35 and p40 were ligated together, linked by a dekapeptide consisting of 2 bovine elastin motifs. The IL-12 cDNA and the cDNA encoding luciferase reporter gene were cloned into appropriate pTRUE Tet-On vector. The constructed plasmid was stably transfected into BHK Tet-On cell line, which offers the possibility for inducible expression of the IL-12 upon addition of Doxycycline to the cell culture medium.

2. Using luciferase assays and immunofluorescent detection with polyclonal goat anti-firefly luciferase of the reporter gene in the transfected cell clones, two cell lines were selected as stably transfected for further propagation. These cell lines demonstrated an induction index of more than 200 each and both showed 100 % of positive cells after immunofluorescent labeling of the luciferase protein.

3. With help of IFN-γ ELISA, the biological activity of the transfected canine single-chain IL-12 was confirmed in Doxycycline induced as well as in Doxycycline non-induced cells. Furthermore, the data from these experiments indicated a basal level of gene transcription from the inserted plasmid. Neutralization experiments with an anti-canine IL-12 antibody demonstrated reversal of the increase in IFN-γ production by IL-2 lymphoblasts incubated with the supernatant of the constructed cell lines. Moreover, the supernatant and the cell lysate of induced cells were analyzed using western blot, demonstrating band of approximately 70 kDa, which is the expected molecular weight of the rcscIL-12.

4. The levels of proliferation and cytotoxicity of canine PBMC, isolated by centrifugation of heparinized blood with a Percoll® gradient, as well as their IFN-γ production were measured upon cscIL-12 stimulation. The data from these experiments indicated that IL-12 stimulates IFN-γ production in canine IL-2 lymphoblasts but not in resting canine PBMC. In addition, IL-2 lymphoblasts showed a very small but non-significant increase in proliferation upon IL-12 incubation, whereas, the increase in cytotoxicity of IL-2 induced lymphoblasts after incubation with IL-12 demonstrated individual variations ranging from 2 up to 75 %.

5. As final conclusion, the established cell lines constitutively express biologically active recombinant canine single-chain IL-12. Thus, this cell line can be used as a source of IL-12 for stimulation of immune effector cells, especially NK or CD8+ cytotoxic T cells. Such stimulated cells can be used as lymphokine activated killer (LAK) cells in an adoptive cancer immunotherapy. Due to the continuous and constant source of IL-12, stimulation of effector cells achieves stimulation conditions, which resemble more closely those found in vivo. Additionally, the cDNA coding for the single chain canine IL-12 can also be used for a gene delivery therapy in tumor patients.

7 Zusammenfassung

1. In dieser Studie wurde eine neue Möglichkeit zur Herstellung von kaninem Interleukin-12 (IL-12) mit einer BHK Tet-On Zellinie geschaffen, welche mit einer für das kanine Einzelketten IL-12 kodierenden cDNA stabil transfiziert wurde. Für die Konstruktion dieser cDNA wurden die beiden einzelnen, für die IL-12 Untereinheiten p35 und p40 codierenden cDNAs ligiert, indem sie durch eine Nukleotid Sequenz verbunden wurden, welche für ein aus 2 bovinen Elastin- Motifs bestehendem Dekapeptid kodiert. Die so konstruierte cDNA wurde zusammen mit der für die Luciferase kodierenden cDNA in den pTRE Tet-On Vektor kloniert. Dieser Vektor wurde anschließend in die entsprechende BHK Tet-On Zelllinie transfiziert, welche nun die induzierbare IL-12 Produktion durch Zugabe von Doxyclin zum Zellkulturmedium ermöglicht.
2. Mit Hilfe des Luciferase Assays und der Immunfluoreszenz, durchgeführt mit einem polyklonalen Ziegen-anti-Luciferase Antikörper zum Nachweis des Reportergens in den transfizierten Zellklonen, wurden zwei Zelllinien als stabil transfizierte Zelllinien für die weiteren Untersuchungen ausgewählt. Jede dieser beiden Zelllinien wies einen Induktionsindex von über 200 sowie die Expression des Reporter-Gens in 100 % der Zellen auf.
3. Durch den IFN-γ ELISA konnte die biologische Aktivität des in den Überständen enthaltenden rcscIL-12 nachgewiesen werden, was sowohl für die Doxycyclin-induzierten als auch für die nicht induzierten Zellen zutraf. Die gewonnenen Daten ließen nämlich erkennen, dass ein basaler Level der Gentranskription des eingebauten Plasmids vorhanden ist. Neutralisierungstests mit einem anti-kaninen IL12-Antikörper (R&D Systems) wiesen klar darauf hin, dass die erhöhte IFN-γ Produktion von zuvor mit IL-2 induzierten Lymphoblasten, welche mit den Überstanden der transfizierten Zellen zuvor inkubiert worden waren, spezifisch auf die Anwesenheit des rcscIL-12 in den Überständen zurückzuführen ist. Darüber hinaus ergab der mit den Überständen und auch mit den Zelllysaten der induzierten Zellen durchgeführte Western Blot die erwartete Bande von annähernd 70 kDa.
4. Das kanine single-chain IL-12 wurde bezüglich stimulierender Einflüsse auf kanine PBMC geprüft, die aus heparinisiertem Blut des Hundes zuvor über einer Percoll®-Gradient isoliert worden waren. Dabei wurden die Proliferationsrate, die IFN-γ-Produktion und die zytotoxische Aktivität der PBMC gemessen. Diese Ergebnisse zeigten, dass das rcscIL-12

nur bei den mit dem IL-2 vorstimulierten, nicht aber bei naiven PBMC die IFN-γ Produktion induzieren kann. Diese Ergebnisse zeigten weiterhin, dass die kaninen PBMC mit IL-2 vorstimuliert werden müssen, um eine signifikante Erhöhung der IFN-γ Produktion durch rcscIL-12 zu induzieren, dass aber dann auch die anti-Tumorantwort der Effektorlymphozyten deutlich erhöht wird. Die mit IL-2 vorstimulierten Lymphoblasten zeigten allerdings nur eine sehr geringe und nicht signifikante Erhöhung ihrer Proliferationsrate nach der Inkubation mit IL-12, während ihre Steigerung der zytotoxischen Aktivität eine hohe individuelle Variation von 2 - 75% zwischen den einzelnen Hunden aufwies.

5. Als Schlussfolgerung kann man feststellen, dass die konstruierten Zelllinien das biologisch aktive Einzelketten IL-12 dauerhaft exprimieren. Dem zu Folge können diese Zelllinien als eine definierte und etablierte Quelle von kaninem IL-12 zur in-vitro Stimulierung von Immuneffektorzellen, insbesondere der NK und der CD8+ zytotoxischen T Zellen als Lymphokin-aktivierte Killer- (LAK) Zellen in einer adoptiven Tumorimmuntherapie verwendet werden. Auf Grund der kontinuierlichen Sekretion des IL-12 kann eine dauerhaften Wirkung auf Effektorzellen in vitro erzielt werden, was den natürlichen Bedingungen in vivo ähnlich ist. Zusätzlich könnte die für die Einzelketten IL-12 kodierende cDNA-Sequenz bei Tumorpatienten für eine Gentherapie sogar als nackter Plasmidvektor eingesetzt werden. Neueste Experimente haben nämlich gezeigt, dass Virusvektoren nicht notwendiger Weise für eine effektive Gentherapie herangezogen werden müssen.

8 Bibliography

ABRAHAM, E.P., CHAIN, E. (1940):
An enzyme from bacteria able to destroy penicillin. Nature 46: 837

ANDERSON R, MACDONALD I, CORBETT T, HACKING G, LOWDELL MW, PRENTICE HG. (1997):
Construction and biological characterization of an interleukin-12 fusion protein (Flexi-12): delivery to acute myeloid leukemic blasts using adeno-associated virus. Human Gene Ther. 8: 1125-1135

BARAJAS, M., MAZZOLINI, G., GENOVE, G., BILBAO, R., NARVAIZA, I., SCHMITZ, V., SANGRO, B., MELERO, I., QIAN, C., PRIETO, J. (2001):
Gene Therapy of Orthotopic Hepatocellular Carcinoma in Rats Using Adenovirus Coding for Interleukin 12. Hepatology 33: 52-61.

BONNEMA, J.D., RIVLIN, K.A., TING, A.T., SCHOON, R.A., ABRAHAM, R.T., LEIBSON, P.J. (1994):
Cytokine-Enhanced Nk Cell-Mediated Cytotoxicity. Positive Modulatory Effects of Il-2 and Il-12 on Stimulus-Dependent Granule Exocytosis. J Immunol 152: 2098-2104.

BRAMSON, J., HITT, M., GALLICHAN, W.S., ROSENTHAL, K.L., GAULDIE, J., GRAHAM, F.L. (1996):
Construction of a Double Recombinant Adenovirus Vector Expressing a Heterodimeric Cytokine: In Vitro and in Vivo Production of Biologically Active Interleukin-12. Hum Gene Ther 7: 333-342.

BRAMSON, J.L., HITT, M., ADDISON, C.L., MULLER, W.J., GAULDIE, J., GRAHAM, F.L. (1996):
Direct Intratumoral Injection of an Adenovirus Expressing Interleukin-12 Induces Regression and Long-Lasting Immunity That Is Associated with Highly Localized Expression of Interleukin-12. Hum Gene Ther 7: 1995-2002.

BRUNDA, M.J., LUISTRO, L., WARRIER, R.R., WRIGHT, R.B., HUBBARD, B.R., MURPHY, M., WOLF, S.F., GATELY, M.K. (1993):
Antitumor and Antimetastatic Activity of Interleukin 12 against Murine Tumors. J Exp Med 178: 1223-1230.

BÜTTNER, M., BELKE-LOUIS, G., RZIHA, H.J., MCINNES, C., KAADEN, O.R. (1998):
Detection, Cdna Cloning and Sequencing of Canine Interleukin 12. Cytokine 10: 241-248.

CARRA, G., GEROSA, F., TRINCHIERI, G. (2000):
Biosynthesis and Posttranslational Regulation of Human Il-12. J Immunol 164: 4752-4761.

CHAN, S.H., KOBAYASHI, M., SANTOLI, D., PERUSSIA, B., TRINCHIERI, G. (1992):
Mechanisms of Ifn-Gamma Induction by Natural Killer Cell Stimulatory Factor (Nksf/Il-12). Role of Transcription and Mrna Stability in the Synergistic Interaction between Nksf and Il-2. J Immunol 148: 92-98.

CHAN, S.H., PERUSSIA, B., GUPTA, J.W., KOBAYASHI, M., POSPISIL, M., YOUNG, H.A., WOLF, S.F., YOUNG, D., CLARK, S.C., TRINCHIERI, G. (1991):
Induction of Interferon Gamma Production by Natural Killer Cell Stimulatory Factor: Characterization of the Responder Cells and Synergy with Other Inducers. J Exp Med 173: 869-879.

CHEHIMI, J., STARR, S.E., FRANK, I., RENGARAJU, M., JACKSON, S.J., LLANES, C., KOBAYASHI, M., PERUSSIA, B., YOUNG, D., NICKBARG, E., ET AL. (1992):
Natural Killer (Nk) Cell Stimulatory Factor Increases the Cytotoxic Activity of Nk Cells from Both Healthy Donors and Human Immunodeficiency Virus-Infected Patients. J Exp Med 175: 789-796.

D'ANDREA, A., RENGARAJU, M., VALIANTE, N.M., CHEHIMI, J., KUBIN, M., ASTE, M., CHAN, S.H., KOBAYASHI, M., YOUNG, D., NICKBARG, E., ET AL. (1992):
Production of Natural Killer Cell Stimulatory Factor (Interleukin 12) by Peripheral Blood Mononuclear Cells. J Exp Med 176: 1387-1398.

DEBLAKER-HOHE, D.F., YAMAUCHI, A., YU, C.R., HORVATH-ARCIDIACONO, J.A., BLOOM, E.T. (1995):
IL-12 Synergizes with Il-2 to Induce Lymphokine-Activated Cytotoxicity and Perforin and Granzyme Gene Expression in Fresh Human Nk Cells. Cell Immunol 165: 33-43.

DUNN, G.P., OLD, L. J., SCHREIBER, R. D.
The three Es of cancer immunoediting. Annu Rev Immunol 22: 329-360.

EBERT, E.C. (2004):
Interleukin-12 up-Regulates Perforin- and Fas-Mediated Lymphokine-Activated Killer Activity by Intestinal Intraepithelial Lymphocytes. Clin Exp Immunol 138: 259-265.

FALKENBERG, F.W., WEICHERT, H., KRANE, M., BARTELS, I., PALME, M., NAGELS, H.O., FIEBIG, H. (1995):
In vitro production of monoclonal antibodies in high concentration in a new and easy to handle modular minifermenter. J Immunol Methods 179: 13-29.

FALKENBERG, F. W. (1998):
Production of monoclonal antibodies in the miniPERM bioreactor: comparison with other hybridoma culture methods. Res Immunol 149:560-570

FOSS, D.L., MOODY, M.D., MURPHY, K.P., JR., PAZMANY, C., ZILLIOX, M.J., MURTAUGH, M.P. (1999):
In Vitro and in Vivo Bioactivity of Single-Chain Interleukin-12. Scand J Immunol 50: 596-604.

FUJITA, T., TIMME, T.L., TABATA, K., NARUISHI, K., KUSAKA, N., WATANABE, M., ABDELFATTAH, E., ZHU, J.X., REN, C., YANG, G., GOLTSOV, A., WANG, H., VLACHAKI, M.T., TEH, B.S., BUTLER, E.B., THOMPSON, T.C. (2007):
Cooperative Effects of Adenoviral Vector-Mediated Interleukin 12 Gene Therapy with Radiotherapy in a Preclinical Model of Metastatic Prostate Cancer. Gene Ther 14: 227-236.

FUNK, J. (2001)
Quantitative Untersuchungen zur Zytotoxizität und Mitogenstimulation isolierter Blutleukozyten von Hunden mit unterschiedlichen Tumoren
Vet Med Diss Gießen

FUNK, J., SCHMITZ, G., BACH, U., FAILING, K., BURKHARDT, E. (2003):
Influence of Different Tumour Types on Natural Cytotoxicity (Nk Cell Activity) and Mitogen-Induced Lymphocyte Proliferation in Isolated Blood Lymphocytes from 110 Dogs with Tumours. Res Vet Sci 74: 129-135.

FUNK, J., SCHMITZ, G., FAILING, K., BURKHARDT, E. (2005):
Natural Killer (Nk) and Lymphokine-Activated Killer (Lak) Cell Functions from Healthy Dogs and 29 Dogs with a Variety of Spontaneous Neoplasms. Cancer Immunol Immunother 54: 87-92.

GAMBOTTO, A., TUTING, T., MCVEY, D.L., KOVESDI, I., TAHARA, H., LOTZE, M.T., ROBBINS, P.D. (1999):
Induction of Antitumor Immunity by Direct Intratumoral Injection of a Recombinant Adenovirus Vector Expressing Interleukin-12. Cancer Gene Ther 6: 45-53.

GATELY, M.K. (1993):
Interleukin-12: A Recently Discovered Cytokine with Potential for Enhancing Cell-Mediated Immune Responses to Tumors. Cancer Invest 11: 500-506.

GATELY, M.K., CARVAJAL, D.M., CONNAUGHTON, S.E., GILLESSEN, S., WARRIER, R.R., KOLINSKY, K.D., WILKINSON, V.L., DWYER, C.M., HIGGINS, G.F., JR., PODLASKI, F.J., FAHERTY, D.A., FAMILLETTI, P.C., STERN, A.S., PRESKY, D.H. (1996):
Interleukin-12 Antagonist Activity of Mouse Interleukin-12 P40 Homodimer in Vitro and in Vivo. Ann N Y Acad Sci 795: 1-12.

GATELY, M.K., DESAI, B.B., WOLITZKY, A.G., QUINN, P.M., DWYER, C.M., PODLASKI, F.J., FAMILLETTI, P.C., SINIGAGLIA, F., CHIZONNITE, R., GUBLER, U., ET AL. (1991):
Regulation of Human Lymphocyte Proliferation by a Heterodimeric Cytokine, Il-12 (Cytotoxic Lymphocyte Maturation Factor). J Immunol 147: 874-882.

GERMANN, T., GATELY, M.K., SCHOENHAUT, D.S., LOHOFF, M., MATTNER, F., FISCHER, S., JIN, S.C., SCHMITT, E., RUDE, E. (1993):
Interleukin-12/T Cell Stimulating Factor, a Cytokine with Multiple Effects on T Helper Type 1 (Th1) but Not on Th2 Cells. Eur J Immunol 23: 1762-1770.

GONDOLF, C. (1994)
Untersuchungen zur Zytotoxizität von Natural Killer (NK)- und Lymphokin-aktivierten Killer (LAK)- Zellen aus dem peripheren Blut des Hundes.
Vet Med Diss Gießen

GONDOLF, C., BURKHARDT, E., FAILING, K., STITZ, L. (1996):
A New Colorimetric Method for Measuring Cell-Mediated Cytotoxicity in Dogs. Vet Immunol Immunopathol 55: 11-22.

GOSSEN, M., BUJARD, H. (1992):
Tight Control of Gene Expression in Mammalian Cells by Tetracycline-Responsive Promoters. Proc Natl Acad Sci U S A 89: 5547-5551.

GOSSEN, M., FREUNDLIEB, S., BENDER, G., MULLER, G., HILLEN, W., BUJARD, H. (1995):
Transcriptional activation by tetracyclines in mammalian cells. Science 268: 1766-1769

GUBLER, U., CHUA, A.O., SCHOENHAUT, D.S., DWYER, C.M., MCCOMAS, W., MOTYKA, R., NABAVI, N., WOLITZKY, A.G., QUINN, P.M., FAMILLETTI, P.C., ET AL. (1991):
Coexpression of Two Distinct Genes Is Required to Generate Secreted Bioactive Cytotoxic Lymphocyte Maturation Factor. Proc Natl Acad Sci U S A 88: 4143-4147.

HASHIMOTO, W., OSAKI, T., OKAMURA, H., ROBBINS, P.D., KURIMOTO, M., NAGATA, S., LOTZE, M.T., TAHARA, H. (1999):
Differential Antitumor Effects of Administration of Recombinant Il-18 or Recombinant Il-12 Are Mediated Primarily by Fas-Fas Ligand- and Perforin-Induced Tumor Apoptosis, Respectively. J Immunol 163: 583-589.

HEINZEL, F.P., HUJER, A.M., AHMED, F.N., RERKO, R.M. (1997):
In Vivo Production and Function of Il-12 P40 Homodimers. J Immunol 158: 4381-4388.

HODGE, D.L., MARTINEZ, A., JULIAS, J.G., TAYLOR, L.S., YOUNG, H.A. (2002):
Regulation of Nuclear Gamma Interferon Gene Expression by Interleukin 12 (Il-12) and Il-2 Represents a Novel Form of Posttranscriptional Control. Mol Cell Biol 22: 1742-1753.

KOBAYASHI, M., FITZ, L., RYAN, M., HEWICK, R.M., CLARK, S.C., CHAN, S., LOUDON, R., SHERMAN, F., PERUSSIA, B., TRINCHIERI, G. (1989):
Identification and Purification of Natural Killer Cell Stimulatory Factor (Nksf), a Cytokine with Multiple Biologic Effects on Human Lymphocytes. J Exp Med 170: 827-845.

LING, P., GATELY, M.K., GUBLER, U., STERN, A.S., LIN, P., HOLLFELDER, K., SU, C., PAN, Y.C., HAKIMI, J. (1995):
Human Il-12 P40 Homodimer Binds to the Il-12 Receptor but Does Not Mediate Biologic Activity. J Immunol 154: 116-127.

MATTNER, F., FISCHER, S., GUCKES, S., JIN, S., KAULEN, H., SCHMITT, E., RUDE, E., GERMANN, T. (1993):
The Interleukin-12 Subunit P40 Specifically Inhibits Effects of the Interleukin-12 Heterodimer. Eur J Immunol 23: 2202-2208.

MAZZOLINI, G., QIAN, C., XIE, X., SUN, Y., LASARTE, J.J., DROZDZIK, M., PRIETO, J. (1999):
Regression of Colon Cancer and Induction of Antitumor Immunity by Intratumoral Injection of Adenovirus Expressing Interleukin-12. Cancer Gene Ther 6: 514-522.

NASTALA, C.L., EDINGTON, H.D., MCKINNEY, T.G., TAHARA, H., NALESNIK, M.A., BRUNDA, M.J., GATELY, M.K., WOLF, S.F., SCHREIBER, R.D., STORKUS, W.J., ET AL. (1994):
Recombinant Il-12 Administration Induces Tumor Regression in Association with Ifn-Gamma Production. J Immunol 153: 1697-1706.

NASU, Y., BANGMA, C.H., HULL, G.W., LEE, H.M., HU, J., WANG, J., MCCURDY, M.A., SHIMURA, S., YANG, G., TIMME, T.L., THOMPSON, T.C. (1999):
Adenovirus-Mediated Interleukin-12 Gene Therapy for Prostate Cancer: Suppression of Orthotopic Tumor Growth and Pre-Established Lung Metastases in an Orthotopic Model. Gene Ther 6: 338-349.

NAUME, B., GATELY, M., ESPEVIK, T. (1992):
A Comparative Study of Il-12 (Cytotoxic Lymphocyte Maturation Factor)-, Il-2-, and Il-7-Induced Effects on Immunomagnetically Purified Cd56+ Nk Cells. J Immunol 148: 2429-2436.

NAUME, B., GATELY, M.K., DESAI, B.B., SUNDAN, A., ESPEVIK, T. (1993):
Synergistic Effects of Interleukin 4 and Interleukin 12 on Nk Cell Proliferation. Cytokine 5: 38-46.

OKANO, F., SATOH, M., YAMADA, K. (1997):
Cloning and Expression of the Cdna for Canine Interleukin-12. J Interferon Cytokine Res 17: 713-718.

PHILLIPS, B.S., PADILLA, M.L., DICKERSON, E.B., LINDSTROM, M.J., HELFAND, S.C. (1999):
Immunostimulatory Effects of Human Recombinant Interleukin-12 on Peripheral Blood Mononuclear Cells from Normal Dogs. Vet Immunol Immunopathol 70: 189-201.

PRESKY, D.H., YANG, H., MINETTI, L.J., CHUA, A.O., NABAVI, N., WU, C.Y., GATELY, M.K., GUBLER, U. (1996):
A Functional Interleukin 12 Receptor Complex Is Composed of Two Beta-Type Cytokine Receptor Subunits. Proc Natl Acad Sci U S A 93: 14002-14007.

RABINOWICH, H., HERBERMAN, R.B., WHITESIDE, T.L. (1993):
Differential Effects of Il12 and Il2 on Expression and Function of Cellular Adhesion Molecules on Purified Human Natural Killer Cells. Cell Immunol 152: 481-498.

RAKHMILEVICH, A.L., TURNER, J., FORD, M.J., MCCABE, D., SUN, W.H., SONDEL, P.M., GROTA, K., YANG, N.S. (1996):
Gene Gun-Mediated Skin Transfection with Interleukin 12 Gene Results in Regression of Established Primary and Metastatic Murine Tumors. Proc Natl Acad Sci U S A 93: 6291-6296.

ROBERTSON, M.J., SOIFFER, R.J., WOLF, S.F., MANLEY, T.J., DONAHUE, C., YOUNG, D., HERRMANN, S.H., RITZ, J. (1992):
Response of Human Natural Killer (Nk) Cells to Nk Cell Stimulatory Factor (Nksf): Cytolytic Activity and Proliferation of Nk Cells Are Differentially Regulated by Nksf. J Exp Med 175: 779-788.

SAIKI, R.K., SCHARF S., FALOONA F., MULLIS K.B., HORN G.T., ERLICH H.A., ARNHEIM, N. (1985):
Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science 230: 1350-1354.

SAMBROOK, J., RUSSEL,W.D. (2001)
Molecular Cloning: A Laboratory Manual. NY: Cold Spring Harbor Laboratory Press

SALDARRIAGA, O. A., PEREZ, L. E., TRAVI, B. L., MELBY, P. C. (2006):
Selective enhancement of the type 1 cytokine response by expression of a canine interleukin (IL)-12 fused heterodimeric DNA. Vet Immunol Immunopathol 110: 377-388.

SCHMITZ, G. (2000)
Qualitative und quantitative Untersuchungen zur zytotoxischen Aktivität aus Hundeblut isolierter Leukozyten gegenüber verschiedenen kaninen Tumorzelllinien.
Vet Med Diss Gießen

SCHMITZ, G., ARMIEN, A.G., FONFARA, S., TEIFKE, J.P., BURKHARDT, E. (2003):
Induction of Apoptosis by Canine Natural Killer Cells. J Vet Med A Physiol Pathol Clin Med 50: 156-159.

SHI, F., RAKHMILEVICH, A.L., HEISE, C.P., OSHIKAWA, K., SONDEL, P.M., YANG, N.S., MAHVI, D.M. (2002):
Intratumoral Injection of Interleukin-12 Plasmid DNA, Either Naked or in Complex with Cationic Lipid, Results in Similar Tumor Regression in a Murine Model. Mol Cancer Ther 1: 949-957.

STRAUSS-AYALI, D., BANETH, G., SHOR, S., OKANO, F., JAFFE, C.L. (2005):
Interleukin-12 Augments a Th1-Type Immune Response Manifested as Lymphocyte Proliferation and Interferon Gamma Production in Leishmania Infantum-Infected Dogs. Int J Parasitol 35: 63-73.

THIERFELDER, W.E., VAN DEURSEN, J.M., YAMAMOTO, K., TRIPP, R.A., SARAWAR, S.R., CARSON, R.T., SANGSTER, M.Y., VIGNALI, D.A., DOHERTY, P.C., GROSVELD, G.C., IHLE, J.N. (1996):
Requirement for Stat4 in Interleukin-12-Mediated Responses of Natural Killer and T Cells. Nature 382: 171-174.

TRINCHIERI, G. (2003):
Interleukin-12 and the Regulation of Innate Resistance and Adaptive Immunity. Nat Rev Immunol 3: 133-146.

WAGNER, U. (1998):
Ermittlung optimaler Parameter für die in vitro-Stimulation von Hundeblutlymphozyten mit Concanavalin A (ConA) und Phytohämagglutinin (PHA) zur Gewinnung von kaninem Interleukin (IL-2) aus Zellkulturüberständen.
Vet Med Diss Gießen

WAGNER, U., BURKHARDT, E., FAILING, K. (1999):
Evaluation of Canine Lymphocyte Proliferation: Comparison of Three Different Colorimetric Methods with the 3h-Thymidine Incorporation Assay. Vet Immunol Immunopathol 70: 151-159.

WATKINS, S.K., EGILMEZ, N.K., SUTTLES, J., STOUT, R.D. (2007):
Il-12 Rapidly Alters the Functional Profile of Tumor-Associated and Tumor-Infiltrating Macrophages in Vitro and in Vivo. J Immunol 178: 1357-1362.

YIN, D.X., ZHU, L., SCHIMKE, R.T. (1996):
Tetracycline-Controlled Gene Expression System Achieves High-Level and Quantitative Control of Gene Expression. Anal Biochem 235: 195-201.

YU, C. R., ORTALDO, J. R., CURIEL, R. E., YOUNG, H. A., ANDERSON, S. K., GOSSELIN, P. (1999):
Role of a STAT binding site in the regulation of the human perforin promoter. J Immunol 162: 2785-90.

ZITVOGEL, L., TAHARA, H., CAI, Q., STORKUS, W.J., MULLER, G., WOLF, S.F., GATELY, M., ROBBINS, P.D., LOTZE, M.T. (1994):
Construction and Characterization of Retroviral Vectors Expressing Biologically Active Human Interleukin-12. Hum Gene Ther 5: 1493-1506.

9 Attachment

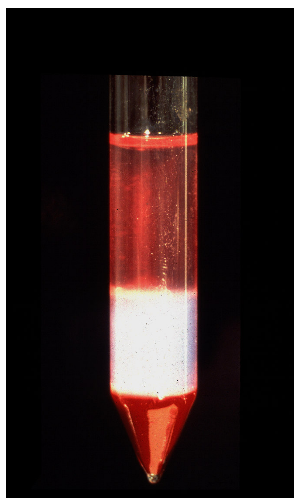

Fig 23: Percoll isolated PBMC (canine).
The white band of cells is located between the clear upper column containing culture medium, blood plasma and platelets, and the lower column of packed red cells and polymorphonuclear leukocytes

Originally measured values of the represented figures:

	+ Dox	**- Dox**
Population 2	6393.9	128.36
Population 3	16.315	4.682
Population 5	45618	724.33
Population 6	44503	634.17

Table 1. Raw values of the luciferase measurement of the initial screening procedure **(fig.9)**

	+ Dox	**- Dox**
Clone 5.1	47172	650.58
Clone 5.2	46889	1038.4
Clone 5.3	46780	567.55
Clone 5.5	46707	698.11
Clone 6.1	46649	2224.6
Clone 6.2	46480	371.27
Clone 6.3	44161	416.56
Clone 6.5	47023	733.78

Table 2. Raw Luciferase values of the luciferase measurement of the first subcloning procedure **(fig.10)**

	+ Dox	- Dox
Clone 6.2.1	46986	456.59
Clone 6.2.2	46860	216.25
Clone 6.2.3	46637	857.2
Clone 6.2.4	46752	302.36
Clone 6.2.5	46997	364.4
Clone 6.3.1	38539	279.61
Clone 6.3.2	29777	161.4
Clone 6.3.3	44718	252.35
Clone 6.3.4	43352	301.56
Clone 6.3.5	45800	207.17

Table 3. Raw Luciferase values of the luciferase measurement of the second subcloning procedure **(fig.11)**

	rcIL-12	negative ctrl.
Probe 1	2.3	0.146
Probe 2	2.25	0.14

Table 4. Values of IFN-γ ELISA measurement in ng/ml **(fig. 14)**

	rcIL-12	negative ctrl.	IFN-gamma (4ng/ml)	IFN-gamma (2ng/ml)	IFN-gamma (1ng/ml)
Probe 1	0.876	0.088	1.57	0.812	0.522
Probe 2	0.886	0.08			

Table 4a. O.D. values of IFN-γ ELISA measurement **(fig. 14)**

	rcIL-12	negative ctrl.
Probe 1	1.8	0.045
Probe 2	1.7	0.055

Table 5. Values of IFN-γ ELISA measurement in ng/ml **(fig. 15)**

	rcIL-12	negative ctrl.	IFN-gamma (4ng/ml)	IFN-gamma (2ng/ml)	IFN-gamma (1ng/ml)
Probe 1	1.276	0.089	2.332	1.478	0.787
Probe 2	1.338	0.116			

Table 5a. O.D. values of IFN-γ ELISA measurement **(fig. 15)**

	IFN-γ (ng/ml)
+ Dox	3.475
- Dox	4
negative ctrl.	0.55

Table 6. Values of IFN-γ ELISA measurement in ng/ml **(fig. 16)**

	O.D.
+ Dox	2.395
- Dox	2.107
negative ctrl.	0.507
IFN-gamma (2ng/ml)	1.405
Blank	0.032

Table 6a. O.D. values of IFN-γ ELISA measurement **(fig. 16)**

Fold Increase	positiv ctrl.	supernatant	supernatant + anti-cIL-12	negative ctrl.
Dog 1	5.1	4.9	0.6	0
Dog 2	10.27	10.31	0.17	0
Dog 3	11.1	10.17	0.08	0

Table 7. Fold increase of the IFN-γ production **(fig.17)**

	positiv ctrl.	supernatant	supernatant + anti-cIL-12	negative ctrl.
Dog 1	3.005	2.95	0.83	0.5
Dog 2	2.818	2.827	0.294	0.25
Dog 3	0.558	0.514	0.05	0.046

Table 7a. O.D. values of IFN-γ ELISA measurement **(fig. 17)**

standard IFN-gamma (2 ng/ml)	1,458
+ rc IL-12	0,053
medium	0,0475
blank	0,0515

Table 8. O.D. values of IFN-γ ELISA measurement **(fig. 18)**

	Dog 1	Dog 2
Standard IFN-gamma (2ng/ml)	2	2
+ cIL-12	0,12	0,06

Table 9. O.D. values of IFN-γ ELISA measurement **(fig. 19)**

	IL-2 + IL-12	IL-2	medium
Probe 1	0,58	0,56	0,124
Probe 2	0,604	0,57	0,145

Table 10. O.D. values of BrdU ELISA measurement **(fig. 20)**

	IL-2 + IL-12	IL-2
Dog 1	32,6	0
Dog 2	97,3	95,29
Dog 3	96,3	84,8
Dog 4	45,7	42,5
Dog 5	70,42	49,84
Dog 6	62	0
Dog 7	92,8	60,4
Dog 8	72,8	0

Table 11. Percentage of cytotoxicity of the PBMC of eight investigated dogs **(fig. 21)**

INSTRUMENTS AND DEVICES

Consort pvba – Turnhout, Belgium

CONSORT E425, Electrophoresis power supply

MJ Research Inc. - Waltham, MA USA

PTC-200 Peltier thermal cycler

Schimadzu Corp. - Kyoto, Japan

UV-VIS 1202 Spectrophotometer

Hereaus Holding - Hanau, Germany

Laborfuge 400R

Laminar air LB-48-C

Incubator

Bachofer – Reutlingen, Germany

Bachofer UV-Lamp (254nm)

Hettich Holding – Tuttlingen, Germany

Centrifuge ROTINA 48 RC

Köttermann GmbH & Co KG - Uetze/Hänigsen, Germany

Waterbath

Titertek – AL, USA

Microplate reader Titertek Multiscan Plus

REAGENTS AND MEDIA

CELL CULTURE

READY MEDIA AND KITS

Alexis Corporation – Lausen, Switzerland

Puromycin dihydrochloride, ALX-380-028-M025

Amersham Biosciences – Piscataway NJ, USA

Percoll® - density gradient medium

Sigma Aldrich, St. Louis, MO, USA

Trizma® hydrochloride, T3253

Sodium dihydrogen phosphate, 20 mM, 82592

Sodium hydrogen carbonate, S5761

PAA Laboratories, GmbH – Pashing, Austria

RPMI1640 cell culture medium E15-885

Fetal Bovine Serum Standard Quality, A15-101

Penicillin/Streptomycin 100x Concentrate, P11-010

Trypsin EDTA (1:250) 1x Concentrate, L11-004

Merck, KAaG – Darmstadt, Germany

Potassium nitrate, 105065

Roche – Penzberg, Germany

BrdU ELISA kit, 114444611001

R&D Systems – Minneapolis, USA

Quantikine canine INF-γ ELISA, CAIF00

Goat anti-canine IL-12 polyclonal antibody, AF1969

Recombinant canine IL-12, 2118-CL

Peprotech EC Ltd – London, Great Britain

Recombinant human IL-12, 200-02

SELF-MADE REAGENTS

Rose Bengal Assay (RBA)

NaCl-PBS, pH 7.4

1.42 g Na_2HPO_4 x 2 H_2O

0.2 g KH_2PO_4

8.0 g NaCl

0.2 g KCl

Ad 1000.0 ml Aqua dest., adjust the pH-value with 1 N HCl

Rose Bengal stock solution

0.25 g Rose Bengal

Ad 100 ml NaCl-PBS

Mix and keep at 4°C

Ethanol-PBS 50% (v/v)

Mix ethanol and NaCl-PBS in 1:1 ratio

WORKING WITH NUCLEIC ACIDS

ROTH – Karlsrue, Germany

Ampiciline, K029.1

Ethanol, 9065.1

IPTG, CN08.2

Isopropanol, 6752.1

LB Agar, X969.2

LB Medium, X968.2

X-Gal, 2315.1

Fermentas – Burlington, Canada

Markers, SM1153, SM1163, SM1133

6X loading dye, R0621

MACHEREY-NAGEL GmbH &Co. KG – Düren, Germany

NucleoSpin, 740615.250

PEGLAB Biotechnologie, GmbH – Erlangen, Germany

Taq Polymerase, 01-1000

Novagen – Madison, WI, USA

KOD HotStart Polymerase, 71086-3

Qiagen – Hilden, Germany

Superfect®, 301307

A-addition kit, 231994

WORKING WITH PROTEINS

SDS PAGE

Anode buffer

200 mM Tris/HCl pH 8,9

Gel buffer

1 M Tris /HCl pH 8,45, 0,1%(w/v) SDS

Cathode buffer

100 mM Tris/HCl pH 8.25, 100 mM Tricin, 0,1%(w/v) SDS

ACKNOWLEDGMENTS

This thesis would not have come to realization without the generous help and unlimited support from a number of people to whom I sincerely thank.

Thus, I would first like to express my gratitude to my mentor, Prof. Dr. Eberhard Burkhardt for guiding me through the exciting field of this thesis and for always having time for a wise advice.

I owe special thanks to Prof. Dr. rer. nat. Norbert Tautz (Department of Virology, Justus-Liebig University- Giessen) for his help on the molecular and cellular aspects of the establishment of the cell line as a central issue of this project.

I also thank Dr. Werner Hecht for being always there with useful advices in overcoming the obstacles, especially on the field of molecular biology.

I thank to Ms. Sandra Preis for introducing to me the techniques of isolation and handling of canine PBMC.

Special thanks to Prof. Dr. Dr. hc. Bernd Hoffmann from the Clinic for Gynecology, Obstetrics and Andrology, and to Prof. Dr. Andreas Moritz from the Small Animal Clinic of the Faculty of Veterinary Medicine - Giessen, as well as to their personnel for providing the beagles as blood donors.

For organizing an excellent and challenging training program in molecular biology through the graduate College 455 “Molekulare Veterinärmedizin”, I thank Prof. Dr. Ernst Petzinger, Prof. Dr. Rolf Bauerfeind and Ms. Jana Heber.

For their help in the life outside the lab and for their friendship I thank my friends Dimitrija and Mira Stojanoski and Daniel Filiposki.

At last, I endlessly thank my family, Nikola, Snezana and Natasha, and to my dear Lidija, for their unlimited love, support and understanding.

This project was financially supported through a research grant from the German Research Council (DFG), to which I sincerely express my gratitude.