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Klinik und Poliklinik für kleine Haustiere
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der Freien Universität Berlin

# Entwicklung und Validierung eines Enzyme-linked Immunosorbent Assays zum Messen von felinem Tumornekrosefaktor alpha in Serum

# Development and Analytical Validation of an Enzyme-linked Immunosorbent Assay for the Measurement of Feline Tumor Necrosis Factor Alpha in Serum

Inaugural-Dissertation
zur Erlangung des Grades eines
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vorgelegt von
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**TABLE OF CONTENT**

## LIST OF ABBREVIATIONS

| | |
|---|---|
| ACTH | adrenocorticotropic hormone |
| °C | degree Celsius |
| µg | microgram |
| µm | micrometer |
| µl | micro liter |
| A | alanine (one letter code) |
| BCA | bicinchoninic acid |
| BSA | bovine serum albumin |
| C | cysteine (one letter code) |
| CAPS | *N*-cyclohexyl-3-aminopropanesulfonic acid |
| cDNA | complementary deoxyribonucleic acid |
| conc. | concentration |
| CPM | counts per minute |
| %CV | coefficient of variation; %CV = (SD/mean)*100 |
| D | aspartic acid (one letter code) |
| $ddH_2O$ | destilled and degassed water |
| DNA | deoxyribonucleic acid |
| EDTA | ethylenediaminetetraacetic acid |
| F | phenylalanine (one letter code) |
| FPLC | fast performance liquid chromatography |
| fTNFα | feline tumor necrosis factor alpha |
| G | glycine (one letter code) |
| g | gram |
| GH | growth hormone |
| GST | glutathione S transferase |
| H | histidine (one letter code) |
| HCl | hydrochloric acid |
| HIV | human immunodeficiency virus |
| HPLC | high performance liquid chromatography |
| hTNFα | human tumor necrosis factor alpha |
| I | isoleucine (one letter code) |
| $^{125}I$ | radioactive iodine |

| | |
|---|---|
| IBD | inflammatory bowel disease |
| IPTG | isopropyl β-D-1-thiogalatopyranoside |
| kb | kilobase |
| KCl | potassium chloride |
| kDa | kilo Dalton |
| $KH_2PO_4$ | potassium dihydrogen phosphate |
| L | leucine (one letter code) |
| L | liter |
| LB | Luria-Bertani agar |
| LPL | lipoprotein lipase |
| M | methionine (one letter code) |
| M | molar (M = moles of solute/liter of solution) |
| mCi | millicurie |
| MES | 2-(N-morpholino)ethanesulfonic acid |
| mg | milligram |
| MHC | mayor histocompatibility complex |
| min | minute |
| ml | milliliter |
| mM | millimolar |
| mRNA | messenger ribonucleic acid |
| N | asparagine (one letter code) |
| N/A | not applicable |
| $Na_2C_4H_4O_6$ | sodium tartrate |
| NaCl | sodium chloride |
| $Na_2CO_3$ | sodium carbonate |
| $NaHCO_3$ | sodium bicarbonate |
| NaOH | sodium hydroxide |
| NB | nonspecific binding |
| NFκB | nuclear factor kappa B |
| nm | nanometer |
| O/E | ratio of observed to expected |
| P | proline (one letter code) |
| p | p-value |
| PBS | phosphate buffer saline |

| | |
|---|---|
| PCR | polymerase chain reaction |
| pg | picogram |
| $PGE_2$ | prostaglandin E2 |
| PVDF | polyvinylidendifluorid |
| pH | -log 10 concentration of $H^+$ ions in solution |
| pI | isoelectric point |
| Q | glutamine (one letter code) |
| R | arginine (one letter code) |
| RIA | radioimmunoassay |
| rfTNFα | recombinant feline tumor necrosis factor alpha |
| RCS | rabbit carrier solution |
| RPM | rounds per minute |
| s | second |
| S | serine (one letter code) |
| SD | standard deviation |
| SDS-PAGE | sodium-dodecyl-sulfate-polyacrylamide gel electrophoresis |
| SELDI-TOF-MS | surface-enhanced laser desorption/ionization time-of-flight mass spectrometry |
| sTNFα | soluble tumor necrosis factor alpha |
| T | threonine (one letter code) |
| TACE | TNFα converting enzyme |
| TB | terrific broth |
| TC | total counts |
| tmTNFα | transmembrane tumor necrosis factor alpha |
| TNFα | tumor necrosis factor alpha |
| Tris | 2-amino-2-hydroxymethyl-1, 3-propanediol |
| TSH | thyroid-stimulating hormone |
| V | valine (one letter code) |
| v/v | volume to volume |
| W | tryptophan (one letter code) |
| WAT | white adipose tissue |
| × g | centrifugal force, expressed as × gravity |

# I. INTRODUCTION

Inflammatory gastrointestinal diseases in the cat are common (NGUYEN et al., 2006). However, even after a diagnosis of an inflammatory gastrointestinal disease has been made, the classification and assessment of disease severity and progression remain challenging. Diagnosis often requires invasive and expensive methods, like endoscopic exploration of the gastrointestinal tract and histologic or immunohistochemical analysis of biopsy specimen. The development and validation of inexpensive, non-invasive, accurate, and objective methods for the evaluation of the patients with inflammatory gastrointestinal diseases are therefore worthwhile.

Tumor necrosis factor α (TNFα) is a cytokine, released mainly by macrophages and other cells of the immune system in response to contact with infectious agents, tissue injury, tumor growth, and during the process of autoimmune diseases. This protein plays a major role in the initiation and enhancement of inflammation, locally as well as systemically. Due to its involvement in numerous diseases, and due to its potential to necrotize certain types of tumors, there has been a great deal of scientific interest in TNFα. The genetic sequences of TNFα from a large number of mammalian species have been identified (SOLLER et al., 2007) and TNFα from different species has been purified. Antibodies raised against TNFα are now used in humans for the treatment of autoimmune diseases like inflammatory bowel disease (IBD), rheumatoid arthritis, and psoriasis (SHEALY and VISVANATHAN, 2008). Also, many different types of assays have been developed for the measurement of the TNFα concentration in biological fluids, such as serum or plasma, from humans, mice, cattle, dogs, and other mammalian species (EGAN et al., 1994; KENISON et al., 1990; KREUZER et al., 1996; SU et al., 1992c; YILMAZ and SENTURK, 2007). To date no such assay has been developed for the measurement of TNFα in the cat and research regarding the cytokine's role in inflammatory diseases of cats is limited.

## 1. Hypothesis and Objectives

The hypothesis of this study was that the measurement of feline TNFα may be a useful tool to assess the severity and progression of inflammatory diseases of the gastrointestinal tract in cats.

For the purpose of proving or disproving this hypothesis, the objectives of this study were to produce recombinant feline TNFα in *Escherichia coli* bacterial cells, purify the protein, develop and analytically validate an enzyme-linked immunosorbent assay for the measurement of feline TNFα in serum, and measure serum concentrations of feline TNFα in cats with chronic inflammatory enteropathies.

# II. LITERATURE REVIEW

## 1. Tumor Necrosis Factor α

Inflammation is essential for the host's defense against invasive organisms. Cells primarily involved in inflammatory responses belong to the monocyte/macrophage lineage (BEUTLER and CERAMI, 1989). These cells produce a variety of substances referred to as cytokines, which play a central role in the body's defense mechanisms (BEUTLER B. and CERAMI A., 1989). Tumor necrosis factor α (TNFα) is among the most abundant substances produced by activated macrophages, especially when these are activated in response to contact with lipopolysaccharide on the surface of gram negative bacteria (CERAMI, 1992; MICHALEK et al., 1980; TRACEY and CERAMI, 1992). Feldmann and Steinman called TNFα "the body's fire alarm" (FELDMANN and STEINMAN, 2005), underlining the molecule's crucial role in several processes, including septic shock (BEUTLER et al., 1985c), inflammatory reactions (SHALABY et al., 1985), infections (JAEAETTELAE, 1991), cachexia (TORTI et al., 1985), and defense against tumor development (CARSWELL et al., 1975). However, TNFα fulfills an even wider variety of functions and might also be involved in the down regulation of the immune system after a successful host defense has taken place (KOLLIAS et al., 1999).
Physiologically, cats do not display any differences in this regard to other mammalian species. Feline TNFα interacts with interleukin 1 (IL1) and interleukin 6 (IL6) as an early inducer of fever, leukocytosis, and the release of acute phase proteins in reaction to the host's contact with potentially pathogenic stimuli (PALTRINIERI, 2008).

### 1.1. Nomenclature

Over time there have been changes in the nomenclature for tumor necrosis factor α. Shortly after its discovery the cytokine was simply named tumor necrosis factor (TNF), due to its capability to induce hemorrhagic necrosis in certain types of tumors in mice and humans (CARSWELL et al., 1975). Later, it was discovered that a factor, already described in the 1960s and named lymphotoxin, after its primary function (GRANGER and WILLIAMS, 1968; RUDDLE and WAKSMAN, 1967), shows a genetic similarity of 35% to TNF (LI et al., 1987). The two proteins are closely related in structure and function (PENNICA et al., 1984). Accordingly, both factors were renamed to TNFα and TNFβ, respectively. Then, in 1998, the organizers of the 7$^{th}$ TNF congress (Hyannis, Ma, USA; May 17-21, 1998) suggested name changes from TNFα back to TNF, and TNFβ to lymphotoxin α, respectively (TRACEY et al., 2008), since more proteins, related to lymphotoxin, had been discovered.

With no consensus found in the scientific community both names (TNF and TNFα) of the protein are now commonly found in the literature (TRACEY et al., 2008). In order to be as specific as possible and to exclude any possibility for confusion, the term used in this manuscript is TNFα.

## 1.2. History and Discovery

It is well known that certain immune mechanisms have the capability to inhibit the progression of tumor growth (BEUTLER and CERAMI, 1989). The hemorrhagic necrosis of tumors that can take place in humans and animals, if infected by gram-negative bacteria while also being affected by a sensitive tumor type, is the most prominent example for the influence of the immune system on tumor growth (BEUTLER and CERAMI, 1989). In 1893, Coley, a surgeon from the Memorial Hospital in New York City, published a report of 23 terminally ill human cancer patients that had tumors that were judged to be non-resectable. These patients also had an accidental infection with *Streptococcus pyogenes* in the region of their neoplasia and experienced either complete remission or a reduction in size of their sarcomas or carcinomas (COLEY, 1893). Coley then inoculated 15 other patients concertedly with killed or live bacteria, or with filtrates derived from bacterial cultures. Of these patients some experienced a temporary reduction of tumor size, or even experienced complete remission of their tumors (COLEY, 1893; COLEY, 1906). This procedure had many negative side effects, mainly uncontrolled infections, some of them leading to death, and the clinical results remained inconsistent (JAEAETTELAE, 1991). It is therefore remarkable that the so called “Coley’s toxin”, a bacterial lysate solution made from *Streptococcos pyogenes* and *Serratia marcescens*, remained the only “known systemic method” for the treatment of cancer for many years, as stated by the American Medical Association in 1934 (OLD, 1985). And it was not until radiation therapy and chemotherapy where introduced, that the use of Coley’s toxin diminished (KRIEGLER et al., 1988). However, others kept working on the isolation of the bacterial product that could be held responsible for the phenomenon of tumor necrosis.

Shear and his group purified a factor from gram-negative bacteria that is now known as lipopolysaccharide (LPS), which was found to effectively induce tumor necrosis in certain transplanted tumors in mice (SHEAR, 1944; SHEAR and ANDERVONT, 1936). However, subsequent work led to the discovery that a factor in serum, whose production was stimulated by the presence of LPS, and not the endotoxin itself, induced necrosis and could lead to the

lysis of numerous tumor cell lines *in vitro* (CARSWELL et al., 1975). This factor was therefore named tumor necrosis factor (TNF) (CARSWELL et al., 1975).

With the production of recombinant human TNFα (rhTNFα), intensive studies of the protein's biological functions became possible (JAEAETTELAE, 1991). Interestingly, the protein was revealed to have many more crucial biologic functions than the necrosis of tumor cells, including effects on immunity, inflammation, sepsis, and cachexia (BEUTLER and CERAMI, 1988).

Other groups were working with a supposedly different type of protein. Cerami and Rouzer were the first to observe a marked hypertriglyceridemia in rabbits, infected with *Trypanosoma brucei brucei* (ROUZER and CERAMI, 1980). This parasitic protozoal species causes African trypanosomiasis (also known as "sleeping sickness") in humans and animal trypanosomiasis (also known as "nagana") in vertebrates in Africa (BARRETT et al., 2003). The increase of serum triglyceride concentrations occurred despite a progression of anorexia and wasting. These researchers discovered that a systemic deficiency of lipoprotein lipase (LPL), an enzyme responsible for the hydrolysis of triglycerides, was responsible for this phenomenon (ROUZER and CERAMI, 1980). In further experiments, carried out with endotoxin-sensitive mice, it could then be shown that the same suppression of LPL was present when these animal were challenged with LPS and that endotoxin resistant mouse strains did not show the same reaction (KAWAKAMI and CERAMI, 1981). A 17-kilo Dalton (kDa) protein was then purified and characterized, and owing to its ability to suppress LPL expression named "cachectin" (BEUTLER et al., 1985b).

The N-terminal amino acid sequences of cachectin and human TNFα were found to be highly homologous (AGGARWAL et al., 1985; BEUTLER et al., 1985a; PENNICA et al., 1984). Serological and molecular cloning studies, as well as direct comparison of biological activities ultimately proved that the two molecules were identical (BEUTLER et al., 1985a; CAPUT et al., 1986; PENNICA et al., 1984; SHIRAI et al., 1985; WANG et al., 1985).

# 2. Structure and Function

## 2.1. Molecular Structure of TNFα

TNFα is a member of a large family of cytokines, now known as the "TNF ligand family". With the exception of lymphotoxin (LT), which is secreted solely by lymphocytes and released from the cell surface directly, most members of this protein family, including TNFα, are first produced as a type II transmembrane protein and need to be cleaved by a metalloproteinase before they can be released from the cell surface (PENNICA et al., 1984). LT and TNFα show an amino acid sequence homology of only 35% (AGGARWAL et al., 1985, Li et al., 1987) and share some biological functions, such as cytotoxicity for L292 cells, which were historically used for TNFα bioactivity assays. The molecules also share the ability to cause hemorrhagic necrosis in certain tumor types (GRAY et al., 1984).

### 2.1.1. The Monomer of TNFα

The gene of human TNFα was the first TNFα encoding gene to be cloned (OLD, 1985). Four exons were identified, which code for a precursor protein with a length of 233 amino acids. After cleavage of the proximal sequence, the subsequent protein has a length of 157 amino acids (PENNICA et al., 1984). The TNFα monomer has a molecular weight of 17 kDa. Crystallographic studies have shown that the protein is composed of 2 packed anti-parallel β-pleated sheets, forming a sandwich structure (ECK and SPRANG, 1989). Each of the sheets contains 4 anti-parallel oriented β-strands (BANNER et al., 1993; ECK et al., 1992). The formation of the sandwiches is also called "jellyroll β-structure", because looking at the β-strands and their connections from one to the other, the whole molecular structure actually resembles a jellyroll (Figure 1) with a narrow peak and a slightly wider base (CHELVANAYAGAM et al., 1992). The jellyroll formation designates the TNFα protein as member of the TNF ligand family (BAZAN, 1993).

Two cysteine residues at positions 69 and 101, form a single intra-molecular disulphide bridge. It is also noteworthy that the molecule does not contain any methionine residues (AGGARWAL et al., 1985). The protein also contains 3 α-helices, each with the length of only one single turn at positions 106-110, 138-142, and 145-150 (ECK and SPRANG, 1989).

### 2.1.2. Active Trimeric Form of TNFα

When 3 monomers of TNFα molecules form a trimer, a truncated pyramid with a vertical 3-fold symmetrical axis is generated (ECK and SPRANG, 1989). In the central core of this timeric molecule, hydrophobic interactions connect the monomers. In every monomer the β-pleated sheet of the jellyroll formation orientated toward the protein's center is responsible for the interaction with other monomers, while the outer sheet forms the surface of the TNFα trimer (ECK and SPRANG, 1989). The interaction between the 3 monomers is based on an edge-to-face contact of the inner sheets, where the edge of one monomer's jellyroll is tightly bound to the front-side of its neighbor (JONES et al., 1989).

At the base of each monomer there are two locations which are involved in the formation of the protein binding sites for the TNFα receptors (ECK and SPRANG, 1989). In the biologically active trimeric protein, one of the two locations from each monomer is facing one location at the base of another monomer (ECK and SPRANG, 1989). This way the two locations from two monomers together form a single functional receptor binding side (ECK and SPRANG, 1989). It is therefore suggested that one of the receptor's protein binding sites reacts with two adjacent monomers at the same time, by inter-subunit-binding (ECK and SPRANG, 1989). Conclusively, each trimer contains 3 distinct analogous binding sites, of which each is formed by two adjacent monomers (BANNER et al., 1993).

All studies analyzing the molecular structure of TNFα were performed on human TNFα (SOLLER et al., 2007). However, the high amount of structural conservation and coherent comparable essential properties of the TNFα protein in mammals allows the inferences to other mammalian species (SOLLER et al., 2007).

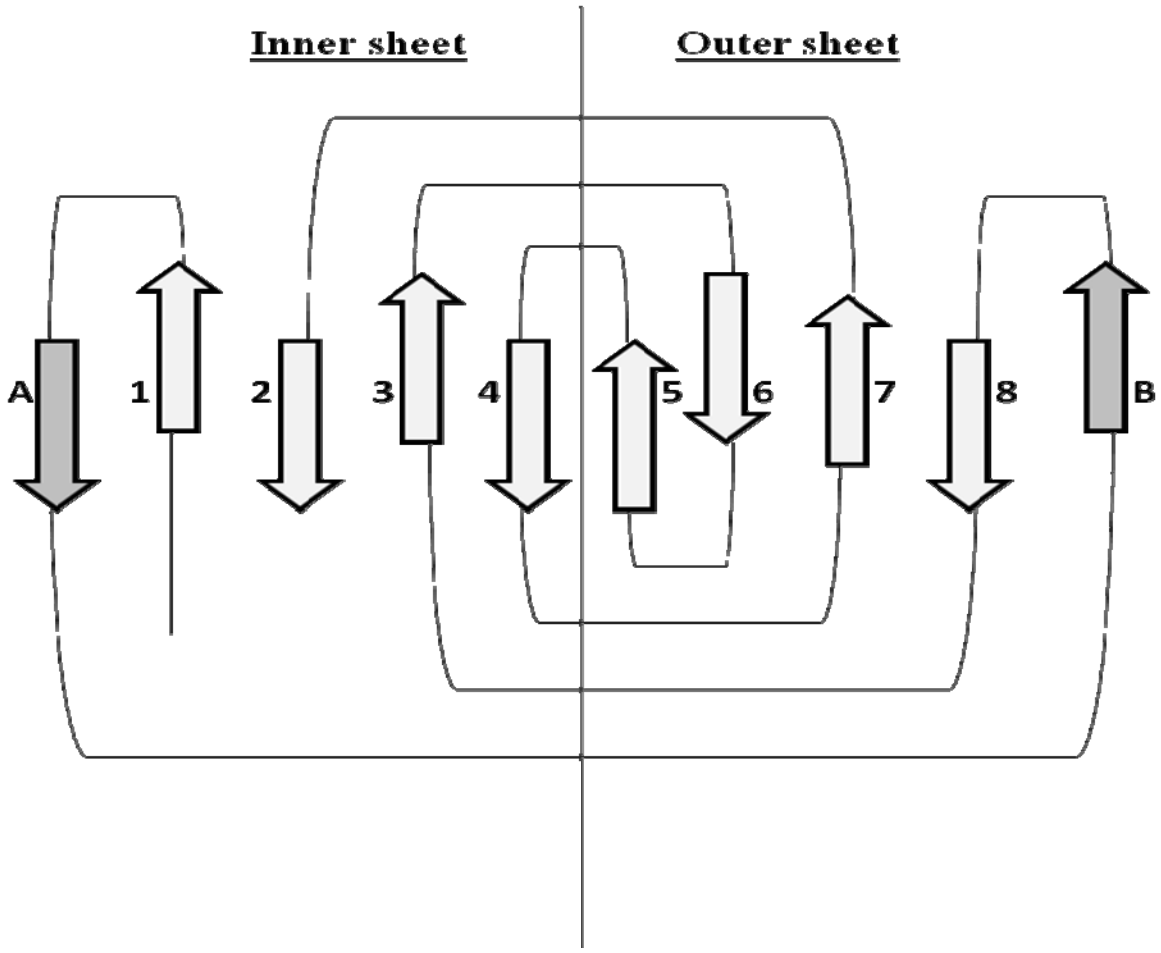


**Figure 1:** Jellyroll formation within the TNFα β-sandwich.

The TNFα monomer contains a β-sandwich structure which is typical for proteins from the TNF ligand family. The so called "jellyroll" core of the β-sandwich is assembled of 2 sheets of 4 anti-parallel oriented β-strands, each (1 to 4, and 5 to 8, respectively). The inner sheet (β-strands 1 to 4), including an additional β-strand (A) serves as the contact surface which allows the formation of a trimeric molecule between 3 monomers. The opposite sheet (formed by β-strands 5 to 8), together with the additional strand (B), forms the outside of the trimeric molecules. This Figure was modified after a pattern published in *The Journal of Biological Chemistry, Vol. 264 (29), M. J. Eck and S. R. Sprang, "The structure of tumor necrosis factor-alpha at 2.6 A resolution. Implications for receptor binding.", pages 17595-605.*

### 2.1.3. Similarities of TNFα between Species

A high genetic conservation of TNFα exists between mammals (Figure 2). In humans the TNFα gene is located on chromosome 6, within the chromosomal segment known as HAS 6p21, a region that contains codes for molecules of the major histocompatibility complex (NEDWIN et al., 1985; SPIES et al., 1986). The gene has a size of approximately 3 kilobases (kb) and consists of 4 encoding exons and 3 introns (NEDWIN et al., 1985). By comparing the available data on TNFα genes from the National Center for Biological Information (NCBI) from different species, including human, canine, murine, porcine, bovine, equine, ovine, rat and feline, Soller et al. found the same genetic composition of the gene (4 exons, 3 introns) in all of these mammalian species (SOLLER et al., 2007).

The similarity and alignment results of the amino acid sequences of the TNFα monomer are shown in table 1 and summarized in table 2. Table 1 is followed by a color code table and another table which explains the one letter code for the amino acids. These tables serve as add-ons to Table 1. The protein sequences were taken from the NCBI database. They were aligned and compared to each other, using a sequence analysis software tool, *ClustalW2*.

The alignment tool *ClustalW2* is publicly available through the following webpage: http://www.ebi.ac.uk/Tools/clustalw2/index.html

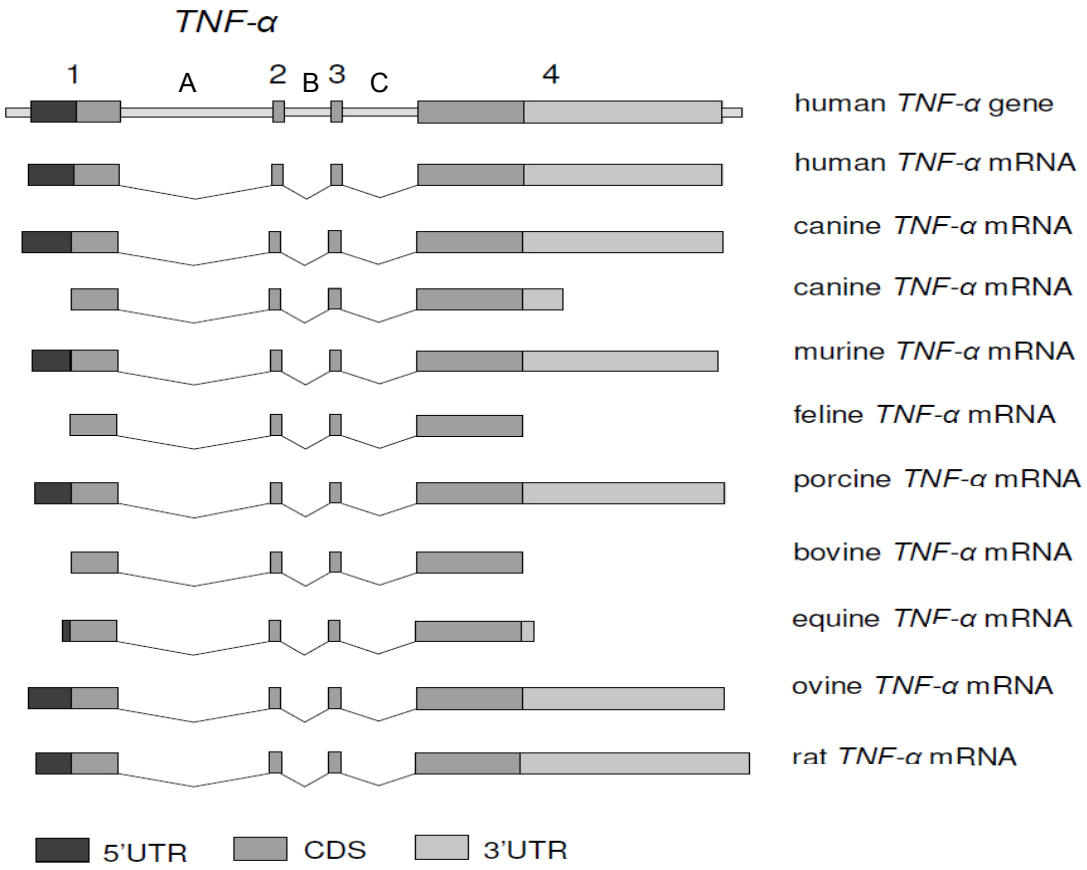


**Figure 2:** Inter-species comparison of TNFα gene transcripts.

The inter-species comparison revealed that the TNFα gene transcripts are highly conserved among different species. All transcripts are composed of 4 exons (labeled 1, 2, 3, and 4) and 3 introns (labeled A, B, C). The feline, bovine, equine, and canine sequences are missing either parts of or the entire 5' untranslated region (5'UTR) or the 3' untranslated region (3'UTR) of the gene. These two regions are part of the exons, but do not contain coding sequences. The fact that they are missing in some sequences analyzed, is most likely due to incomplete characterization of the mRNA transcripts, and does not reflect true biological circumstances. The coding sequences (CDS) are complete in all species evaluated. Human as well as canine mRNA sequences a shown twice in this figure, because the sequences were analyzed and published by two distinct research groups, each. *Reprinted from the Journal of Heredity, Vol 98 (5),J. T. Soller, H. Murua-Escobar, S. Willenbrock, M. Janssen, N. Eberle, J. Bullerdiek and I. Nolte," Comparison of the Human and Canine Cytokines IL-1 (α/β) and TNF-α to Orthologous Other Mammalians", pages 485-490, © 2007, with permission from Oxford Journals.*

**Table 1:** Aligned amino acid sequences of TNFα from 13 different mammalian species.

```
Cat             MSTESMIRDVELAEEALPKKAGGPQGSGRCLCLSLFSFLLVAGATTLFCLLHFGVIGPQR 60
Dog             MSTESMIKDVELAEEPLPKKAGGPPGSRRCFCLSLFSFLLVAGATTLFCLLHFGVIGPPE 60
Human           MSTESMIRDVELAEEALPKKTGGPQGSRRCLFLSLFSFLIVAGATTLFCLLHFGVIGPQR 60
Chimpanzee      MSTESMIRDVELAEEALPKKTGGPQGSRRCLFLSLFSFLIVAGATTLFCLLHFGVIGPQR 60
Cattle          MSTKSMIRDVELAEEVLSEKAGGPQGSRSCLCLSLFSFLLVAGATTLFCLLHFGVIGPQR 60
Sheep           MSTKSMIRDVELAEEVLSNKAGGPQGSRSCWCLSLFSFLLVAGATTLFCLLHFGVIGPQR 60
Pig             MSTESMIRDVELAEEALAKKAGGPQGSRRCLCLSLFSFLLVAGATTLFCLLHFEVIGPQK 60
Horse           MSTESMIRDVELAEEELAKKAGGPQGSRRCLCLSLFSFLLVAGATTLFCLLHFGVIGPQR 60
Rabbit          MSTESMIRDVELAEGPLPKKAGGPQGSKRCLCLSLFSFLLVAGATTLFCLLHFRVIGPQE 60
Mouse           MSTESMIRDVELAEEALPQKMGGFQNSRRCLCLSLFSFLLVAGATTLFCLLNFGVIGPQR 60
Rat             MSTESMIRDVELAEEALPKKMGGLQNSRRCLCLSLFSFLLVAGATTLFCLLNFGVIGPNK 60
Woodchuck       MSTESMIRDVELAEEALPKEAWGPQGSSRCLCLSLFSFLLVAGATTLFCLLHFGVIGPQR 60
Guinea-pig      MSTESMIRDVELAEEQLPKKAGGPQGSRRCWCLSLFSFLLVAGATTLFCLLHFGVIGPQR 60
                ***:***:******  *.::  *  .*  *  *******:***********:* **** .

Cat             EE-LPHGLQLINPLP--QTLRSSSRTPSDKPVAHVVANPEAEGQLQRLSRRANALLANGV 117
Dog             EE-LPNGLQLISPLA--QTVKSSSRTPSDKPVAHVVANPEAEGQLQWLSRRANALLANAV 117
Human           EE-SPRDLSLISPLA--QAVRSSSRTPSDKPVAHVVANPQAEGQLQWLNRRANALLANGV 117
Chimpanzee      EE-FPRDLSLISPLA--QAG-SSSRTPSDKPVAHVVANPQAEGQLQWLNRRANALLANGV 116
Cattle          EEQSPGGPSINSPLV--QTLRSSSQASSNKPVAHVVADINSPGQLRWWDSYANALMANGV 118
Sheep           EEQSPAGPSFNRPLV--QTLRSSSQASNNKPVAHVVANISAPGQLRWGDSYANALMANGV 118
Pig             EE-FPAGPLSINPLA--QGLRSSSQTS-DKPVAHVVANVKAEGQLQWQSGYANALLANGV 116
Horse           EEQLPNAFQSINPLA--QTLRSSSRTPSDKPVAHVVANPQAEGQLQWLSGRANALLANGV 118
Rabbit          EEQSPNNLHLVNPVAQMVTLRSASRALSDKPLAHVVANPQVEGQLQWLSQRANALLANGM 120
Mouse           DEKFPNGLPLISSMAQTLTLRSSSQNSSDKPVAHVVANHQVEEQLEWLSQRANALLANGM 120
Rat             EEKFPNGLPLISSMAQTLTLRSSSQNSSDKPVAHVVANHQAEEQLEWLSQRANALLANGM 120
Woodchuck       EE-FLNNLPLSP-QAQMLTLRSSSQNMNDKPVAHVVAKNEDKEQLVWLSRRANALLANGM 118
Guinea-pig      EEQFSSGPPFRP-LAQTLTLRSASQNDNDKPVAHVVANQQAEEELQWLSKRANALLANGM 119
                :*                  *:*:   :**:*****. .   :*   .  ****:**.:

Cat             ELTDNQLKVPSDGLYLIYSQVLFTGQGCPSTHVLLTHAISRFAVSYQTKVNLLSAIKSPC 177
Dog             ELTDNQLIVPSDGLYLDSSQVLFKGQGCPSTHVLLTHTISRFAVSYQTKVNLLSAIKSPC 177
Human           ELRDNQLVVPSEGLYLIYSQVLFKGQGCPSTHVLLTHTISRIAVSYQTKVNLLSAIKSPC 177
Chimpanzee      ELRDNQLVVPSEGLYLIYSQVLFKGQGCPSTHVLLTHTISRIAVSYQTKVNLLSAIKSPC 176
Cattle          KLEDNQLVVPADGLYLIYSQVLFRGQGCPSTPLFLTHTISRIAVSYQTKVNILSAIKSPC 178
Sheep           ELKDNQLVVPTDGLYLIYSQVLFRGHGCPSTPLFLTHTISRIAVSYQTKVNILSAIKSPC 178
Pig             KLKDNQLVVPTDGLYLIYSQVLFRGQGCPSTNVFLTHTISRIAVSYQTKVNLLSAIKSPC 176
Horse           KLTDNQLVVPLDGLYLIYSQVLFKGQGCPSTHVLLTHTISRLAVSYPSKVNLLSAIKSLA 178
Rabbit          KLTDNQLVVPADGLYLIYSQVLFSGQGCRS-YVLLTHTVSRFAVSYPNKVNLLSAIKSPC 179
Mouse           DLKDNQLVVPADGLYLVYSQVLFKGQGCPD-YVLLTHTVSRFAISYQEKVNLLSAVKSPC 179
Rat             DLKDNQLVVPADGLYLIYSQVLFKGQGCPD-YVLLTHTVSRFAISYQEKVSLLSAIKSPC 179
Woodchuck       ELIDNQLVVPANGLYLVYSQVLFKGQGCPS-YVLLTHTVSRFAVSYQDKVNLLSAIKSPC 177
Guinea-pig      GLSDNQLVVPSDGLYLIYSQVLFKGQGCPS-YLLLTHTVSRLAVSYPEKVNLLSAIKSPC 178
                 * **** ** :****  ***** *:** .  ::***::**:*:**  **.:***:** .

Cat             QRETPEGAEAKPWYEPIYLGGVFQLEKGDRLSTEINLPAYLDFAESGQVYFGIIAL      233
Dog             QRETPEGTEAKPWYEPIYLGGVFQLEKGDRLSAEINLPNYLDFAESGQVYFGIIAL      233
Human           QRETPEGAEAKPWYEPIYLGGVFQLEKGDRLSAEINRPDYLDFAESGQVYFGIIAL      233
Chimpanzee      QRETPEGAEAKPWYEPIYLGGVFQLEKGDRLSAEINRPDYLDFAESGQVYFGIIAL      232
Cattle          HRETPEWAEAKPWYEPIYQGGVFQLEKGDRLSAEINLPDYLDYAESGQVYFGIIAL      234
Sheep           HRETLEGAEAKPWYEPIYQGGVFQLEKGDRLSAEINLPEYLDYAESGQVYFGIIAL      234
Pig             QRETPEGAEAKPWYEPIYLGGVFQLEKDDRLSAEINLPDYLDFAESGQVYFGIIAL      232
Horse           NTESPEQAEAKPWYEPIYLGGVFQLEKGDQLSAEINQPNYLDFAESGQVYFGIIAL      234
Rabbit          HRETPEEAEPMAWYEPIYLGGVFQLEKGDRLSTEVNQPEYLDLAESGQVYFGIIAL      235
Mouse           PKDTPEGAELKPWYEPIYLGGVFQLEKGDQLSAEVNLPKYLDFAESGQVYFGVIAL      235
Rat             PKDTPEGAELKPWYEPMYLGGVFQLEKGDLLSAEVNLPKYLDITESGQVYFGVIAL      235
Woodchuck       PKESLEGAEFKPWYEPIYLGGVFELQKGDRLSAEVNLPSYLDFAESGQVYFGVIAL      233
Guinea-pig      QKETPEGAERKPWYEPIYLGGVFQLQKGDRLSAEVNLPQYLDFADSGQIYFGVIAL      234
                  :: * :*  .****:* ****:*:*.* **:*:* * *** ::***:***:***
```

In this table amino acid sequences of the TNFα proteins from 13 different mammalian species are aligned. Names to the left indicate the species. A number at the end of each row marks the position of the last amino acid of this row in the context of the whole protein. In the process of aligning the sequences, the software automatically shifts the sequence of a species if necessary for a better alignment of the sequences. Gaps resulting from shifting are marked with "–" within the sequences.

Underneath each column consensus symbols are used to demonstrate the degree of similarity observed between the amino acids of species at this exact point of the protein sequence.

Consensus symbols:

"*" = all amino acids in this column are identical in all sequences aligned

":" = conserved substitutions are observed (amino acids are replaced by amino acids with similar chemical properties; as grouped in the color code table below)

"." = semi-conserved substitutions are observed (amino acids are replaced by amino acids with a similar steric conformation, but with different chemical properties)

**Color Code Table:** Different colors of the amino acids in the alignment represent distinct groups of amino acids with specific chemical and steric properties.

| **Amino acids** | **Color** | **Amino acid group represented** |
|---|---|---|
| A, V, F, P, M, I, L, W | Red | small hydrophobic and/or aromatic |
| D, E | Blue | acidic |
| R, K | Magenta | basic |
| S, T, Y, H, C, N, G, Q | Green | hydroxyl , sulfhydryl, amine, and G |

**Letter Code Table:** Every letter in the alignment and the color code tables equals one amino acid, according to the internationally used one-letter-abbreviations for amino acids.

| Letter code | Amino acid | Letter code | Amino acid |
|---|---|---|---|
| **A** | Alanine | **M** | Methionine |
| **C** | Cysteine | **N** | Asparagine |
| **D** | Aspartate | **P** | Proline |
| **E** | Glutamate | **Q** | Glutamine |
| **F** | Phenylalanine | **R** | Arginine |
| **G** | Glycine | **S** | Serine |
| **H** | Histidine | **T** | Threonine |
| **I** | Isoleucine | **V** | Valine |
| **K** | Lysine | **W** | Tryptophan |
| **L** | Leucine | **Y** | Tyrosine |

**Table 2:** Percentages of similarity of amino acid sequences of TNFα between species.

| | Cat | Dog | Human | Chimp. | Pig | Horse | Rabbit | Guinea pig | Mouse | Wood-chuck | Cattle | Sheep | Rat |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Cat** | 100 | 90 | 89 | 89 | 87 | 87 | 81 | 80 | 79 | 79 | 79 | 79 | 78 |
| **Dog** | | 100 | 88 | 88 | 84 | 85 | 79 | 80 | 78 | 76 | 77 | 77 | 78 |
| **Human** | | | 100 | 99 | 86 | 86 | 80 | 80 | 79 | 77 | 80 | 79 | 78 |
| **Chimpanzee** | | | | 100 | 85 | 86 | 79 | 80 | 79 | 78 | 78 | 79 | 79 |
| **Pig** | | | | | 100 | 86 | 76 | 78 | 77 | 77 | 84 | 84 | 78 |
| **Horse** | | | | | | 100 | 82 | 80 | 78 | 77 | 78 | 77 | 77 |
| **Rabbit** | | | | | | | 100 | 82 | 77 | 77 | 75 | 73 | 77 |
| **Guinea pig** | | | | | | | | 100 | 82 | 82 | 74 | 76 | 81 |
| **Mouse** | | | | | | | | | 100 | 81 | 73 | 72 | 94 |
| **Woodchuck** | | | | | | | | | | 100 | 72 | 73 | 80 |
| **Cattle** | | | | | | | | | | | 100 | 92 | 73 |
| **Sheep** | | | | | | | | | | | | 100 | 72 |
| **Rat** | | | | | | | | | | | | | 100 |

All data are expressed as a percentage (%) of homology; Chimp. = Chimpanzee

### 2.1.4. Synthesis

Initially monocytes/macrophages were considered to be the only cells to produce TNFα (MATTHEWS, 1981; OETTGEN et al., 1980). In fact, they are the major source of this cytokine, but other types of immune cells are also capable of producing TNFα, including natural killer cells, and B cells (BECKER et al., 1990), T cells (POLI et al., 1990), mast cells, and granulocytes (JAEAETTELAE, 1991). Non-immune cells, like fibroblasts, neurons, keratinocytes, and smooth and cardiac muscle cells also have this ability (BRADLEY, 2008; JAEAETTELAE, 1991; TRACEY et al., 2008). However, the amount of TNFα produced by cell lines other than macrophages is considered minute regarding to the overall biological effect on the body (JAEAETTELAE, 1991). LPS is the major stimulus for TNFα production by macrophages and augments the TNFα gene transcription in these cells 3-fold, the mRNA content 100-fold, and the amount of assembled and secreted protein 10,000-fold (BEUTLER et al., 1986). However, other stimuli like tumor cell membrane components (HASDAY et al., 1990), parasites (ROUZER and CERAMI, 1980), fungi (DJEU et al., 1988; JOHNSON et al., 2009), certain viruses (ADERKA et al., 1986; TAKANO et al., 2009), and components of the bacterial cell wall other than LPS (BARNES et al., 1990; MICHALEK et al., 1980) also have the ability to either induce or increase the secretion of TNFα.

Cells first produce a 26 kilo Dalton (kDa) precursor protein. Subsequently, 3 of these monomers form one homotrimer, which remains bound to the cell surface as transmembrane TNFα (tmTNFα) (PENNICA et al., 1984). When tmTNFα is cleaved by a matrix metalloproteinase, TNFα-converting enzyme (TACE; also known as ADAM-17), soluble TNFα is released from the cell surface. Soluble TNFα (sTNFα) is a homotrimer consisting of three 17 kDa subunits, and a total molecular mass of approximately 51 kDa (PENNICA et al., 1984). Both forms, monomers and the homotrimers, display biological activity *in vitro*. However, in studies with a binding assay, it has been shown that the trimer is approximately 8-fold more active than the single protein (SMITH and BAGLIONI, 1987) and has the ability to interact with two distinct receptors, TNF receptor 1 and TNF receptor 2. These receptors are expressed by different cell types, demonstrate unequal affinities for TNFα, and differ in their cytoplasmatic structures, as well as in their intracellular signaling mechanisms (TRACEY et al., 2008) (described in detail in chapter "2.1.5. Receptors and Signal Transduction Pathways").

In humans, the soluble protein was found to have a length of 157 amino acids (PENNICA et al., 1984). However, the part of the precursor protein that is cleaved by TACE still has a

length of 76 amino acids, which is unusually long for a pre-sequence, as the average length of pre-sequences is 20 to 30 amino acids (WATSON, 1984). The pre-sequence region shows a higher homology between human and mouse than the secreted protein itself (86% versus 79% homology for precursor and secreted protein, respectively) (MARMENOUT et al., 1985). This high conservation of the pre-sequence points toward a potentially important biological function of this region (MARMENOUT et al., 1985). Kriegler et al. were the first to report a cytotoxic transmembranal function of tmTNFα on the surface of activated monocytes, which is deliverd through the pre-sequence, serving as an intracellular domain of the transmembrane protein (KRIEGLER et al., 1988). It is therefore suspected that tmTNFα and sTNFα may serve distinct biological roles (BRADLEY, 2008).

### 2.1.5. Receptors and Signal Transduction Pathways

The complex pathways of molecular action of TNFα are not fully understood to date. In 2004 Bouwmeester et al. performed a proteomic analysis and composed a map of over 30 known components. The group identified about 220 molecular associations and discovered 80 previously unknown molecules involved in the TNFα-nuclear factor kappa B (TNFα-NFκB) pathway, one of the major pathways of TNFα's action (BOUWMEESTER et al., 2004). Still, many details remain unknown and the existence of several other less important pathways and activated proteins complicate further understanding (BOUWMEESTER et al., 2004).

It has been shown that TNFα affects cells through the interaction with two distinct receptors: TNF receptor 1 (TNFR1; also known as CD120a) and TNF receptor 2 (TNFR2; also known as CD120b). TNFR1 has a molecular weight of approximately 55 kDa and was therefore given the alternative name p55 (BROCKHAUS et al., 1990). This receptor is ubiquitously expressed. TNFR2 has a size of approximately 75 kDa (it is therefore also called p75) (BROCKHAUS et al., 1990; HELLER et al., 1990b). This receptor is mainly expressed on the surface of immune cells and cells of the vascular endothelium. Its expression is tightly regulated (WAJANT et al., 2003). The extracellular domains of both receptors contain 4 repetitive sequences, containing 6 characteristic cysteine-rich motifs each (SMITH et al., 1990). These domains identify them as members of the nerve growth factor/TNF receptor superfamily (SMITH et al., 1990).

One of the most important substances activated by different TNFα-induced cascades in cells is a transcription factor, nuclear factor kappa B (NFκB) (LI and LIN, 2008). This factor induces the expression of genes involved in inflammation and immunity, as well as genes

responsible for apoptotic and anti-apoptotic effects, cell proliferation and stress responds (LI and LIN, 2008).

#### 2.1.5.1. TNFR1

TNFR1 is a type 1 transmembrane protein (CHEN and GOEDDEL, 2002). It is continually synthesized and stored in the Golgi-apparatus of inactive cells, from where it can be quickly released if needed (CHEN and GOEDDEL, 2002). The significance of this pool of proteins in the Golgi-apparatus is not known, but mobilized receptors from the Golgi-apparatus become transmembrane proteins on the cell surface, where they form homotrimers by association of 3 subunits through pre-ligand assembly domains (PLAID) (CHEN and GOEDDEL, 2002). The interaction of TNFα with the TNFR1 initiates the majority of TNFα's biological functions, including pro-inflammatory stimulation and induction of apoptosis, but also initiation of wound healing and angiogenesis. This has been demonstrated by several research groups (CHEN and GOEDDEL, 2002; JOHNSTON et al., 2009; LI and LIN, 2008).

Binding of TNFα to TNFR1 induces the release of an inhibitory protein from the intracellular domain of the receptor-subunits called the silencer of death domain (SODD), which, when attached to the receptor, prevents spontaneous signaling (JIANG et al., 1999). The exposed domains are then recognized by a protein, containing a death domain, the TNFR-associated death domain (TRADD) (HSU et al., 1996). Once activated, this protein recruits 3 more proteins: the Fas-associated death domain (FADD), TNFR associated factor 2 (TRAF2, a E3 ubiquitin ligase, without death domains (HSU et al., 1996)), and receptor interacting protein (RIP), a serine/threonine kinase (HSU et al., 1996; TING et al., 1996). These proteins activate further signaling proteins that belong to 3 different major pathways (CHEN and GOEDDEL, 2002).

One of these pathways is the caspase-8 pathway, which ultimately leads to apoptosis of the cell by cleavage and activation of pro-caspase 3, which further activates caspases 6, 7, and 9 (BOLDIN et al., 1996). All these caspases contribute to the destruction of the cell nucleus and fragmentation of the DNA (SLEE et al., 2001). They also affect mitochondrial processes, which also contributes to cell death (LAKHANI et al., 2006).

Alternatively, TRAF2 and RIP induce pathways leading to the activation of proteins that are members of either the transcription factor activating protein 1 (AP1) or the NFκB protein families (LI and LIN, 2008). Within these cascades, the phosphorylation-dependent ubiquitination and degradation of the inhibitors of NFκB (IκB) takes place (GHOSH and

BALTIMORE, 1990). Briefly, mitogen-activated protein kinase kinase kinases (MAP3K) are recruited by RIP and their activity leads to the phosphorylation of the α- and β-subunits of the IκB kinase (IKK) (GHOSH and BALTIMORE, 1990). Then, RIP and TRAF2, together ubiquitinate the third subunit of the IKK complex, the NFκB essential modulator (NEMO), which completes the activation of the complex. The IKK complex has a kinase activity capable of degrading IκB. IκB in turn suppresses NFκB activity in resting cells (LI and LIN, 2008). Once ubiquitinated and phosphorylated by the IKK complex, IκB molecules degrade and release NFκB (GHOSH and BALTIMORE, 1990). NFκB can then induce gene transcription (Figure 3).

Not every single protein described above is crucial to NFκB activation, and alternative pathways do exist (LI and LIN, 2008). One example for such an alternative pathway is the dispensability of the RIP within the pathway (BLONSKA et al., 2004). It is believed that other members from the protein family of MAP3Ks can be activated alternatively through TNFR1 interaction and fulfill RIP's functions of recruiting and modifying further proteins of the pathway (BLONSKA et al., 2004; BLONSKA et al., 2005).

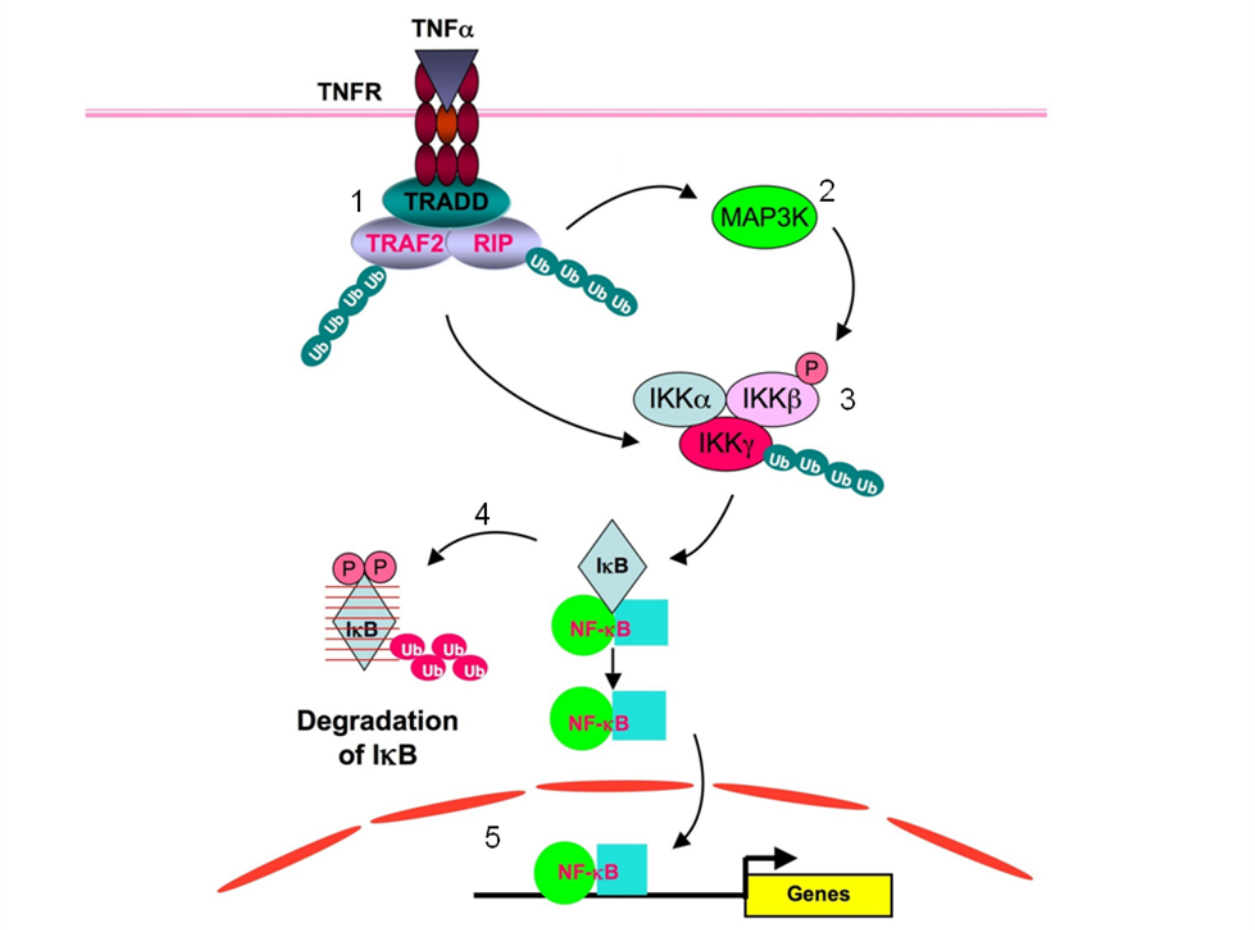


**Figure 3:** Overview of the TNFα induced pathway, leading to NFκB activation.
After dissociation of SODD from the TNFR (not shown), TRAF2 and RIP are recruited by the activated receptor (1). This triggers the ubiquitination of these two proteins. RIP then activates MAP3K proteins (2), which are responsible for the phosphorylation, and therefore activation, of the IKKα and β subunits (3). Also, RIP and TRAF2 cooperate to activate the third subunit of IKK, NEMO (not shown). Now, the ubiquitinated and phosphorylated complex of IKKα/β and NEMO uses its kinase activity to degrade the NFκB suppressing IκB (4). Subsequently, released NFκB activates the transcription of various genes (5). The recruitment and activation of caspase-8 by the FADD protein and the activation of AP-1 by RIP and TRAF2 are not shown. *Reprinted from Cytokine, Vol 41, H. Li and X. Lin, "Positive and negative signaling components involved in TNFα-induced NF-κB activation", pages 1-8, © 2007, with permission from Elsevier.*

#### 2.1.5.2. TNFR2

The more tightly regulated and less frequently expressed TNFR2 and the consequences of its interaction with TNFα have been less well described than those for TNFR1. It is known that the receptor partly fulfills the same biologic functions as does TNFR1, and that it also has specific functions in some tissues, including the promotion of tissue repair and activation of cell migration by chemotaxis in human tonsils (CORCIONE et al., 1997). In experiments with mice, which over-expressed two alleles, coding for human TNFR2, it was demonstrated that this receptor is also involved in the development of chronic inflammation, since the animals studied displayed a severe generalized inflammation, affecting mainly liver, pancreas, lung, and kidneys (DOUNI and KOLLIAS, 1998). TNFR2 does not contain an intracellular death domain, but interacts with the TNFR associated factor 1 (TRAF1) and TRAF2 (ZHAO et al., 2007). These proteins are capable of forming homo- and heterocomplexes, which might be one explanation for the fact that the TNFα receptors share certain functions on one hand and have different ones on the other (ZHAO et al., 2007). Another observation is that interaction between TNFα and TNFR2 can actually suppress receptor signaling, by inducing an ubiquitination-dependent degradation of TRAF2, which terminates the activity of certain MAP3Ks and leads to interruptions in the pathway (ZHAO et al., 2007). Lastly, TNFR2 also activates a TRAF-independent reaction. A cytosolic kinase, the endothelial/epithelial tyrosine kinase (Etk) is associated with TNRF2 in its inactive form (ZHANG et al., 2003). The binding of TNFα is thought to induce unfolding and activation of Etk (ZHANG et al., 2003). This kinase is involved in adhesion, migration, and proliferation processes of epithelial cells (ZHANG et al., 2003).

#### 2.1.5.3. Receptor Cooperation

At low physiological concentrations of TNFα, TNFR2 supports TNFR1 by a mechanism, whose existence was questioned for a long time, known as ligand passing (SLOWIK et al., 1993; TARTAGLIA et al., 1993). TNR2 “traps” TNFα, thus increasing the protein’s concentration on the cell surface (LEEUWENBERG et al., 1995). Then TNFα dissociates and binds with a higher affinity to TNR1 (SLOWIK et al., 1993).

A second receptor-efficacy enhancing mechanism has been observed by which TNFR1 and TNFR2 form ligand-induced heterocomplexes (PINCKARD et al., 1997). This formation is triggered by binding of TNFα to TNFR2. The heterocomplexes only exist temporarily, for approximately 3 minutes, before the receptors dissociate again (PINCKARD et al., 1997).

### 2.1.6. Regulation

On a molecular level the production of TNFα is induced and enhanced by other cytokines, in particular interleukin 1 (IL1), interleukin 2 (IL2), interferon gamma (IFNγ), and granulocyte-macrophage colony-stimulating factor (GM-CSF) (KASID et al., 1990). It has also been shown that the presence of TNFα boosts its own production (KASID et al., 1990). Other influences inducing and enhancing the protein's production are multifaceted and include stimulation by LPS, osmotic stress, and radiation (VAN DEVENTER, 1997).

The expression of TNFα is not only enhanced, but also tightly regulated by suppression, and TNFα is hardly detectable in resting cells (TRACEY et al., 2008). As with other proteins, the presence of mRNA encoding for TNFα does not equal the amount of protein that is truly assembled and secreted from the cells (BEUTLER et al., 1986). IFNγ, for example, is a substance capable of inducing TNFα mRNA production, but its presence alone does not stimulate the biosynthesis of the TNFα protein (COLLART et al., 1986). Instead, further stimuli are required (COLLART et al., 1986). Interleukin 4 (IL4) is a cytokine that has been shown to suppress IL2-driven activation of TNFα mRNA production in the cells as well as protein secretion (KASID et al., 1990). Also, IL10, interferon beta (IFNβ), prostaglandins, and corticosteroids were found to suppress TNFα mRNA production (TRACEY et al., 2008).

It is believed that the regulation of the expression of TNFα mainly takes place at a post-translational level (CAPUT et al., 1986). At the 3'-untranslated region of TNFα mRNA a structural element is found, which contains multiple repeats of the sequence UUAUUUAU (CAPUT et al., 1986). These, so called AU-rich elements (ARE) are also found in other cytokine mRNAs, including the ones coding for IL1, IFNγ, and GM-CSF, and are known to control translation (CAPUT et al., 1986). Furthermore, AREs destabilize the mRNA by serving as a recognition signal (SHAW and KAMEN, 1986). Once recognized by specialized proteins a cascade leading to the degradation of the mRNA, containing this sequence, is activated (SHAW and KAMEN, 1986). This cascade involves an mRNA-specific ribonuclease, the working rate of which depends on the number of AU-sequence copies within an mRNA molecule, and which is essential for post-translational regulation of TNFα (BEUTLER et al., 1988).

Even after TNFα has been secreted from the cells, regulatory mechanisms take action. A soluble protein, which is equal to the extracellular domain of the TNFR2 (HELLER et al., 1990a), and which competes with the cell surface receptors for the binding of TNFα, was found in the urine of healthy individuals (ENGELMANN et al., 1989). A binding protein was

also discovered in the serum of human cancer patients (GASTANAGA et al., 1990). It is also thought to be the soluble equivalent to the extracellular domain of one of the TNF receptors, since antibodies against this protein were also able to compete with TNFα for binding sides at cell-surface receptors (SCHALL et al., 1990). Since proteins like this were not present in the serum of healthy individuals, it was assumed that the shedding of the outer parts of cell surface receptors, and therefore a higher concentration of these binding molecules in serum, could be a mechanism of cell defense against the cytotoxic influence of TNFα during disease processes (SCHALL et al., 1990). The shedding of these outer parts of cell surface receptors is most likely dependent on the systemic concentration of TNFα (SCHALL et al., 1990).
It is also known, that TACE, the same metalloproteinase that is responsible for cleaving tmTNFα into sTNFα and the pre-sequence, also cleaves the extracellular domains of the receptors and is therefore involved in the neutralization of TNFα activity (WANG et al., 2003). Other substances can also induce receptor shedding. Among those are GM-CSF, histamine, and the complement component C5a (PORTEU and NATHAN, 1990; WANG et al., 2003).

### 2.1.7. Cell-Types influenced by TNFα

TNFα affects a wide variety of different cell types (Tab. 3). The protein has many, very different roles, including cell growth, regeneration, cell death and destruction (WAJANT et al., 2003). These different functions are possible, because of the dependance of TNFα activity on tissue type, exact cellular context, and receptor availability, as well as timing and duration of TNFα exposure (WAJANT et al., 2003). At low concentrations the cytokine usually has beneficial effects, including the enhancement of mechanisms of host defense against infections. At high concentrations it may show the opposite effect and lead to excess inflammation and therefore to organ injury (TRACEY et al., 2008).

**Table 3:** Effect of TNFα on a variety of cell types.

| **Tissue/Cell type** | **Effect by TNFα** | **References** |
|---|---|---|
| Macrophages | Maturation and activation | (TRINCHIERI et al., 1986) |
| Neutrophils | Enhancement of phagocytosis<br>Respiratory burst<br>Adhesion to endothelial cells | (KLEBANOFF et al., 1986) |
| Eosinophils | Enhancement of toxicity to certain parasites | (BEUTLER et al., 1985a) |
| T lymphocytes | Enhancement of growth<br>Maturation by induction of IFNγ secretion<br>Expression of IL-2 receptors | (HACKETT et al., 1988)<br>(ABOTT et al., 1981)<br>(SCHEURICH et al., 1987) |
| B lymphocytes | Enhancement of growth and immunoglobulin production | (JELINEK and LIPSKY, 1987) |
| Natural killer cells | Enhancement of cytolytic activity | (OSTENSEN et al., 1987) |
| Endothelial cells | Activation, modulation, expression of adhesion molecules and of MHC I<br>Increased permeability by induction of actin filament clumping and subsequent development of extracellular gaps<br>Procoagulative activity | (SLOWIK et al., 1993) and (POBER et al., 1986)<br>(MARK et al., 2001)<br><br>(BEVILACQUA et al., 1986) |
| Epithelial cells | Induction of barrier impairment in the area of tight junctions | (MIYAUCHI et al., 2008) |

| | | |
|---|---|---|
| Adipocytes | Suppression of morphological maturation and differentiation<br>Suppression of LPL, acetyl-CoA carboxylase, and fatty acid synthetase | (PEKALA et al., 1984)<br><br>(TORTI et al., 1985) |
| Fibroblasts | Stimulation of growth<br>Collagenase and $PGE_2$ secretion | (VILCEK et al., 1986)<br>(DAYER et al., 1985) |
| Muscle cells | Stimulation of glucose-uptake<br>Lowering of the transmembrane potential in skeletal muscle cells<br>Production of $PGE_2$ and interleukins in smooth muscle cells | (LEE et al., 1987)<br>(TRACEY et al., 1986)<br><br>(WARNER and LIBBY, 1989) |
| Hepatocytes | Production of acute phase proteins | (PERLMUTTER et al., 1986) |
| Astrocytes | Proliferation | (LACHMAN et al., 1987) |
| Central nervous system | Pyrogenic effects | (BEUTLER et al., 1985d) |
| Pituitary cells | Stimulation of ACTH, and inhibition of GH and TSH secretion | (MILENKOVIC et al., 1989) |
| Synovial cells | Enhancement of growth<br>Collagenase, gelatinase, stromelysin, and $PGE_2$ secretion | (BUTLER et al., 1988)<br>(DAYER et al., 1985) and (MACNAUL et al., 1990) |
| Osteoclasts | Stimulation and subsequent bone resorption | (BERTOLINI et al., 1986; SAKLATVALA, 1986) |
| Chondroclasts | Resorption of proteoglycan | (SAKLATVALA, 1986) |

## 3. TNFα and Diseases

In healthy individuals TNFα serum concentrations are sparsely detected (KWIATKOWSKI et al., 1990). Increased serum or tissue levels during infection or inflammation correlate well with the severity of an infection in most diseases (KWIATKOWSKI et al., 1990; WAAGE et al., 1987). Due to TNFα's important role in inflammatory responses of the body, the protein is involved in the development of a wide variety of diseases, including sepsis, cancer, type 2 diabetes, osteoporosis, psoriasis, rheumatoid arthritis, and inflammatory bowel disease (CHEN and GOEDDEL, 2002). It is mainly an inappropriate TNFα production or, alternatively, an inappropriate activation of TNFα signaling pathways, which contributes to the development of the diseases (CHEN and GOEDDEL, 2002). This chapter provides an overview of the most relevant diseases that TNFα has been linked to.

### 3.1. Tumors

TNFα is capable of inducing hemorrhagic necrosis in certain tumor types, but also serves as an initiator and promoter of tumor growth in other tumor types, such as epithelial cancers of the skin (MOORE et al., 1999). It is also noteworthy that the action of TNFα is most likely dose-dependent (TRACEY et al., 2008). While chronic exposure to low concentrations of TNFα might promote tumor growth, high doses administered once can lead to tumor destruction (MOORE et al., 1999). However, TNFα is not used as a therapeutic agent for cancer treatment, due to cardiovascular side effects seen at very high dosages (ASHER et al., 1987).

### 3.2. Human Acquired Immunodeficiency Syndrome

An example of a disease that has triggered scientific investigations of TNFα is human acquired immunodeficiency syndrome (AIDS). It had been observed that TNFα concentrations are increased in patients with AIDS (POLI et al., 1990). Furthermore, the concentration of circulating TNFα correlates with disease severity (LAHDEVIRTA et al., 1988). It was also shown that TNFα enhances the replication of the human immunodeficiency virus (HIV) by stimulating the transcription of the viral genes via the NFκB pathway in primary T cell cultures (GRIFFIN et al., 1991; SUZUKI et al., 1989). It is suspected that TNFα plays a major role in the development of AIDS and the opportunistic infections seen with this syndrome. Particularly, those infections which are associated with lymphoid cell

expansion, including the largest cause of death in HIV positive humans: tuberculosis (MATSUYAMA et al., 1991; RANJBAR et al., 2009).

### 3.3. Insulin Resistance in Obesity

Obesity can be defined as a condition in which body fat has excessively accumulated to a point that the individual's health is likely to be adversely affected (KOPELMAN, 2000). It is well known that white adipose tissue (WAT) acts as an endocrinologically active, multifunctional organ (TRAYHURN and WOOD, 2004). WAT produces a wide variety of hormones, chemokines, and some of the "classical" cytokines, like IL1, 6, 8, and 10, transforming growth factor β, and TNFα (TRAYHURN and BEATTIE, 2001; TRAYHURN and WOOD, 2004). Obesity is furthermore associated with an increased risk for the development of type 2 diabetes (insulin resistance) in humans, cats (HOENIG, 2002), and dogs (HOENIG, 2002; LUND et al., 2006). In humans (FLIER, 1995), dogs (GAYET et al., 2004) and rats (HOTAMISLIGIL et al., 1993), increased concentrations of TNFα were linked to the development of insulin resistance. Different effects of TNFα on insulin were described, including the inhibition of insulin signaling (HOTAMISLIGIL, 2000; HOTAMISLIGIL, 2003). Also, when rats were treated with TNFα blocking substances, they required 2 to 3 times more glucose in order to maintain normal blood glucose concentrations because of a significantly higher glucose uptake in response to insulin (HOTAMISLIGIL et al., 1993). This effect on the insulin sensitivity of cells strongly suggests an involvement of TNFα in the development of diabetes mellitus in obese subjects (HOTAMISLIGIL et al., 1993). It has further been shown that weight loss in markedly obese humans (MANCO et al., 2007) and dogs (GERMAN et al., 2009) decreases circulating concentrations of TNFα and other pro-inflammatory cytokines produced by WAT, and therefore reverses the chronic, subclinical inflammation of the obese state.

### 3.4. Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a chronic, immune-mediated, inflammatory disease process (SAKLATVALA, 1986). RA involves synovial tissue and leads to cartilage and bone destruction, by the enhanced resorption and inhibition of proteoglycan synthesis (SAKLATVALA, 1986). TNFα is among the most important cytokines involved in the development of this autoimmune disease, as discussed in depth elsewhere (AREND and DAYER, 1990). The main sources of TNFα in inflamed joints are macrophages and the

concentration of TNFα correlates well with disease severity (TAK et al., 1997). As shown previously in table 3 TNFα enhances the activity of osteoclasts and chondroclasts and thereby contributes to tissue destruction (BERTOLINI et al., 1986; SAKLATVALA, 1986).

### 3.5. Inflammatory Bowel Disease

A wide variety of cytokines are involved in any inflammatory disease process. One of those inflammatory conditions where TNFα plays a role is Inflammatory Bowel Disease (IBD). This disease is thought to be caused by a combination of genetic predisposition as well as immunological and environmental influences (WITTIG and ZEITZ, 2006). In humans two different types of diseases are summarized under the term IBD: Crohn's disease (CD), a multi-focal, transmural, granulomatous inflammation, possibly involving any part of the gastrointestinal tract and ulcerative colitis (UC), where the inflammation is limited to the large bowel and usually involves the superficial layers of the epithelium (PAPADAKIS and TARGAN, 2000).

In CD T1 helper cells are primarily involved, which secrete mainly interferon gamma (IFNγ) and TNFα. This leads to a delayed-type hypersensitivity reaction, including macrophage activation and granuloma formation (SARTOR, 1994). TNFα, together with other cytokines, such as IL1 and IFNγ, up-regulates the expression of adhesion molecules and chemokines in the intestinal epithelium, which in turn attracts inflammatory cells from the blood circulation by chemotaxis and amplifies the ongoing inflammation (PAPADAKIS and TARGAN, 2000). Also, by activation of the NFκB pathway in the macrophages and T cells within the mucosa the transcription of genes of even more pro-inflammatory cytokines, including TNFα itself and IL1, 6, and 12 is promoted (NEURATH et al., 1998). Ma et al. showed that TNFα, in addition to its immune modulating effects, alters the formation of tight junctions between the epithelial cells, which leads to an alteration of the barrier function of the gastrointestinal epithelium (MA et al., 2005). Several investigators have found that TNFα concentrations are significantly increased in serum, stool (BRAEGGER et al., 1992), and within the tissue of the intestinal wall (MURCH et al., 1993) in both, patients with CD and UC. Concentrations correlated well with the disease severity (MURCH et al., 1993).

Diseases closely related to human IBD also exist in other species. One of the animal models of the human disease, for example, is ulcerative colitis in cottontop tamarins. These New-world monkeys show a spontaneously developing ulcerative colitis, which shows many

similarities to human UC, one of which is the increased concentrations of TNFα found in the stool from diseased animals (WATKINS et al., 1997).

Compared to the vast amount of knowledge gained in the field of human IBD, little is known about the development of IBD in dogs (ALLENSPACH, 2007) and cats (JERGENS, 2002). A lymphocytic-plasmacytic enteropathy (LPE) is the most frequently recognized form of IBD in the dog, which is characterized by the infiltration of the lamina propria with lymphocytes and plasma cells in any part of the small or large intestine (HAYDEN and VAN KRUININGEN, 1982). German et al. have identified a cytokine mRNA profile within the mucosa of German Shepherd dogs that suggests a bias towards the T1 helper cell (Th1) controlled immune reaction, similar to the one seen in human CD patients (GERMAN et al., 2000). A newer study could, however, not confirm the finding of a Th1 profile bias (PETERS et al., 2005), and Sauter et al. even noted that the mRNA concentrations showed no significant correlation with actual cytokine concentrations within the mucosa (SAUTER et al., 2006). According to another study, TNFα seems not to be increased systemically during IBD in dogs. This study failed to show increased concentrations of this cytokine in the serum from 16 dogs (MCCANN et al., 2007).

## 3.6. Other Inflammatory Diseases of the Gastrointestinal Tract

### 3.6.1. Gastric Inflammation

In humans an increased production of TNFα within the gastric mucosa appears to be part of the cytokine profile in patients with chronic gastritis (MOSS et al., 1994). Two groups were able to demonstrate increased TNFα concentrations within mucosal biopsies taken from patients with *Helicobacter pylori* associated stomach ulcers (MOSS et al., 1994), and also in the supernatant from *in vitro* cultured human antral mucosal cells (CRABTREE et al., 1991). It was also shown that the concentration of TNFα mRNA is increased in *Helicobacter pylori* negative patients with chronic gastritis (ISHIHARA et al., 1996). In the rat, it was shown, that gastric motility was down-regulated by increased systemic concentrations of TNFα (HERMANN et al., 1999). TNFα overcomes the blood brain barrier via a saturable, specific transport system (GUTIERREZ et al., 1993) and therefore has the ability to centrally influence vagal, descending fibers, involved in the control of gastric motility (HERMANN et al., 1999).

### 3.6.2. Pancreatitis

Several studies have revealed the cytokine profile in serum from human patients with acute pancreatitis, and have reported increased concentrations of TNFα, IL1, and IL6 (BLANCHARD et al., 2000). It was further shown that different cell types of the pancreas are capable to produce and react to TNFα, namely ductal epithelium (BLANCHARD et al., 2000) and acinar cells (GUKOVSKAYA et al., 1997). Using a rat model researchers found a moderate improvement of pancreatitis by neutralizing TNFα, possibly due to the reduction of apoptosis within the organ (GUKOVSKAYA et al., 1997). With the exception of studies in mice and rats (as models for the human disease) and one study in dogs (RUAUX et al., 1999) there has been no further research conducted, regarding the concentrations and involvement of TNFα in acute or chronic pancreatitis in other species. However, Ruaux et al. found a strong correlation between disease severity and TNFα activity when using the bioactivity assay L292 (discussed in detail in the chapter "5.1. Bioactivity assay"). In plasma samples from dogs with severe pancreatitis the activity was the highest. However, protein concentrations measured by a dot-blot immunoassay did not correlate with the severity of disease in the same study population. This result must be interpreted with caution, as an anti-rat TNFα antibody was used for the dot-blot immunoassay, which may not have cross-reacted with canine TNFα and a species-specific antibody may have led to different results (RUAUX et al., 1999).

### 3.6.3. Hepatitis

Not surprisingly, high serum concentrations of TNFα were found in human patients infected with the hepatitis B (HBV) (SHERON et al., 1991) or hepatitis C (HCV) (LARREA et al., 1996). The level of production of TNFα correlated well with serum concentrations of HBV DNA (SHERON et al., 1991). However, a correlation of TNFα activity and hepatic damage during the course of disease could not be demonstrated (HOLTMANN et al., 2002). To the author's knowledge this still holds true to date.

Other liver diseases in humans, including type 1 (classic) autoimmune hepatitis in adults (COOKSON et al., 1999) and primary biliary cirrhosis (GORDON et al., 1999), have been linked to a genetic polymorphism in the promoter region of the TNFα gene. Once again, research in animals is limited, with the exception of rodents.

## 4. TNFα and Cats

Less research has been done in cats than in other species, such as humans or rats. Various studies, however, have evaluated mRNA expression of feline TNFα in whole blood (GELAIN et al., 2006) and more specifically, in monocytes/macrophages in diseased (LIN and BOWMAN, 1993; OTTO and RAWLINGS, 1995) and healthy animals (KIPAR et al., 2001). But as described earlier, the expression of mRNA does not necessarily correlate well with the synthesis of protein (BEUTLER et al., 1986). In 1990, McGraw et al. published the DNA sequence for feline TNFα (MCGRAW et al., 1990) and Rimstad et al. reported the successful production of biologically active recombinant feline TNFα (rfTNFα) in *Escherichia coli*, with the goal to facilitate studies of the role of TNFα in cats infected with feline immunodefiency virus (FIV), aimed at serving as a model for HIV infection (RIMSTAD et al., 1995). Similar to HIV, increased systemic concentrations of TNFα were described for cats with an FIV infection (LAWRENCE et al., 1992; LEHMANN et al., 1992). Further research led to the discovery that high serum concentrations of hTNFα correlated strongly with an enhanced virus replication of the HIV and that the same relationship exists between fTNFα concentrations and FIV expression (KRAUS et al., 1996).
Another disease for which TNFα expression shows similarities between humans and cats is obesity. A higher concentration of fTNFα mRNA was found in the fat tissue of obese cats (HOENIG et al., 2006), compared to lean individuals. Miller et al. found high concentrations of the protein itself within the fat tissue of obese cats (MILLER et al., 1998). Hoenig et al. hypothesized that fTNFα could be involved in the process of redistributing fatty acids from fat to muscles, for either deposit or use as an energy source (HOENIG et al., 2006). It was suggested that fTNFα might suppress the adipocyte lipoprotein lipase in this context.

IBD is among the most common intestinal diseases of the cat (CAVE, 2003). Nguyen et al. demonstrated that the concentration of TNFα mRNA and mRNA from other pro-inflammatory cytokines is significantly increased in intestinal mucosal biopsies from cats with IBD compared to biopsies from animals that showed no clinical signs of a gastrointestinal disease (NGUYEN et al., 2006). Other chronic gastrointestinal diseases and their association with fTNFα have been poorly studied. For example, chronic gastritis is not fully understood, but cases that are not related to parasitic infections, could be part of the IBD complex and may be caused by adverse reactions to bacterial antigens and/or food ingredients (NGUYEN et al., 2006). Chronic gastritis can cause reduced gastric motility (WYSE et al., 2003) and an involvement of TNFα, as shown for rats, can not be excluded.

## 5. Anti-TNFα Therapy

In recent years, anti-TNFα antibodies and soluble receptors, capable of suppressing the protein's cellular action, have proven to be effective in controlling the activity of several chronic inflammatory diseases in humans, including rheumatoid arthritis (SHEALY and VISVANATHAN, 2008) and inflammatory bowel disease (SANDS and KAPLAN, 2007). Most prominent examples of such anti-TNFα agents include Infliximab, Adalimumab, Etanercept, and thalidomide.

Infliximab is an immunoglobulin G (IgG) antibody, genetically engineered from a human constant region and a murine variable region of a monoclonal anti-human TNFα antibody (SHEALY and VISVANATHAN, 2008). Infliximab binds soluble as well as transmembranal TNFα molecules, thereby inhibiting protein-receptor interaction (SHEALY and VISVANATHAN, 2008). With a long half-life of 9.5 days, single injections often help to improve the patients' well being for several weeks. Drawbacks of anti-TNFα therapy include side effects like antibody dependent cytotoxicity and the induction of apoptosis in monocytes (LUGERING et al., 2001). Short term application in humans is considered safe (VULTAGGIO et al., 2008). However, less data exists regarding long term application and its side effects, the therapy is generally considered to be safe and only rarely accompanied by relatively harmless side effects (VULTAGGIO et al., 2008). One concern is the development of antibodies against the chimeric antibodies, potentially leading to the development of autoantibodies, and drug-induced lupus-like diseases (ELEZOGLOU et al., 2007; SANDBORN and HANAUER, 1999). Other anti-TNFα antibodies have been developed, including Adalimumab, a non-chimeric human antibody with a similar clinical profile as Infliximab.

Another anti-TNFα therapeutic, commonly used in humans, is Eternacept, which is a fusion protein between TNFR2 and the constant Fc portion of IgG. Eternacept is only capable of binding soluble TNFα (SANDBORN et al., 2001). This is a possible explanation as to why the product is successfully deployed in patients suffering from rheumatoid arthritis, but remains without effect in patients with IBD (SANDBORN et al., 2001).

Infliximab and Adalimumab, together with Eternacept, are to date the only TNFα antagonists registered in Europe and the United States. But several other antagonists have been developed for the same purpose. Certolizumab for example, which has been registered in the United States since the beginning of 2009, is a fragment of an antibody that has been coupled with polyethylene glycol and mainly displays similar affinity and biologic function as the intact

antibodies (SHEALY and VISVANATHAN, 2008).

A different approach from binding TNFα and reducing its activity, is the use of thalidomide, a drug that has been rediscovered for its ability to effectively suppress inflammation (HOLTMANN et al., 2002). It destabilizes TNFα mRNA, decreasing its half life, in *in vitro* experiments and thereby reducing the synthesis of TNFα in monocytes (MOREIRA et al., 1993).

## 6. Assay Systems for TNFα

### 6.1. Bioactivity Assays

In order to evaluate feline TNFα it was necessary to develop assays specifically for the measurement of this protein in biological samples from cats. One traditional assay system for the measurement of bioactive human and murine TNFα uses the protein's cytotoxic effect on cells of the mouse fibrosarcoma cell line L929 (MEAGER et al., 1989; NARGI and YANG, 1993). In order to sensitize these cells, they had to be pretreated with actinomycin D. The interaction of TNFα with the TNF receptor 1 on the cell surface provokes a cytolytic effect in the cells (NARGI and YANG, 1993). One activity unit of TNFα equals a concentration of 20 pg/ml of the cytokine and is defined as the induction of the half maximum cytotoxicity under predefined, standardized conditions (GRELL and SCHEURICH, 1997). Another commonly used bioactivity assay is based on the murine fibrosarcoma cell line WEHI 164 clone 13. This assay is considered over 100 times more sensitive than the L292 assay (ESPEVIK and NISSEN-MEYER, 1986).

However, both bioactivity assays have disadvantages limiting their usefulness. One is the unpredictability of the influence of other substances, which may affect the cells in a similar fashion to TNFα (MEAGER et al., 1989). For example, Meager et al. (1989) found that lymphotoxin also has a cytotoxic effect on L292 cells. Therefore, the reliability of measurements of TNFα in biological fluids, like serum, using these biological assays is limited. Further, biological assays are incapable of measuring the total amount of TNFα in a sample, because part of the protein might be biologically inactive, since it may be bound by inhibitors, such as free portions of TNFα-receptors (ELBORN et al., 1995). Others have reported further disadvantages, including poor reproducibility due to variation in the cells' responsiveness to the cytokine, possibly as a consequence of repeated cell passages in order to produce more cells for one assay type (MEAGER et al., 1989). The assays are also associated with high costs and a relatively small sample capacity (FOMSGAARD, 1988).

### 6.2. Immunoassays

Immunoassays have several advantages over bioactivity assays, including lower costs, higher reproducibility, higher sample throughput, and an especially high specificity due to the use of monospecific antibodies (ABE et al., 1989; ELBORN et al., 1995).

Two different kinds of immunoassays are commonly used: radioimmunoassays (RIAs) (YALOW and BERSON, 1960) and enzyme-linked immunosorbent assays (ELISAs)

(ENGVALL and PERLMANN, 1971). The RIA is a competitive assay in which radioactively labeled target protein competes with the protein from the sample for the binding to an antigen-specific antibody (YALOW and BERSON, 1960). The higher the protein concentration in the sample, the more antibodies will bind to it, and the weaker the measurable radioactive signal will be.

Direct sandwich ELISAs are usually the more sensitive method to detect an antigen (CROWTHER, 1995). Monospecific antibodies are immobilized at the bottom of the wells of microtiter plates. The protein from the sample then binds to these antibodies and a second antibody is used as a reporter antibody, which binds to the protein. A measurable colorimetric signal is ultimately produced, because the secondary antibodies are coupled with a reporter substance, often biotin, which reacts with other chemicals to produce color (CROWTHER, 1995).

TNFα specific RIAs and ELISAs for different species have been developed, and an overview of some of these assays is given in table 4. It is important to note that a direct comparison of concentrations measured with different immunoassays should not be attempted, since immunoassays are not truly quantitative for the analyte (KREUZER et al., 1996). Instead, the quantity of immunoreactivity is measured, which in turn depends on the labeling of the analyte. Many factors influence the labeling process, including the specificity and affinity of the antibodies, as well the kind of reagents, and the incubation times used. This results in different reference intervals and different results when the same sample is measured in different assay systems (KREUZER et al., 1996).

**Table 4:** List of TNFα immunoassays.

| Assay | Sample type | Species | Detection limit | Reference |
|---|---|---|---|---|
| ELISA (mono) | Plasma | human | 80 pg/ml | (MCLAUGHLIN et al., 1990) |
| ELISA (mono/poly) | Serum | | 39 pg/ml | (SCUDERI et al., 1986) |
| ELISA (mono/poly) | Serum | | 100 pg/ml | (FREEMAN, 1990) |
| ELISA | Serum | | N/A | (MURCH and MACDONALD, 1990) |
| RIA | Serum | | 7 pg/ml | (MAURY, 1989) |
| ELISA (poly) | Serum | | 15 pg/ml | (ABE et al., 1989) |
| RIA | Serum | | 150 pg/ml | (GIRARDIN et al., 1988) |
| ELISA (mono/poly) | Plasma | | 28 pg/ml | (NORMAN et al., 1991) |
| RIA<br>ELISA<br>ELISA | Plasma | | N/A | (PARSONS et al., 1992) |
| RIA | Plasma | | 50 pg/ml | (SUTER et al., 1989) |
| ELISA | Serum/ Plasma | | 0.1 pg/ml | (HEDAYATI et al., 2001) |
| ELISA (mono/poly) | Tissue/ Serum | porcine | 400 pg/ml | (SU et al., 1992) |
| ELISA (mono/poly) | Serum | equine | 100 pg/ml | (SU et al., 1992) |
| ELISA (mono/poly) | Serum | ovine | 0.24 ng/ml | (EGAN et al., 1994) |
| RIA | Plasma | bovine | 4 pg/ assay tube | (KENISON et al., 1990) |
| ELISA (Rat TNFα) | Serum | canine | N/A | (YILMAZ and SENTURK, 2007) |

N/A = not applicable

If known, parentheses behind the word "ELISA" in the first column indicate whether the assay was developed using monoclonal or polyclonal antibodies. Further, sample type used for the analysis, species tested, and the limit of detection for each assay are given, if known.

# III. MATERIALS AND METHODS

## 1. Materials

### 1.1. Chemicals and Reagents

| | |
|---|---|
| 3,3',5,5'-tetramethylbenzidine solution (TMB) | Pierce Chemical CO, Rockford, IL, USA |
| Acetic acid | Sigma Chemicals, St. Louis, MO, USA |
| Ampicillin | Sigma Chemicals, St. Louis, MO, USA |
| BCA™ protein assay kit | Pierce Chemical CO, Rockford, IL, USA |
| Biotin (EZ-Link® Sulfo-NHS-LC-Biotin) | Pierce Chemical CO, Rockford, IL, USA |
| Bovine serum albumin | Sigma Chemicals, St. Louis, MO, USA |
| CAPS | Sigma Chemicals, St. Louis, MO, USA |
| Carbonate-bicarbonate buffer pack (BupH™) | Pierce Chemical CO, Rockford, IL, USA |
| Chloramin T | Sigma Chemicals, St. Louis, MO, USA |
| Coomassie Brilliant Blue G-250 | Pierce Chemical CO, Rockford, IL, USA |
| Dimethyl sulfoxide | Sigma Chemicals, St. Louis, MO, USA |
| EAM solution | BioRad, Hercules, CA, USA |
| Ethanolamine hydrochlorid | Sigma Chemicals, St. Louis, MO, USA |
| Glutathione | Sigma Chemicals, St. Louis, MO, USA |
| Glycine | Sigma Chemicals, St. Louis, MO, USA |
| Hydrochloric acid, 37% | Sigma Chemicals, St. Louis, MO, USA |
| IEF anode buffer | Invitrogen, Carlsbad, CA, USA |
| IEF calibration markers | Invitrogen, Carlsbad, CA, USA |
| IEF cathode buffer | Invitrogen, Carlsbad, CA, USA |
| IEF electrophoresis gel, pH range 3-10 | Invitrogen, Carlsbad, CA, USA |
| IEF sample buffer | Invitrogen, Carlsbad, CA, USA |
| Immuno Pure Streptavidin HRP-conjugated | Pierce Chemical CO, Rockford, IL, USA |
| Isopropyl β-D-1-thiogalactopyranoside | EMD Chemicals Inc., Gibbstown, NJ, USA |
| LB-Agar Medium | MP Biomedicals,LLC, Solo,OH, USA |
| Molecular weight markers – Mark 12 | Invitrogen, Carlsbad, CA, USA |
| NoWeigh™ HABA/Avidin Premix | Pierce Chemical CO, Rockford, IL, USA |
| NuPAGE 10% Bis-Tris gel | Invitrogen, Carlsbad, CA, USA |
| NuPAGE MES SDS running buffer | Invitrogen, Carlsbad, CA, USA |
| NuPAGE sample reducing agent | Invitrogen, Carlsbad, CA, USA |

| | |
|---|---|
| NuPAGE antioxidant | Invitrogen, Carlsbad, CA, USA |
| NuPAGE LDS sample buffer | Invitrogen, Carlsbad, CA, USA |
| p-Aminobenzamidine | Sigma Chemicals, St. Louis, MO, USA |
| Phosphate buffered saline pack (BupH™) | Pierce Chemical CO, Rockford, IL, USA |
| Polyoxyethylene sorbitan monolaurate (Tween™-20) | Pierce Chemical CO, Rockford, IL, USA |
| Precipitating Solution | Siemens, Los Angeles, CA, USA |
| Rabbit carrier solution | Sigma Chemicals, St. Louis, MO, USA |
| Silver stain kit | Pierce Chemical CO, Rockford, IL, USA |
| Sodium azide | Sigma Chemicals, St. Louis, MO, USA |
| Sodium chloride | Sigma Chemicals, St. Louis, MO, USA |
| Sodium citrate | Sigma Chemicals, St. Louis, MO, USA |
| Sodium hydroxide | Sigma Chemicals, St. Louis, MO, USA |
| Sodium metabisulfite | Sigma Chemicals, St. Louis, MO, USA |
| Sodium phosphate, dibasic | Sigma Chemicals, St. Louis, MO, USA |
| Sodium phosphate, monobasic | Sigma Chemicals, St. Louis, MO, USA |
| Sulfuric acid | Sigma Chemicals, St. Louis, MO, USA |
| Superblock®Blocking buffer in PBS | Thermo Scientific, Rockford, IL, USA |
| Terrific Broth (Difco™Terrific Broth) | Becton, Dickinson & Company, Sparks, MD, USA |
| Thrombin | Sigma Chemicals, St. Louis, MO, USA |
| Trichloroacetic acid | Sigma Chemicals, St. Louis, MO, USA |
| Triton x-100 | BioRad, Hercules, CA, USA |
| Trizma™- HCl | Sigma Chemicals, St. Louis, MO, USA |

## 1.2. Instrumentation

| | |
|---|---|
| 2470 Automatic Gamma Counter Wizard$^{2}$$_{TM}$ | Perkin Elmer, Waltham, MA, USA |
| Centrifuge 5810R | Eppendorf, Hamburg, Germany |
| Centrifuge GS6R | Beckman, Palo Alto, CA, USA |
| Centrifuge rotor F 34-6-38 | Eppendorf, Hamburg, Germany |
| Centrifuge rotor GH 3.8 | Beckman, Palo Alto, CA, USA |
| Chromatography column – HiTrap$^{TM}$ Benzamidine FF | GE Healthcare Bio-Sciences AB, Uppsala, Sweden |
| Chromatography column - HiTrap$^{TM}$ NHS-activated HP | GE Healthcare Bio-Sciences AB, Uppsala, Sweden |
| Chromatography column PD-10, Sephadex G-25 M | Amersham Biosciences, Piscataway, NJ, USA |
| Electrophoresis chamber (Novex Mini Cell) | Invitrogen, Carlsbad, CA, USA |
| FPLC ÄKTApurifier | GE Healthcare Bio-Sciences AB, Uppsala, Sweden |
| Fraction collector Frac-900 | GE Healthcare Bio-Sciences AB, Uppsala, Sweden |
| Heating block - Incu BlockTM | Denville Scientific Inc., Metuchen, NJ, USA |
| Hotplate/ Stirrer | VWR, West Chester, PA, USA |
| Incubator/shaker , 311 DS Labnet | Labnet International, Inc., Woodbridge, NJ, USA |
| Multipitte Finnpipette® 300 µl | Thermo Scientific, Rockford, IL, USA |
| Multipitte Finnpipette® Multistepper | Thermo Scientific, Rockford, IL, USA |
| Orbital Shaker | VWR, West Chester, PA, USA |
| pH-meter - model 8010 | VWR, West Chester, PA, USA |
| Pipettors P-10, P-20, P-100 | Rainin, Woburn, MA, USA |
| Pipettors P-250, P-1000, P-5000 | Rainin, Woburn, MA, USA |
| Plate incubator/shaker Stat Fax©-2200 | Awareness Technology Inc., Palm State, FL, USA |

| | |
|---|---|
| PowerEase 500 | Invitrogen, Carlsbad, CA, USA |
| ProteinChip®SELDI System (Personal Edition) | BioRad, Hercules, CA, USA |
| Spectrophotometer, Ultrospec 2000 | GE Healthcare Bio-Sciences AB, Uppsala, Sweden |
| Spectrophotometer, Nanodrop 1000 | Thermo Scientific, Rockford, IL, USA |
| Statistical software package Prism 3.0 | GraphPad Software Inc., San Diego, CA |
| Ultrasonic homogenizer, Model 300 V/T | BioLogics, Inc., Manassas, VA, USA |
| Vortex mixer | Fisher Scientific, Pittsburgh, PA, USA |

### 1.3. Disposables

| | |
|---|---|
| Centrifugal filter devices - Centripep YM-10 | Amicon Bioseparations, Bedford, MA, USA |
| Centrifugal filter devices - Centripep YM-3 | Amicon Bioseparations, Bedford, MA, USA |
| Centrifugal filter devices -Amicon Ultra - 4 5,000 MWCO | Millipore Corporation, Bedford, MA, USA |
| Culture tubes (Polypropylene, 12 x 75 mm) | VWR, West Chester, PA, USA |
| Culture tubes (Polystyrene, 12 x 75 mm) | VWR, West Chester, PA, USA |
| Dialysis cassettes - Slide-A-Lyser 2K | Pierce, Rockford, IL, USA |
| Disposable glass culture tubes (12 x 75 mm) | VWR, West Chester, PA, USA |
| ELISA basins | Fisher Scientific, Pittsburgh, PA, USA |
| Finntip® pipette tips (300 & 1000 µl) | Thermo Scientific, Rockford, IL, USA |
| Glutathione-agarose | Sigma Chemicals, St. Louis, MO, USA |
| Magnetic Stir Bar | VWR, West Chester, PA, USA |
| Membrane filters 0.2 µm - 0.45 µm | Pall - Gelmann Sciences, Ann Arbor, MI, USA |
| Microtiter Assembly (EB, 1 x 8) | Thermo Scientific, Rockford, IL, USA |
| Petri dish (Polystyrene, 100 x 15 mm) | VWR, West Chester, PA, USA |
| Pipettman, manual pipette tips (2, 20, 100, & 200 µl) | Thermo Scientific, Rockford, IL, USA |
| Pleated dialysis tubing - Snakeskin 10k MW | Thermo Scientific, Rockford, IL, USA |
| ProteinChip®NP20 Array | BioRad, Hercules, CA, USA |
| Purelab Ultra (Laboratory water purifier) | ELGA LLC, Woodbridge, NJ, USA |
| Rainin® pipette tips ( 10, 250, & 1000 µl) | Thermo Scientific, Rockford, IL, USA |
| Self-Standing centrifugal tubes, polypropylene, 50 ml | Corning Incorporated, Corning, NJ, USA |
| EZ Flip™ centrifugal tubes, 15 ml | Nalge Nunc International, USA |
| Blue Max™ Jr., conical polypropylene tube, 15 ml | Falcon, Franklin Lakes, NJ, USA |
| ProSorb™, PVDF membrane | Perkin Elmer, Foster City, CA, USA |

## 2. Methods

### 2.1. Overexpression and Purification of Recombinant Feline Tumor Necrosis Factor α

#### 2.1.1. Preparation and Culture of Bacteria

In 1995, Rimstad et al. extracted mRNA, encoding for feline TNFα (fTNFα) from feline macrophage cultures, and synthesized complementary DNA for amplification by polymerase chain reaction (PCR) (RIMSTAD et al., 1995). Complementary DNA (cDNA) is DNA, which does not include the introns of the original gene sequences. The template for its synthesis is the intron-free mRNA. TNFα mRNA codes for four exons for the cytokine (described in detail in chapter "2.1.3. Inter-Species Similarities" in part "II. Literature review"). The mRNA for the 4 exons is linked in direct sequence following cleavage of the 3 introns. Subsequent to the isolation of mRNA, cDNA was amplified by PCR. The PCR products were then cloned into the plasmid vector pGEX-2T (Pharmacia, Uppsala, Sweden) as described elsewhere (RIMSTAD et al., 1995). The pGEX-2T vector is a plasmid vector that has previously been described to be functional (REID et al., 1991). The plasmid DNA was then used to transfect the *Escherichia coli* strain XL1-blue. As a result, these bacteria produced a fusion protein composed of recombinant feline tumor necrosis factor α (rfTNFα) and the affinity tag Glutathione-S-transferase (GST). A stock of transformed bacteria was generously provided to the Gastrointestinal Laboratory by Espen Rimstad et al., Department of Pharmacology, Microbiology and Food Hygiene, Norwegian College of Veterinary Medicine, Olso, Norway.

The stock of bacterial cells was kept frozen at -80°C. Bacteria were separated by scraping off material from the surface of the frozen batch, using a sterile inoculation loop. The collected bacteria were then streaked onto an agar-plate containing Luria-Bertani (also known as lysogeny broth or LB) medium, which contained ampicillin in a concentration of 75 mg/l. The plate was incubated at 37°C for 12 hours, allowing bacterial colonies to grow. Subsequently, the plate was stored at 4°C until further use. Bacterial colonies stored under refrigeration kept their ability to proliferate and could be utilized for up to two weeks. A single bacterial colony was picked by use of a sterile inoculation loop, and was transferred to a 500 ml glass flask containing 100 ml of terrific broth (TB) and ampicillin (50 mg/l TB). Both, the glass flask and the TB were autoclaved beforehand and ampicillin was added after autoclaving. This starter culture was then incubated at 37°C, again for 12 hours. Continuous shaking at 250 rounds per minute (RPM) ensured aerobic conditions, which are necessary for

growth of the bacteria. A 25 ml aliquot of the starter culture was subsequently added to 225 ml TB containing 50 mg/l ampicillin. This procedure was repeated 3 times. The 4 bacterial subcultures were then inoculated at 37°C for 4 to 6 hours. The distribution of the starter culture to 4 subcultures was necessary in order to provide the bacteria with enough nutrients and "quorum sensing" to continue their growth phase. The absorbance of the cultures was controlled hourly during the incubation by the use of a spectrophotometer at a wavelength of 280 nanometer (nm) and TB/ampicillin medium was used as a blank. An exponential increase in bacterial numbers was observed over a period of 4 to 5 hours. Once a plateau in growth was reached, the bacterial over-expression of the GST-rfTNFα fusion protein was induced by adding 75 µl of 1 M Isopropyl β-D-1-thiogalactopyranoside (IPTG) to each of the four 250 ml cultures (final IPTG concentration: 0.3 mM). The cultures were subsequently incubated at 30°C for 12 hours while continuously shaking at 180 RPM. Thereafter, the bacteria-containing broth was transferred into 50 ml polypropylene tubes and was centrifuged at 4000 RPM at 4°C for 15 minutes. After centrifugation, the supernatant was carefully discarded. The pellets containing the bacteria were combined into a single propylene tube and centrifuged again at 4000 RPM and 4°C for 40 min, after which the clear supernatant was discarded. The pellet containing the bacteria was stored at -80°C until further use.

### 2.1.2. Bacterial Cell Lysis

The frozen bacterial pellet was allowed to thaw slowly for 30 min on ice. Then, 15 ml of 1X phosphate buffered saline solution (PBS; 140 mM NaCl, 2.7 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$, pH 7.2) were added. The tube was gently inverted until the pellet was completely thawed and suspended in the buffer. Then, the suspension was sonicated in order to lyse the bacterial cells. To avoid overheating of the solution and denaturation of proteins, a 50 percent (%) intensity (or 150 Watts) of the sonicator were not exceeded and the sample was kept on ice during the entire process. The sonication was repeated 5 times for 15 seconds each. Subsequently, Triton X-100, a nonionic detergent, was added to the solution with a final concentration of 1% volume to volume (v/v). The mixture was gently inverted and incubated on ice for 30 minutes, followed by centrifugation at 10,000 × g for 10 min to pellet cell debris. The supernatant containing the fusion protein was transferred to a new polypropylene tube and centrifuged again at 12,000 × g for 50 min, thus yielding an even purer supernatant. Supernatant and pellet were separated and the supernatant was filtered through a 0.45 µm-pore size filter and subsequently through a filter with a pore size of 0.2 µm.

### 2.1.3. Cleavage of the Fusion Protein

Three self-packed columns (8 × 40 mm) of 10 ml bed volume each and equipped with filter frits, were packed with glutathione-agarose beads. The beads were prepared according to the manufacturers' instructions. Briefly, for each 15 ml of fusion protein containing solution, which originated from 1 liter of the original bacterial culture, 140 mg of lyophilized bead powder were used. After equilibrating the columns with 5 column volumes (cv) of equilibration buffer (PBS), the protein solution, containing the fusion protein as well as other bacterial products, was subdivided into three aliquots that were loaded onto the 3 columns. The columns were then sealed and incubated at 4°C, while slowly (approximately 30 times per minute) being inverted on a plate rocker for 12 hours. This procedure allowed the agarose bead-bound glutathione to bind the GST part of the fusion protein. After incubation, the supernatant was eluted by gravity in 1 ml fractions and evaluated by sodium-dodecyl-sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). If the supernatant was free from fusion protein it was discarded.

The resin was washed with 5 cv of a 1X PBS-Triton X-100 buffer (Triton x-100: 1% v/v) followed by a PBS buffer to wash out the Trition X-100, which interferes with spectrophotometric measurements. The wash steps were repeated until the absorbance of the eluent, measured at 280 nm, approached zero. Thereafter, 10 µl of bovine thrombin (1 activity unit/µl) were added to each column in order to cleave the rfTNFα portion from the bead-bound GST portion of the fusion protein. Theoretically, one unit of thrombin can cleave 100 µg of protein. Therefore, the usage of 10 µl (i.e., 10 activity units) guarantees the availability of sufficient amounts of active enzyme in each column. Additionally, 3 ml of PBS were loaded onto each column to resuspend the resin. To allow thrombin cleavage, the columns were then incubated at 4°C for 4 hours while constantly inverting the columns slowly on a plate rocker. Then, PBS was loaded onto the column in order to elute rfTNFα and thrombin. The resin was then washed with PBS and fractions were collected until the absorbance measured at 280 nm returned to zero. The GST part of the fusion protein was eluted from the column by incubation in elution buffer (7 mM glutathione, 50 mM Tris-HCl, pH 8.0) at 4°C for 12 hours. After a cleansing procedure according to the manufacturer's instructions, the beads could be re-used up to 4 times, without a marked loss of binding capacity. The columns were stored at 4°C in storage buffer (2 M NaCl, 1 mM sodium azide, pH 7.0) in between uses.

### 2.1.4. Removal of Thrombin by HiTrap™ Benzamidin FF Chromatography

To remove thrombin from the otherwise pure rfTNFα protein solution, the buffer of the protein solution was exchanged to a binding buffer (50 mM Tris-HCl, 0.5 M NaCl, pH 7.4) by cartridge dialysis. After equilibrating the column according to the manufacturer's instruction, the protein solution was loaded onto the HiTrap$^{TM}$Benzamidine FF column (1.0 ml/min) by use of a 3 ml syringe. This column specifically binds trypsin-like serine proteases such as thrombin. The eluent containing the pure rfTNFα protein and was collected in 1 ml fractions. Before assessing the overall success of the purification by SDS-PAGE, the buffer was once again exchanged, this time to PBS. The pure rfTNFα solution was stored at -80°C, and remained stable for at least 4 months. Thrombin was eluted from the column by applying 1.0 ml/min of elution buffer (20 mM p-aminobenzamidine in binding buffer). The column remained re-usable for up to 10 purifications.

### 2.1.5. Sodium-Dodecyl-Sulfate-Polyacrylamide Gel Electrophoresis

The purity of the obtained protein was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. Precast, 10% polyacrylamide vertical gels with 1 mm gel thickness were used in accordance with the manufacturer's instructions. The gel cassette was inserted into the electrophoresis chamber and a wedge piece forced all the components tightly together. The inner and outer chambers were filled with MES running buffer (1 M 2-[N-morpholino] ethanesulfonic acid, 1 M Tris-base, 70 mM SDS, 21 mM EDTA, pH 7.3). Fivehundred µl of NuPAGE® antioxidant were added to the inner chamber. The samples were prepared by adding 6.5 µl of sample (ideal protein concentration of approximately 0.03 mg/ml) to 1 µl NuPAGE® sample reducing agent and 2.5 µl of NuPAGE® LDS sample buffer (4 g sucrose, 0.68 g Tris-base, 0.67 g Tris-HCl, 0.8 g SDS, 0.006 g EDTA, 0.75 ml Serva blue G250, 0.25 ml phenol red, and ultra pure water added for a total volume of 10 ml). Samples with a concentration greater than 0.03 mg/ml were loaded onto the gel after dilution with distilled and degassed water ($ddH_2O$) to approximately 0.03 mg/ml. The sample-buffer mixtures were incubated at 68°C for 7 min in order to denaturate the proteins. Ten µl of each sample-buffer mixture were subsequently loaded into the wells of the gel. Ten µl of a standard protein solution were loaded into the first well. The standard protein solution contained 12 different proteins with a known molecular mass: myosin (200.0 kDa), β-galactosidase (116.3 kDa), phosphorylase (97.4 kDa), bovine serum albumin (66.3 kDa), glutamic dehydrogenase (55.4 kDa), lactate

dehydrogenase (36.5 kDa), carbonic anhydrase (31.0 kDa), trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa), aprotinin (6 kDa), insulin B chain (3.5 kDa), and insulin A chain (2.5 kDa). The electrophoresis was performed at a constant voltage of 200 V for 35 minutes at room temperature. Immediately after the electrophoresis, the gel was removed from the cassette, washed and stained with a silver stain kit (GelCode® SilverSNAP™ Stain Kit II) according to the manufacturer's instructions.

Briefly, the gel was fixated by incubating it in 30% ethanol/ 10% acetic acid solution for 15 min and subsequently in 10% ethanol for 5 min at room temperature. It was then washed by incubating it in $ddH_20$ for 5 min. Next, the gel was sensitized by placing it in sensitizer solution (50 µl of the sensitizer solution from the kit were added to 25 ml $ddH_20$) for 1 min. Subsequently, the gel was washed by incubating it in $ddH_20$ for 2 min and exchanging the water after 1 min. The gel was then stained with a staining solution (0.5 ml of the enhancer solution from the kit were added to 25 ml of stain from the kit) for 30 min. After washing the gel by rinsing it carefully with $ddH_20$ for 20 s, the gel was placed in the developer working solution (0.5 ml of enhancer solution were added to 25 ml of developer solution). The development took between 2 to 3 min, and was stopped after protein-bands became visible. As a stopping solution 5% acetic acid was used and the gel was incubated in the solution for 10 min in order to completely stop the progression of the development.

## 2.2. Partial Characterization of Recombinant Feline Tumor Necrosis Factor α

### 2.2.1. Determination of the Specific Absorbance

The specific absorbance of rfTNFα at a wavelength of 280 nm was determined based on the total amount of protein in solution as estimated by the BCA™ protein assay. The bicinchoninic acid (BCA) protein assay represents a colorimetric method, which is based on a shift in absorbance from 465 nm to 595 nm, as the dye Coomassie Brilliant Blue G-250 forms complexes with proteins in acidic solution. Thus, the amount of dye-protein complexes reflects the protein concentration of the solution, and is estimated by measuring the absorbance of the sample at 562 nm.

The assay was performed using a microplate. Eight standard solutions were prepared, using a concentrated solution (2 mg/ml) of pure bovine serum albumin (BSA). The standards prepared in PBS ranged from 2000 µg/ml to 25 µg/ml of BSA (25, 125, 250, 500, 750, 1000, and 2000 µg/ml). Purified rfTNFα, with a known spectrophotometric absorbance at 280 nm, was serially diluted in PBS, using dilutions from 1 in 2 to 1 in 16. The absorbance of the

dilutions was measured at 280 nm and the exact values were noted. The assay reagent was produced by agitating 50 volume parts of BCA™ reagent "A" ($Na_2CO_3$, $NaHCO_3$, bicinchoninic acid, and $Na_2C_4H_4O_6$ in 0.1 M NaOH (molarities not given by manufacturer)) with 1 part of BCA™ reagent "B" (4% cupric sulfate). Subsequently, 15 µl of each standard were loaded into the microplate wells in duplicates. Also, 15 µl of the protein dilutions, as well as pure PBS, serving as a blank (non-specific binding) were loaded into the microplate wells in duplicates. Then, 200 µl of the BCA™ reagent (mixture of reagents "A" and "B") were added to each well. After incubation for 30 minutes at 37ºC the absorbance was measured at 562 nm using an automated plate reader. The absorbance of standards and samples was corrected by subtracting the mean absorbance of the blank wells from the absorbance of all other wells. A standard curve was generated by plotting the mean absorbance of the duplicates for each standard against the respective concentration in µg/ml. For the curve fit a 3$^{rd}$ degree polynomial regression analysis was performed and the protein concentration of each unknown sample was interpolated from the standard curve.
To calculate the specific absorbance, the absorbance of the pure rfTNFα solution measured at 280 nm was divided by the protein concentration determined by the BCA™ protein assay. The specific absorbance was determined for each of the 4 dilutions of rfTNFα (1 in 2, 4, 8, and 16) and then the average value was calculated. This value equals the specific absorbance of rfTNFα.

### 2.2.2. Estimation of Relative Molecular Mass by SELDI-TOF-MS

The molecular weight of rfTNFα was estimated by the Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS). This method represents the combination of retentate chromatography and mass spectrometry. The ProteinChip®NP20 Array (NP = Normal Phase) was chosen for the size analysis of rfTNFα. The silicon dioxide ($SiO_2$) surface groups of the chip allow water-soluble proteins, such as rfTNFα, to bind to the surface through hydrophilic and charged amino acids, including serine, threonine, and lysine, on the protein surface. The NP20 array is relatively non-selective and the protein does therefore not need to be exchanged to a specific binding buffer prior to the analysis.
A predefined spot on the NP20 ProteinChip® was primed by adding 2 consecutive volumes of 5 µl of dd$H_2O$. After each load of water the spot was allowed to air-dry for 5 minutes at room temperature. Remaining water droplets were carefully removed using absorbent paper,

without contacting the chip surface. Then, 5 µg of rfTNFα in 5 µl PBS buffer (1mg/ml) were applied and incubated on the spot and the spot was then allowed to air-dry for 10 min at room temperature. The spot was subsequently rinsed by applying 5 µl of $ddH_2O$ and carefully shaking the chip for 30 s. The water was then carefully removed using absorbent paper. This procedure was repeated twice. The spot was then allowed to air-dry for 15 min at room temperature. The washing process ensured the formation of a homogenous layer of co-crystallized proteins and residues of the chip surface ($SiO_2$). Then, 0.5 µl of a saturated solution of ProteinChip® energy absorbing molecules (EAM; 50% satured sinapinic acid (3,5-dimethozy-4-hydroxycinnamic acid), in 50% acetonitrile and 0.5% trifluorioacetic acid; ProteinChip® SELDI Starter Kit, BioRad) were applied onto the spot twice and the spot was allowed to air-dry at room temperature for 5 min after each step.

Hereafter, the sample was analyzed using the SELDI-TOF-MS ProteinChip® Reader. For analysis a laser power of 2000 nano Joules (nJ) was used and the mass-to-charge ratio (m/z) range was set to 0 to 20,000. The focus mass was set to 17,000 m/z, as this equaled the approximate size (17 kDa) at which we expected to find rfTNFα. The run was normalized for the factor 1. Normalization is the process of linearity scaling of the intensities of a set of spectra to account for spectrum-to-spectrum variations due to differing amounts of overall protein sample, degradation over time, or instrument variation. It makes measurements of different runs comparable to each other. Calibration of the machine by using standards made of bovine insulin is part of the routine maintenance of the machine, but was not performed specifically before the analysis of the rfTNFα sample.

### 2.2.3. Preparation for N-terminal Amino Acid Sequencing

Using the method of electro-transfer, 50 µg of purified rfTNFα were transferred onto a polyvinyliden-difluoride (PVDF) membrane with a pore size of 0.45 µm. A mini-gel blotting sandwich cassette was used to perform the transfer. Briefly, an SDS-PAGE was run under the conditions described in detail in chapter 2.1.5 (Sodium-Dodecyl-Sulfate-Polyacrylamide Gel Electrophoresis). The gel was not stained, but only carefully rinsed with $ddH_2O$ and then equilibrated in transfer buffer (10 mM N-cyclohexyl-3-aminopropanesulfonic acid (CAPS) with 10 v/v % methanol, pH 11) for 30 min. The PVDF membrane was pre-wetted by immersion in 100% methanol for 30 s, followed by equilibration in transfer buffer for 15 min. After carefully inserting the gel and the PVDF membrane into a blotting cassette according to the maufacturer's instructions, a constant electric current of 100 milli amperes was applied

over a time period of 2 hours at room temperature (approximately 22-25 $C^o$). The membrane was subsequently stained using Coomassie blue stain in order to localize the protein bands on the membrane. Protein-containing bands were cut out of the membrane using a razor blade. The amino acid sequence analysis of the first 11 N-terminal amino acids of the rfTNFα molecule was performed using the Edman degradation method at the Protein Chemistry Laboratory, Department of Biochemistry and Biophysics at Texas A&M University under the direction of Dr. Lawrence J. Dangott.

The analysis of 11 amino acids was sufficient for the identification of feline TNFα and the sequence obtained was compared to the published protein sequence available through the NCBI database (MCGRAW et al. 1990).

### 2.2.4. Estimation of the Isoelectric Point

The isoelectric point was estimated by polyacrylamide gel electrophoresis using a NOVEX® Pre-Cast vertical IEF gel (5% polyacrylamide, 2% ampholytes, pH range: 3-10). A mixture of proteins with known isoelectric points (pI) was used to generate a standard curve. The protein mixture contained 9 proteins: cytochrome C (horse, heart; pI: 10.7), ribonuclease A (bovine, pancreas; pI: 9.5), lectin (Lens culinaris; pIs: 8.3, 8.0, and 7.8), myoglobin (horse, muscle; pIs: 7.4 and 6.9), carbonic anhydrase (bovine, erythrocytes; pI: 6.0), β-lactoglobulin (bovine, milk; pIs: 5.3 and 5.2), trypsin inhibitor (soybean; pI: 4.5), glucose oxidase (*Aspergillus niger*; pI: 4.2), and amyloglucosidase (*Aspergillus niger*; pI: 3.5).
The sample was prepared by thoroughly mixing one part of sample with one part of NOVEX® IEF sample buffer. One part of NOVEX® IEF cathode buffer was added to nine parts of $ddH_2O$ in order to produce the buffer for the cathode and this buffer was filled into the upper buffer chamber. The anode buffer was prepared by adding one part of NOVEX® IEF anode buffer to 49 parts of $ddH_2O$. This solution was filled into the lower buffer chamber. Samples were loaded into the wells of the gel and a constant voltage of 100 V was applied for 1 hour, followed by a constant voltage of 200 V for another hour, and a constant voltage of 500 V for 30 min at room temperature. Thereafter, the gel was incubated in a fixation solution (17.3 g sulphosalicylic acid, 57.3 g trichloroacetic acid, in 500 ml of $ddH_2O$) for 30 minutes. The gel was stained using a silver stain kit (GelCode® SilverSNAP™ Stain Kit II) according to the manufacturer's instructions. A standard curve was generated by measuring the migration distance from the well to the protein bands of the markers.

## 2.3. Production of anti-rfTNFα Antiserum

The production of anti-rfTNFα antiserum was performed by Lampire Biological Laboratories, Pipersville, Pennsylvania, USA. Briefly, two New Zealand White rabbits (*Oryctolagus cuniculus*) were injected subcutanously with 200 µg of rfTNFα protein in 0.5 ml of PBS, mixed with 0.5 ml complete Freund's adjuvant. To booster the immune-response to the antigen, the animals were re-inoculated with 150 µg of rfTNFα in 0.5 ml of PBS mixed with 0.5 ml of incomplete Freund's adjuvant on days 21 and 42. One of the animals received a third injection of 150 µg of rfTNFα in 0.5 ml of PBS mixed with 0.5 ml of incomplete Freund's adjuvant on day 63. Blood samples were collected 10 days after each injection, and a serum titer was estimated by radioimmunoassay to assess the production of antibodies against the injected protein. The animals were exsanguinated after a total of 3 and 4 injections, respectively. The sera were used for the development of an immunoassay.

## 2.4. Production of Tracer for Radioimmunoassay

To determine the titer of anti-rfTNFα antibodies in the rabbits, pure rfTNFα was radio-labeled with $^{125}I$ using the chloramine-T method following the method first described by Hunter (HUNTER and GREENWOOD, 1962). Briefly, a 5 ml polystyrene tube was placed above a stir plate and a miniature magnet was inserted. Then, 10 µl of a 0.25 M sodium phosphate solution, pH 7.5 and 10 µl of $^{125}I$ (approximately 0.1 mCi/µl at the time of production) were added. The mixture was allowed to incubate for 1 min at room temperature. Then, the following was added in fast succession: 10 µl rfTNFα in PBS with a concentration of 1 mg/ml, 10 µl chloramine-T solution (2 mg/ml chloramine-T in 0.05 M sodium phosphate buffer), 100 µl sodium metabisulfite solution (0.4 mg/ml $Na_2S_2O_5$ in 0.05 M sodium phosphate buffer), and 860 µl potassium iodide solution (2 mg/ml KI in 0.05 M sodium phosphate buffer). Ten µl of this mixture were subsequently pipetted into 2 polypropylene tubes, each. This solution was used to measure the total radioactivity. To separate free $^{125}I$ from the iodinated protein, the remaining 980 µl of the solution were loaded onto a disposable PD-10 desalting column (Sephadex™ G-25M; 14.5×50 mm). In order to prevent unspecific protein binding to the dextran resin of the column, the column was equilibrated with 5 cv of a concentrated BSA solution (50 mM monobasic sodium phosphate solution and 50 mM dibasic sodium phosphate solution were mixed to reach a pH of 7.5, then 100 g/l BSA were added) beforehand. The eluent of the 980 µl of solution loaded onto the column was collected in a polystyrene tube. Then, 1 ml of RIA buffer (0.05 M sodium phosphate solution,

0.2 g/l $NaN_3$, 5 g/l BSA, pH 7.5) was applied onto the column and the eluent was collected in a polystyrene tube. This procedure was repeated 14 times and the single fractions were numbered 2 to 15. Ten µl of each solution, as well as 10 µl from a mixture of 10 µl of the unfiltered isotope solution, diluted with 990 µl of RIA buffer, were used to determine the total radioactivity applied and the radioactivity of each fraction. The radioactivity was recorded in counts per minute (CPM).

Fractions 4 and 5, as well as fractions 10 to 13 showed the highest radioactivity, based on the CPM measured. Since the iodinated protein has a higher molecular weight than free $^{125}I$ and because molecules of a higher molecular mass pass through a gel chromatography column faster than low molecular mass molecules we concluded that the iodinated protein eluted in the earlier fractions. Fraction 4 was chosen for the production of the tracer. Using RIA buffer, fraction 4 was diluted to approximately 40,000 CPM per 100 µl.

## 2.5. Antibody Titer Analysis by Radioimmunoassay

To estimate the antibody titer in the sera of the two rabbits, a titer RIA was performed. Twenty µl of serum were diluted in 180 µl of RIA buffer (0.25 M sodium phosphate solution, 0.2 g/l $NaN_3$, 5 g/l BSA, pH 7.5), and 20 µl of this dilution were further diluted in 980 µl of RIA buffer. This 1 in 500 dilution was the initial solution for a 2-fold serial dilution of the serum. The dilutions were 1 in 1,000, 1 in 2,000, 1 in 4,000, 1 in 8,000, 1 in 16,000, 1 in 32,000, 1 in 64,000, 1 in 126,000, and 1 in 256,000. One hundred µl from each of the dilutions were aliquoted into two 5 ml polypropylene tubes each, and 100 µl of RIA buffer, as well as 100 µl of the previously produced tracer were added to each tube. Also, 200 µl of RIA buffer and 100 µl of tracer were mixed in a separate tube and served as a control for non-specific binding (NB). In order to measure the total counts (TC) of the tracer, 100 µl of tracer only, were pipetted into two polypropylene tubes. All samples were run in duplicates. After 2 hours of incubation at room temperature 100 µl of Rabbit Carrier Solution (RCS; consisting of 1 ml normal rabbit serum (i.e., from an animal that did not receive any injections of specific antigen) and 99 ml of RIA buffer) and 1 ml of a commercially available precipitation solution were added to each tube, except the TC tubes. The precipitation solution contains specific anti-rabbit-antibody antibodies and therefore binds to the complexes of rabbit derived antibodies and protein, which led to the precipitation of these complexes. The tubes were subsequently centrifuged at 3,800 RPM and 4°C for 30 min, and the supernatants were carefully decanted (except TC). Using an automated gamma counter

with a counting efficiency of 82%, the radioactivity of the remaining pellets was measured. For each sample, the counts were measured for 120 seconds. The unit for the measurement of the radioactivity was Counts per minute (CPM). The results were expressed as percentage of binding of the anti-rfTNFα antibodies to radiolabled protein:
%binding = (mean of CPM - mean of NB)/(mean of TC - mean of NB)*100.
A titre curve was generated by blotting the results, with the x-axis showing the dilution of the serum and the y-axis showing the %binding.

## 2.6. Purification of anti-rfTNFα Antibodies

### 2.6.1. Preparation of the *N*-Hydroxysuccinimide-Affinity Chromatography Column

Affinity chromatography was used for the purification of polyclonal anti-rfTNFα antibodies from the antiserum from one of the two rabbits. The antiserum that showed a higher titer of anti-rfTNFα antibodies was chosen. An *N*-hydroxysuccinimide (NHS)-affinity column was prepared. First, 1 mg of pure rfTNFα in 1 ml of PBS was buffer-exchanged against a ligand buffer (200 mM $NaHCO_3$, 500 mM NaCl, pH 8.3) using cartridge dialysis. After equilibration of the column with 3 × 2 cv of ice-cold HCl at a flow rate of 1 ml/min, the rfTNFα containing ligand solution was applied onto the column. The column was then incubated at 4°C for 12 hours to allow permanent binding of the protein to the NHS resin. The column was then washed with 6 ml (equals 3 × 2 cv) of buffer A (500 mM ethanolamine, 500 mM NaCl, pH 8.3), then with 6 ml of buffer B (100 mM sodium acetate, 500 mM NaCl, pH 4.0), followed by equilibration with 6 ml of buffer A. The column was then incubated for 30 min at room temperature and was again washed with 6 ml of buffer B, 6 ml of buffer A, and once again 6 ml buffer B. Finally, the column was equilibrated with PBS and stored in storage buffer (50 mM $Na_2HPO_4$, 0.1% $NaN_3$, pH 7.0) at 4°C until further use.

### 2.6.2. Affinity Chromatography

In preparation for the purification of the anti-rfTNFα antibodies, 20 ml of rabbit anti-serum were thawed and dialyzed against the starting buffer (buffer C; 75 mM Tris-HCl, 150 mM NaCl, pH 8.0) using dialysis tubing. The initial dialysis buffer was exchanged once after 2 hours of dialysis, a second time after 12 hours, and a third time after 2 more hours. The dialysis was performed at 4°C.

The NHS-affinity column was connected to a fast performance liquid chromatography (FPLC) system and was equilibrated with 5 cv of buffer C. Two ml of the dialyzed antiserum were applied onto the column. The absorbance of the eluent was monitored at a wavelength of 280 nm using a software program, especially designed for automated FPLC. The column was washed with buffer C until the absorbance measured in the eluent fractions returned to baseline. Subsequently, an elution buffer (buffer D; 100 mM glycine, 500 mM NaCl, pH 2.7) was applied, and 2 ml fractions of the eluent were collected into glass tubes, containing 600 µl of 1 M Tris-HCl, pH 8.0, in order to neutralize the low pH of the elution buffer. The fractions collected during the detection of an elution peak (fractions, which showed an absorbance at 280 nm above the baseline) were pooled, concentrated, and the buffer was changed to PBS, using a centrifugal filter device with a molecular weight cut-off (MWCO) of 10,000. The purity of the antibody solution was assessed by SDS-PAGE. Aliquots of 250 µl, with a concentration of 1 mg/ml, were stored frozen at -80°C until further use.

### 2.7. Biotinylation of anti-rfTNFα Antibodies

In order to produce secondary antibodies for an enzyme-linked immunosorbent assay, 2 mg of the NHS-purified polyclonal antibodies in PBS (1 mg/ml) were mixed with a 20-fold molar excess of EZ-Link® Sulfo-NHS-LC tagged biotin, according to the manufacturer's instruction. This allowed the NHS esters to react with primary amino acids of the antibodies, leading to the formation of stable amide linkages. The mixture was incubated at room temperature for 30 min and then the buffer was exchanged against PBS, using a centrifugal filter device with an MWCO of 10,000.

The efficacy of the biotinylation process was determined by use of a 2-4'-hydroxyazonbenzene bencoic acid (HABA)/avidin assay kit. The No-Weigh™ HABA/avidin premix was equilibrated to room temperature, 100 µl of ultrapure water were added, and the solution was mixed carefully with a pipette tip. Then 800 µl of PBS were pipetted into a 1 ml cuvette, and were used to zero the spectrophotometer at a wavelength of 500 nm. The 100 µl of the HABA/avidin mixture were then pipette into the cuvette and the mixture was gently inverted several times. The absorbance of the mixture at 500 nm was measured and recorded. Finally, 100 µl of the antibody solution were added and the absorbance was measured again. After the measured value remained constant for more than 15 s the absorbance was recorded. Avidin is a natural ligand to biotin. Thus, the measured absorbance allowed for assement of formation of complexes and thus the calculation of the biotinylation coefficient according to

the instructions of the manufacturer of the biotinylation kit (EZ-Link® Sulfo-NHS-LC-Biotinylation Kit).
A biotinylation coefficient between 4.0 and 8.0 mmol Biotin/mmol protein was considered optimal. These values are solely based on good experiences we made with antibodies in this range in our laboratory. The biotinylated antibodies were stored in aliquots of 250 µl at -80°C until further use.

## 2.8. Enzyme-Linked Immunosorbent Assay for the Measurement of feline TNFα in Serum

An enzyme-linked immunosorbent assay (ELISA) for the measurement of fTNFα in serum samples from cats was developed. For performing the assay, a 96-well flat-bottom enhanced binding ELISA plate was used. Each well was coated with 200 ng of the purified anti-rfTNFα antibodies in 100 µl of 0.2 M carbonate-bicarbonate buffer, pH 9.4. The plates were incubated at 37° for 1 hour, while constantly shaking using an automated plate incubator. The wells were subsequently washed 4 times with 200 µl/well of PBS with Tween (0.05% v/v), pH 7.2. To block the remaining binding sites of the plastic surface of the wells, the plates were incubated for 1 hour at 37°C with 200 µl/well of a commercially available Superblock® solution, while shaking continuously. The wells were then washed as described above. For the standard solutions of fTNFα, a serial 2-fold dilution of the purified rfTNFα protein was prepared in sample buffer (PBS, 1 v/v % BSA, 0.05 v/v % Tween) at concentrations of 1250, 625, 312.5, 156.3, 78.1, and 39.1 ng/l rfTNFα. Aliquots of 250 µl of each standard solution were stored at -80°C until further use. One set of these standards was thawed immediately prior to an assay run. Also, three different controls (low, medium, and high range of the assay) were prepared. The controls contained purified rfTNFα protein in sample buffer at concentrations of 300, 600, and 900 ng/l and aliquots of 250 µl of each control were stored at -80°C until further use. Controls were used in order to assure a constant quality and precision of the assay during each run. Controls and unknown samples were prepared in a 1 in 2 dilution in sample buffer. Standards, as well as controls and samples were then loaded in duplicates of 100 µl per well. One hundred µl of the sample buffer served as a negative control (blank). After loading the samples, the plates were incubated and washed as described above. Then 100 ng biotinylated anti-rfTNFα antibodies, diluted in 100 µl of sample buffer, were added to each well. Following one hour of incubation and washing of the plate as described above, 8 ng of horseradish peroxidase-labeled streptavidin in 100 µl sample buffer

were added to each well. Plates were again incubated and washed as described. Then, 100 µl of stabilized 3, 3', 5, 5'-tetramethylbenzidine (1-Step™ Ultra-TMB-ELISA) were added to each well and the plate was covered with aluminium foil and incubated at room temperature for 20 minutes. The 3, 3', 5, 5'-tetramethylbenzidine-reaction was stopped by adding 100 µl/well of a stopping solution (4 M acetic acid, 0.5 N sulfuric acid). Finally, the absorbance was measured at a wavelength of 450 nm, using an automated plate reader.
A 5-parameter logistic curve fit, using the following mathematical equation, was used to calculate the standard curve:

Y = (A-D)/(1+[X/C]^B)^E + D.

This 5-parameter logistic curve fit equation represents an extension of the well-known 4-parameter logistic curve fit equation (BAUD, 1993). The latter assumes that a curve is symmetrical around its midpoint. The 5-parameter logistic curve fit equation, however, adds one more parameter, which allows for the calculation of an asymmetrical curve (BAUD, 1993). In this equation, A equals the y-value that corresponds to the asymptote of the x-axis at low values, D equals the y-value that corresponds to the asymptote of the x-axis at high values, B describes how rapidly the curve transits from the asymptotes in the center of the curve, and C is the x-value that corresponds to the midpoint between A and D. The fifth parameter, E, is a measure of the asymmetry of the curve (BAUD, 1993).
The value for each unknown sample was calculated by extrapolating the mean absorbance of each sample duplicate on the calculated standard curve using a computer program (Synergy 2, Biotek, Winooski, VA, USA).

### 2.8.1. Analytical Validation of the ELISA for fTNFα

Data were analyzed using a statistical software package (Prism 5, GraphPad Software, Inc., La Jolla, CA, USA). Detection limit, dilutional parallelism, spiking recovery, intra-assay variability, and inter-assay variability of the fTNFα ELISA were determined in order to analytically validate the ELISA for the measurement of fTNFα concentrations in serum samples from cats.
The detection limit of the assay also was defined as the concentration of fTNFα calculated from the absorbance equal to the mean absorbance of 10 duplicates of the negative control (i.e., pure sample buffer) plus 3 times its standard deviation (SD).

For dilutional parallelism, four different feline serum samples were diluted and evaluated. The dilutions for each sample were: 1 in 2, 1 in 4, 1 in 8, and 1 in 16. Dilutional parallelism is a test for the linearity of an assay and observed to expected ratios (O/E) were calculated and the percentage by which the observed value differed from the expected value was recorded for each sample and dilution.

Spiking recovery was determined by adding 19.5, 39.1, 78.1, 156.3, 312.5, and 625.0 ng/l of rfTNFα each to 4 different serum samples with a known concentration of fTNFα. Then O/E for the measured values were calculated and the resulting percentage of the difference between the observed and the expected values describe the accuracy of the assay.

Intra-assay variability was determined by evaluation of 4 different serum samples run 10 times in duplicates within the same assay run. Subsequently, the mean as well as the standard deviation (SD) of the ten values were calculated for each separate sample. Then, the coefficient of variation (%CV) was calculated as follows:

%CV = (SD/mean)*100.

Inter-assay variability was determined by evaluating 4 different serum samples during 10 consecutive assay runs. Subsequently, the mean, as well as the SD of the 10 values for each sample were calculated and the %CV was calculated.

### 2.8.2. Serum fTNFα Concentration from Healthy Cats

A total of 20 serum samples were collected from healthy pet cats. The collection of blood from these animals was reviewed and approved by the Clinical Research Review Committee of the Texas A&M University, College of Veterinary Medicine and Biomedical Sciences. Each animal underwent a physical examination and had to be dewormed and vaccinated on a regular basis for inclusion into this study. Serum fTNFα was measured by ELISA in all of the 20 cats.

The dataset was tested for normality by the Shapiro-Wilk test. Then the dataset obtained was analyzed by determining the median and the range. A reference interval for serum fTNFα concentration was calculated using the central $95^{th}$ percentile for these 20 cats.

## 2.9. Serum fTNFα Concentration from Cats with Gastrointestinal Disease

A total of 39 cats were enrolled in this preliminary clinical study. For this part of the study left-over serum samples from the Gastrointestinal Laboratory that had been submitted for testing of other parameters were used. The referring veterinarians submitting the samples were asked to fill out a questionnaire concerning the clinical history of the cat whose serum sample had been evaluated for the study.

All animals included in this study showed one or more clinical signs of a chronic enteropathy, predominantly diarrhea, vomiting, and/or weight loss, but also anorexia, abdominal pain, fecal incontinence, and/or depression. All serum samples showed decreased serum concentrations of cobalamin (<290 ng/l; reference interval 290 – 1499 ng/l), which is consistant with chronic diseases of the distal small intestine. In addition, all samples from animals enrolled in this study had serum concentrations of trypsin-like immunoreactivity (fTLI) and pancreatic lipase immunoreactivity (fPLI) within their respective reference intervals (fTLI: 12-82 µg/l and fPLI: 0.1-3.5 µg/l, respectively) allowing the exclusion of concurrent diseases of the exocrine pancreas, such as exocrine pancreatic insufficiency or pancreatitis.

Based on the clinical presentation the cats were grouped into two subgroups. Group 1 (n = 23) included cats that showed clinical signs of a chronic enteropathy, but did not have a history of diarrhea, group 2 (n = 16) included cats with a chronic enteropathy that were reported to have diarrhea. The serum fTNFα concentration was measured by the newly developed ELISA in all 39 cats. Data were analyzed using a statistical software package (Prism 5, GraphPad Software, Inc., La Jolla, CA, USA).. The data were tested for normal distribution using the Shapiro-Wilk test, and the Mann-Whitney test for non-parametric comparisons was used. For comparison of proportions, contingency tables were constructed and a Fisher's exact test was used. Statistical significance for all tests was set at $p < 0.05$.

# IV. RESULTS

## 1. Purification of the GST-rfTNFα Fusion Protein

In order to monitor the successful production of the Glutathione-S-transferase-recombinant feline tumor necrosis factor α (GST-rfTNFα) fusion protein in *Escherichia coli* bacteria, as well as the subsequent separation of the GST-rfTNFα from the bacterial cell debris and other bacterial proteins, and the separation of the rfTNFα protein from the GST part of the fusion protein, gel electrophoresis with SDS-PAGE was performed.

The expected molecular mass of the GST-rfTNFα fusion protein was approximately 43 kilo Daltons (kDa). This was based on the fact that the GST protein has a molecular mass of 27 kDa and the rfTNFα of approximately 17 kDa. Samples containing pure rfTNFα (Figure 4), as evaluated by SDS-PAGE, were pooled and concentrated.

A digital picture of the SDS-PAGE was taken and evaluated, using the Quantity One software, which allows a relative quantification of protein bands within one lane of the gel picture. The results calculated by the software program represent the relative purity of the protein within a sample. It was calculated that all final samples of rfTNFα in this study showed a purity of greater than 95.5%.

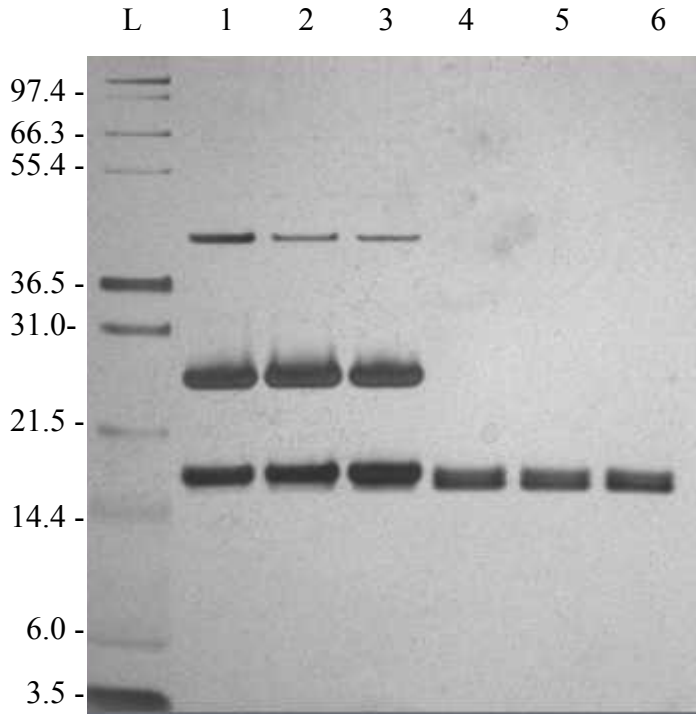


**Figure 4:** SDS-PAGE, demonstrating different stages of rfTNFα purification.

This figure shows a typical SDS-PAGE stained with a silver stain kit. The lane marked with "L" contains visible bands of the marker proteins (from top to bottom: phosphorylase (97.4 kDa), bovine serum albumin (66.3 kDa), glutamic dehydrogenase (55.4 kDa), lactate dehydrogenase (36.5 kDa), carbonic anhydrase (31.0 kDa), trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa), aprotinin (6 kDa), and insulin B chain (3.5 kDa)). The numbers at the left of the image indicate the molecular mass of these marker proteins in kDa. Lanes 1 to 3 contain one visible protein band with a size of approximately 43 kDa, each. These bands represent the GST-rfTNFα fusion protein. Another band with a size of approximately 27 kDa is also present in lane 1 to 3, and corresponds to the size of the GST part of the fusion protein. A third protein band of approximately 17 kDa in size can be observed in lanes 1 to 6 and represents rfTNFα. This band actually consists of 2 bands in very close proximity. These minor differences in molecular mass can be explained by minor size variations of the rfTNFα protein (see also chapter 4. N-terminal Amino Acid Sequencing of this section). Lanes 4 to 6 contain pure rfTNFα after completing the purification protocol. No contaminations with other proteins are visible.

## 2. Determination of Specific Absorbance

The extinction coefficient (EC) for rfTNFα was determined by the BCA™ assay and calculated to be 1.75 (Table 5).

**Table 5:** Values for the calculation of the specific absorbance of rfTNFα.

| Dilutions | Absorbance at 280 nm | Protein concentrations as measured by the BCA assay (mg/ml) | Extinction coefficient (EC) |
|---|---|---|---|
| 1 in 2 | 2.13 | 1.25 | 1.71 |
| 1 in 4 | 1.28 | 0.68 | 1.89 |
| 1 in 8 | 0.69 | 0.38 | 1.80 |
| 1 in 16 | 0.4 | 0.25 | 1.62 |
| **Mean EC** | | | **1.75** |

This table shows the results of the BCA™ assay. PBS was used to dilute each of the samples. The undiluted sample was too high in concentration to be reliably measured. Therefore, the dilutions 1 in 2 to 1 in 16 were used for this part of the study. The mean of the calculated extinction coefficients for the four dilutions was calculated to be 1.75.

### 3. Estimation of the Relative Molecular Mass

After the purification of rfTNFα, the surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) method was used for a more accurate determination of the molecular mass of the protein. Figure 5 shows that the pure protein solution consists of 2 proteins with a slightly different molecular mass. As demonstrated in chapter 4. (N-terminal Amino Acid Sequencing) of this section, both proteins represent rfTNFα.

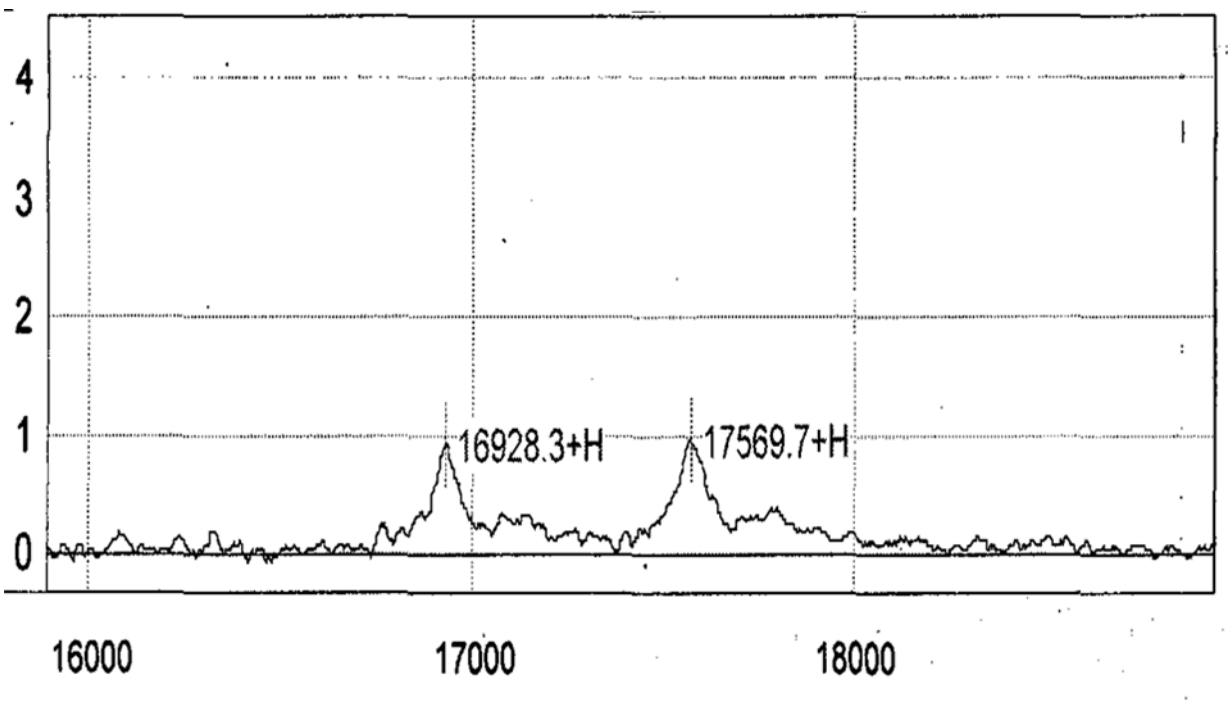


**Figure 5:** SELDI-TOF-MS performance derived graph, demonstrating molecular mass of rfTNFα**.**

This graph was generated after running one sample, containing pure rfTNFα, on the SELDI-TOF-MS system. The x-axis is labeled with the mass to charge ratio (m/z). The m/z was calculated as follows:

m/z = (mass + charge)/charge.

The m/z values are close to equal to the molecular mass in Dalton. The y-axis gives information about the size of the peaks, but the intensity is reported without a unit. One peak was calculated to represent a protein of the size of 16.9283 kDa. A second one was located at 17.5697 kDa. Both peaks were theorized to represent slightly different variants of rfTNFα.

## 4. N-terminal Amino Acid Sequencing

In order to determine whether the purification protocol had lead to pure rfTNFa and whether the 2 proteins identified by both SDS-PAGE and SELDI-TOF-MS represented the same protein (rfTNFα), N-terminal amino acid sequencing was performed. Results for N-terminal amino acid sequencing are shown in Table 6.

**Table 6:** N-terminal amino acid sequence of rfTNFα.

| | Amino acid sequence<br>1-2-3-4-5-6-7-8-9-10-11 |
|---|---|
| Sequence A | S-S-S-R-T-P-S-D-K-P-V |
| Sequence B | R-T-P-S-D-K-P-V-A-H-V |

This table shows the N-terminal amino acid sequence for two proteins purified and hypothesized to be rfTNFα. The one letter code is being used to display the sequence: D= Aspartate, K= Lysine, P= Proline, R= Arginine, S= Serine, T= Threonine, V= Valine
Both of these sequences are part of the complete amino acid sequence published for fTNFα (Figure 6). The recombinant protein did not consist of the complete sequence, since its coding DNA was derived originally from mRNA most likely coding only for the 157 amino acids containing sTNFα. The variation of the N-terminal of the protein strand in parts explains the slight difference in the relative molecular mass determined on SDS-PAGE and SELDI-TOF-MS.

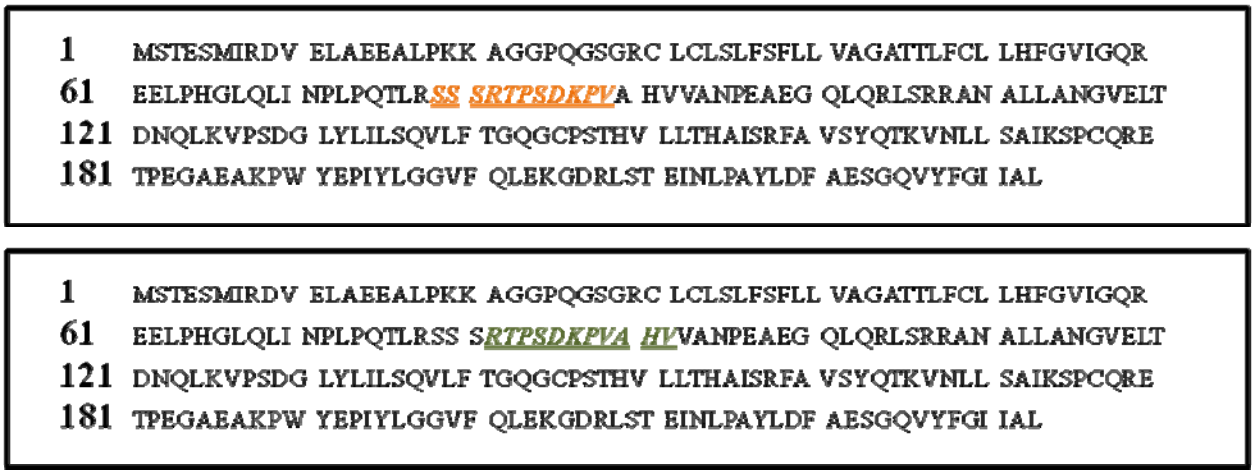


**Figure 6:** Complete amino acid sequence of fTNFα and designation of identified N-terminal amino acid sequences.

The boxes shown display the complete amino acid sequence published for fTNFα in the NCBI (MCGRAW et al., 1990). In the first box N-terminal amino acid sequence A is highlighted in orange, while in the second box N-terminal amino acid sequence B is highlighted in green.

Sequences are shown using the international one-letter code for amino acids.

| Letter code | Amino acid | Letter code | Amino acid |
|---|---|---|---|
| **A** | Alanine | **M** | Methionine |
| **C** | Cysteine | **N** | Asparagine |
| **D** | Aspartate | **P** | Proline |
| **E** | Glutamate | **Q** | Glutamine |
| **F** | Phenylalanine | **R** | Arginine |
| **G** | Glycine | **S** | Serine |
| **H** | Histidine | **T** | Threonine |
| **I** | Isoleucine | **V** | Valine |
| **K** | Lysine | **W** | Tryptophan |
| **L** | Leucine | **Y** | Tyrosine |

## 5. Estimation of the Isoelectric Point

The isoelectric point of rfTNFα, as determined by polyacrylamide gel electrophoresis using a NOVEX® Pre-Cast vertical IEF gel, was estimated at 5.3.

## 6. Production of anti-rfTNFα Antiserum and Antibody Titer Analysis

Resulting antibody titers from injections in New Zealand White rabbits were analyzed by titer radioimmunoassay (RIA) and figures 7 and 8 demonstrate the increase in binding over the period of 3 injections in rabbit 1, and over the period of 4 injections in rabbit 2, respectively.

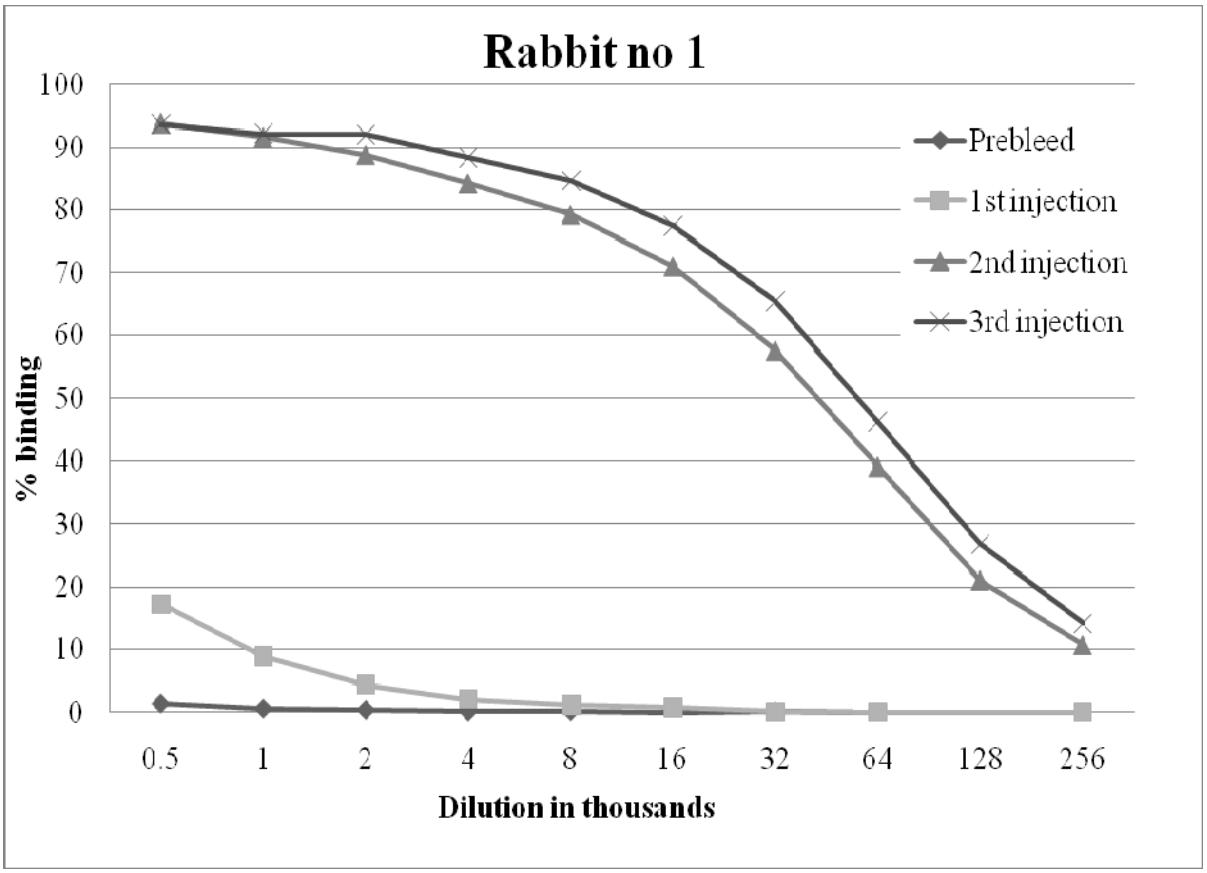


**Figure 7:** Titer curves for rabbit number 1 after injections with antigen.

The figure shows the serum anti-rfTNFα antibody titer at 4 different time points for rabbit 1. The pre-bleed sample is a baseline sample, which was taken before the first injection of the antigen. The other samples were collected approximately 10 days after the 1st, 2nd, and 3rd injection, respectively.

The x-axis shows the different dilutions of the antibodies in RIA buffer, which were measured in the RIA. The dilutions ranged from 1 in 500 to 1 in 256,000. The y-axis represents the percentage of binding of the anti-rfTNFα antibodies to radiolabled protein.

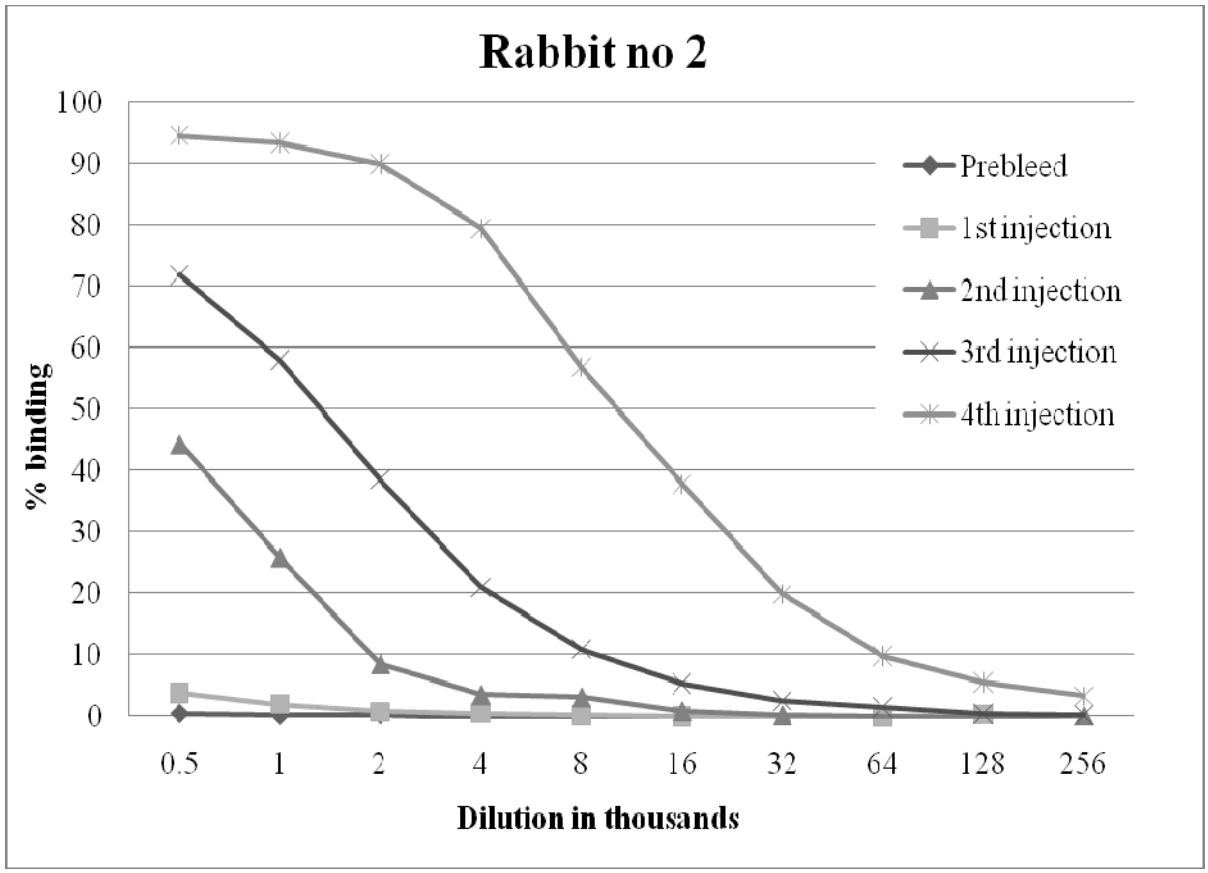


**Figure 8:** Titer curves for rabbit number 2 after injections with antigen.

The figure shows the serum anti-rfTNFα antibody titer at 5 different time points for rabbit 2. The pre-bleed sample is a baseline sample that was taken before the first injection of antigen. The other samples were collected approximately 10 days after the 1st, 2nd, 3rd, and 4th injection, respectively.

The x-axis shows the different dilutions of the antibodies in RIA buffer, which were measured in the RIA. The dilutions ranged from 1 in 500 to 1 in 256,000. The y-axis represents the percentage of binding of the anti-rfTNFα antibodies to radiolabled protein.

## 7. Affinity Chromatography

The monospecific polyclonal anti-rfTNFα antibodies were purified by affinity chromatography, in order to develop an fTNFα-specific ELISA. A total of 12.3 mg IgG were purified from 20 ml of rabbit serum. Figure 9 shows a chromatogram of the affinity purification of the anti-rfTNFα antibodies, as generated by the FPLC software. The purity of the anti-rfTNFα antibodies was verified by SDS-PAGE, as shown in figure 10.

Two mg of the pure antibodies were biotinylated for use as a secondary antibody in the ELISA. The biotinylation coefficient was estimated at 6.4 mmol biotin/mmol protein by use of the No-Weigh$^{TM}$ HABA/avidin according to the manufacturer's instuctions.

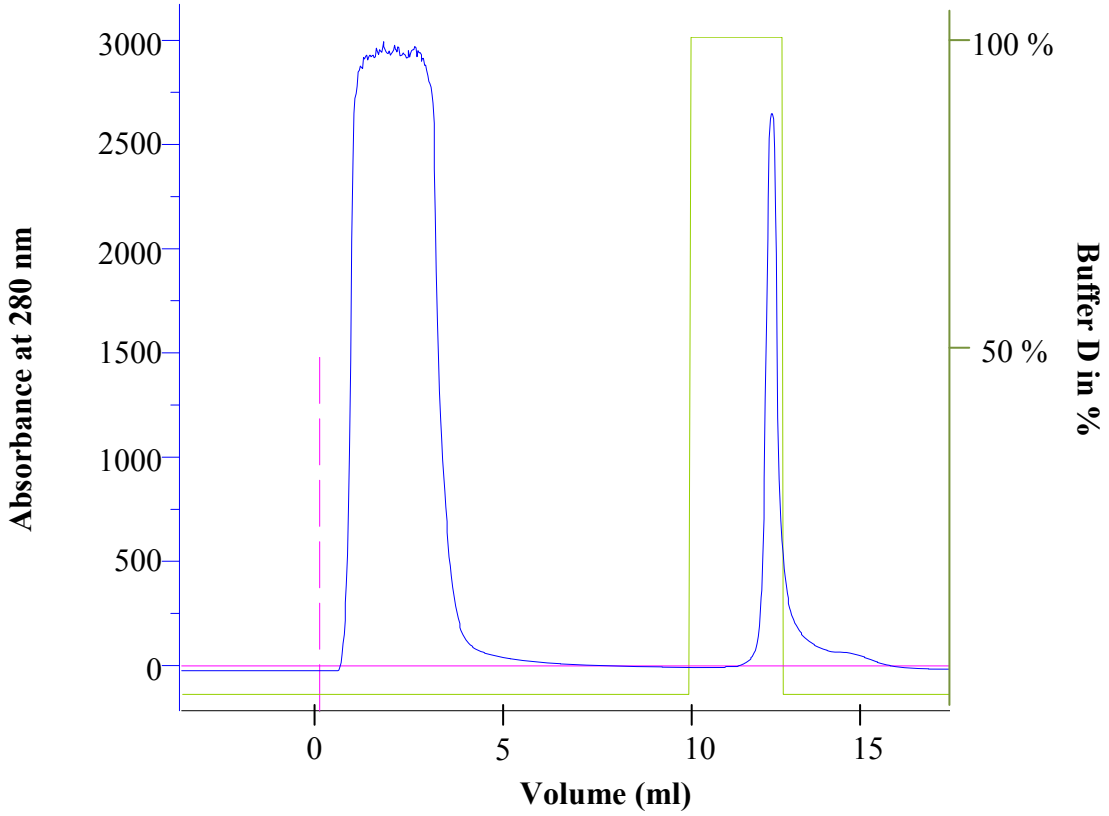


**Figure 9:** Affinity chromatography of anti-rfTNFα antiserum.

This figure shows the elution chromatogram during the purification of anti-rfTNFα antibodies. In this chromatogram the absorbance at 280 nm is shown on the blue y-axis to the left. The two distinct peaks (solid blue line) represent protein elution. The first peak was generated by proteins that did not bind to the column, and therefore were washed out immediately. The second peak was generated by monospecific polyclonal anti-rfTNFα antibodies, which did bind to the column, and were washed off by changing the buffer to 100% of elution buffer (buffer D, 100 mM glycine, 500 mM NaCl, pH 2.7), as indicated by the solid green line. The vertical interrupted pink line indicates the time point of sample injection onto the column. The solid pink line represents an absorbance of 0 nm and serves as a helpful indicator as to when the absorbance approached zero after a peak was seen.

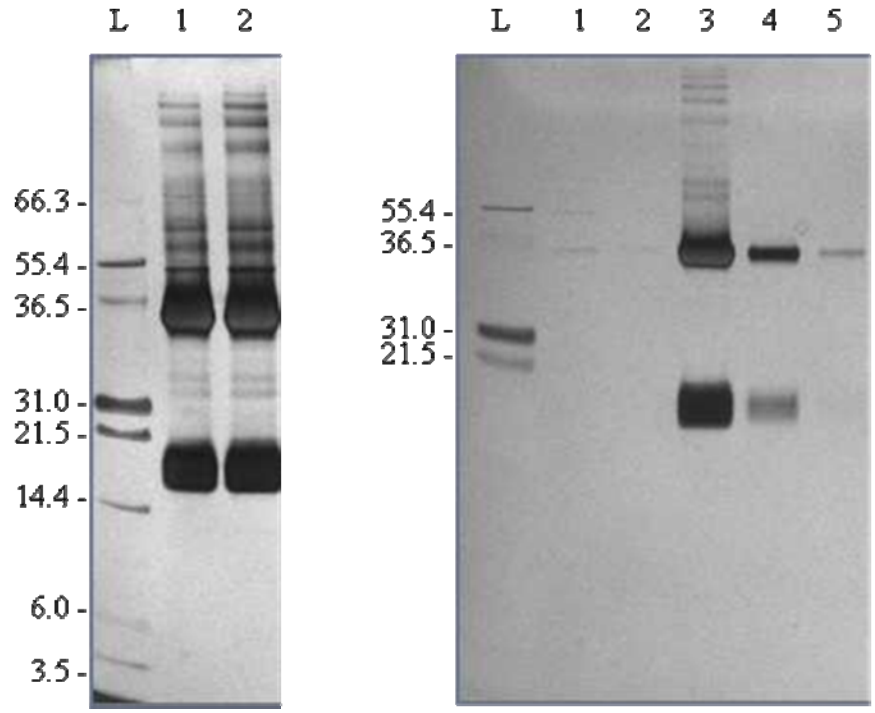


**Figure 10:** SDS-PAGE of anti-rfTNFα antibodies.

The SDS-PAGE gels displayed were stained with silver staining. The gel on the left shows 3 lanes. The lane marked "L" contains visible bands of marker proteins of a known molecular mass (as indicated at left side in kDa). Lanes 2 and 3 contain multiple bands each. These bands represent a mixture of proteins, which did not bind to the affinity column and were washed out as a 1st peak as demonstrated in figure 9.

In the gel on the right 6 lanes are shown. The 1st lane once again, contains marker proteins and their size is marked to the left of the picture in kDa. Lanes 1 and 2 do not display any bands. Here, fractions were loaded onto the gel, which were eluted from the column while no peak was visible in the accordant chromatogram. Lanes 3, 4, and 5 contain bands that represent the pure anti-rfTNFα antibodies from the fractions, represented by the 2nd peak from figure 9. Only lane 4 contains the antibodies in an optimal concentration (0.03 mg/ml). The antibodies in lane 3 are too concentrated and therefore artifacts of multiple bands can be seen.

Antibodies are always represented by 2 bands on gel chromatography, because light and heavy chains separate during the preparative denaturation process.

## 8. Development and Analytical Validation of an ELISA for the Measurement of fTNFα in Serum

The ELISA protocol described in the chapter 2.8 produced reproducible standard curves with the lowest standard at 39.1 ng/l and the highest at 1,250 ng/l, as shown in Figure 11.

The detection limit of the assay was established by running 10 duplicates of the negative control (PBS, 0.05% Tween, 10% BSA) and determining the mean (±SD) absorbance at 450 nm. The standard deviation (SD) multiplied by three was added to the value of the extrapolation of the mean absorbance of the 10 samples from the standard curve. This value equalled a fTNFα concentration of 19.2 ng/l. Because serum samples were diluted 1:2 prior to running, the calculated detection limit was 38.4 ng/l.

Assay linearity was determined by evaluation of dilutional parallelism of 4 different serum samples. Observed to expected ratios (O/E) ranged from 75.1 to 111.9% with a mean (±SD) of 98.9% (±11.4%) as shown in Table 7.

Accuracy was determined by evaluation of spiking recovery of 4 different serum samples. O/E ranged from 91.3 to 129.7% with a mean of (±SD) of 102.3% (± 7.9%) as shown in Table 8.

Intra-assay variability was evaluated for 4 different serum samples. The variability ranged from 3.9 to 7.6% with a mean (±SD) of 5.0% (± 1.8%) as shown in Table 9.

Inter-assay variability was evaluated for 4 different serum samples. The variability ranged from 7.8 to 12.5% with a mean (±SD) of 9.5% (±2.2%) as shown in Table 10.

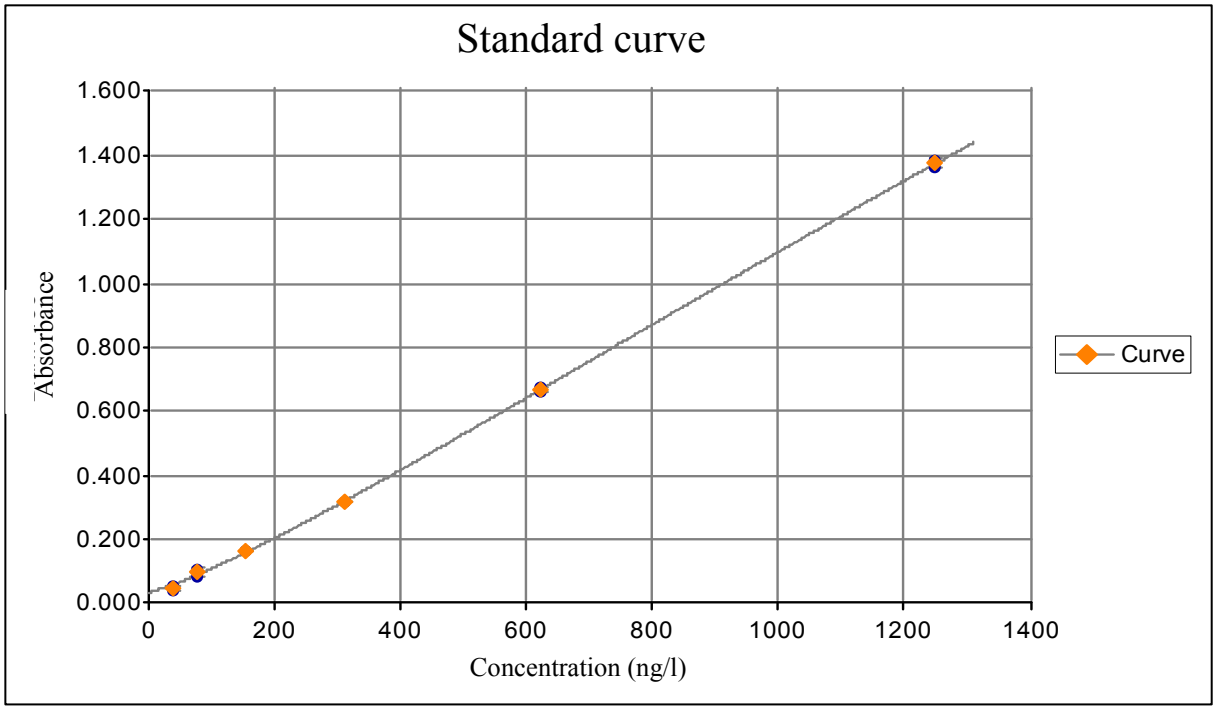


**Figure 11:** Typical standard curve of the fTNFα ELISA.

This figure shows a typical standard curve for the fTNFα ELISA. The concentration of the standards ranged from 39.1 ng/l to 1,250 ng/l (39.1, 78.1, 156.3, 312.5, 625 and 1250 ng/l). The x-axis represents the concentration in ng/l. On the y-axis the absorbance is shown after the subtraction of the blank. A computer software package was used to calculate all 5 parameters of the curve needed to achieve a 5-parameter curve fit (Synergy 2, Biotek, Winooski, VA, USA).

**Table 7:** Dilutional parallelism.

| Sample | Dilution | Observed (ng/l) | Expected (ng/l) | O/E (%) |
|---|---|---|---|---|
| | | | | |
| **1** | **1 in 2** | 480.9 | N/A | N/A |
| | **1 in 4** | 255.3 | 240.5 | **106.2** |
| | **1 in 8** | 124.5 | 120.2 | **103.6** |
| | **1 in 16** | 50.8 | 60.1 | **84.4** |
| | | | | |
| **2** | **1 in 2** | 565.9 | N/A | N/A |
| | **1 in 4** | 292.0 | 282.9 | **103.2** |
| | **1 in 8** | 140.9 | 141.5 | **99.6** |
| | **1 in 16** | 53.1 | 70.7 | **75.1** |
| | | | | |
| **3** | **1 in 2** | 495.5 | N/A | N/A |
| | **1 in 4** | 277.3 | 247.7 | **111.9** |
| | **1 in 8** | 132.6 | 123.9 | **107.1** |
| | **1 in 16** | 53.5 | 61.9 | **86.4** |
| | | | | |
| **4** | **1 in 2** | 466.4 | N/A | N/A |
| | **1 in 4** | 253.6 | 233.2 | **108.7** |
| | **1 in 8** | 120.2 | 116.6 | **103.1** |
| | **1 in 16** | 53.3 | 58.3 | **91.4** |

O/E = (observed / expected)*100; N/A = not applicable

Four different samples were serially diluted in PBS. The dilutions ranged from 1 in 2 to 1 in 16 for each sample. Each dilution was evaluated in duplicates and the mean value was used. (O/E) ratios ranged from 75.1 to 111.9% with a mean (±SD) of 98.9% (±11.4%).

**Table 8:** Spiking recovery.

| Sample | Spiking conc. | Observed (ng/l) | Expected (ng/l) | O/E (%) |
|---|---|---|---|---|
| **1** | 0.0 | 132.9 | N/A | N/A |
| | 19.5 | 147.7 | 152.4 | **96.9** |
| | 39.1 | 169.0 | 171.9 | **98.3** |
| | 78.1 | 218.1 | 211.0 | **103.4** |
| | 156.3 | 303.1 | 289.1 | **104.8** |
| | 312.5 | 459.1 | 445.4 | **103.1** |
| | 625.0 | 862.6 | 757.9 | **113.8** |
| | | | | |
| **2** | 0.0 | 213.5 | N/A | N/A |
| | 19.5 | 257.9 | 233.0 | **110.7** |
| | 39.1 | 259.6 | 252.5 | **102.8** |
| | 78.1 | 294.4 | 291.6 | **101.0** |
| | 156.3 | 384.4 | 369.7 | **104.0** |
| | 312.5 | 522.6 | 526.0 | **99.4** |
| | 625.0 | 808.9 | 838.5 | **96.5** |
| | | | | |
| **3** | 0.0 | 359.3 | N/A | N/A |
| | 19.5 | 367.7 | 378.8 | **97.1** |
| | 39.1 | 376.6 | 398.3 | **94.6** |
| | 78.1 | 425.5 | 437.4 | **97.3** |
| | 156.3 | 496.6 | 515.5 | **96.3** |
| | 312.5 | 679.8 | 671.8 | **101.2** |
| | 625.0 | 964.5 | 984.3 | **98.0** |
| | | | | |
| **4** | 0.0 | 102.5 | N/A | N/A |
| | 19.5 | 111.4 | 122.0 | **91.3** |
| | 39.1 | 139.1 | 141.6 | **98.2** |
| | 78.1 | 234.3 | 180.6 | **129.7** |
| | 156.3 | 260.2 | 258.7 | **100.6** |
| | 312.5 | 449.3 | 415.0 | **108.3** |
| | 625.0 | 778.3 | 727.5 | **107.0** |

O/E = (observed / expected)*100; N/A = not applicable

The spiking recovery rate was determined by adding 19.5, 39.1, 78.1, 156.3, 312.5, and 625.0 ng/l of pure rfTNFα to alliquots of 4 serum samples, each. All samples were assayed in duplicates and the mean values are shown in the table for each sample. The recovery ranged from 91.3 to 129.7% with a mean of (±SD) of 102.3% (± 7.9%).

**Table 9:** Intra-assay variability.

| Sample | Mean (ng/l) | SD (ng/l) | %CV |
|---|---|---|---|
| | | | |
| **A** | 646.8 | 14.9 | 4.6 |
| | | | |
| **B** | 736 | 14.3 | 3.9 |
| | | | |
| **C** | 100.2 | 19.5 | 3.9 |
| | | | |
| **D** | 1470.2 | 55.9 | 7.6 |

SD = standard deviation; CV = coefficient of variation; %CV = (SD/mean)*100

This table displays the intra-assay variability for the fTNFα ELISA. Four different serum samples were analyzed 10 times during the same assay run. All samples were run at a 1 in 2 dilution and, therefore, the values in this table were calculated by multiplying the measured values by 2, since all original samples were loaded onto the assay in a 1:2 dilution with sample buffer. All samples were evaluated in duplicates.

The variability ranged from 4.6 to 7.6% with a mean (±SD) of 5.0% (±1.8%).

**Table 10:** Inter-assay variability of the fTNFa ELISA

| Sample | Mean (ng/l) | SD (ng/l) | CV (%) |
|---|---|---|---|
| | | | |
| **A** | 663 | 41.3 | 12.5 |
| | | | |
| **B** | 752.8 | 36.6 | 9.7 |
| | | | |
| **C** | 2022.8 | 80.6 | 8 |
| | | | |
| **D** | 842 | 31.9 | 7.6 |
| | | | |

SD = standard deviation; CV = coefficient of variation; %CV = (SD/mean)*100

This table shows the inter-assay variability for the fTNFα ELISA. Four different serum samples were analyzed 10 times in consecutive assay runs. All samples were run at a 1 in 2 dilution and, therefore, the values in this table were calculated by multiplying the measured values by 2 since all original samples were loaded onto the assay in a 1:2 dilution with sample buffer. The samples were evaluated in duplicates.

The variability ranged from 7.6 to 12.5% with a mean (±SD) of 9.5% (±2.2%).

## 8.1 Serum fTNFα Concentration from Healthy Cats

A reference interval was determined by measurement of fTNFα in the serum from 20 healthy pet cats. The animals were owned by students or staff at Texas A&M University. According to the questionnaire each cat's owner had to fill out, all animals were current on vaccinations and were recently dewormed. Serum fTNFα was undetectable in 16/20 cats. The reference interval, which was determined by calculation of the central 95$^{th}$ percentile in these 20 cats, was <223.5 ng/l (Figure 12). An overview of the concentrations measured in the serum of the 20 cats is given in Table 11.

**Table 11:** Serum fTNFα concentration in 20 healthy pet cats.

| Cat number | fTNFα (ng/l) |
|---|---|
| 1 | <38.4 |
| 2 | 268.8 |
| 3 | <38.4 |
| 4 | <38.4 |
| 5 | <38.4 |
| 6 | <38.4 |
| 7 | <38.4 |
| 8 | <38.4 |
| 9 | <38.4 |
| 10 | <38.4 |
| 11 | 173.4 |
| 12 | <38.4 |
| 13 | 152.7 |
| 14 | <38.4 |
| 15 | <38.4 |
| 16 | <38.4 |
| 17 | 40.4 |
| 18 | <38.4 |
| 19 | <38.4 |
| 20 | <38.4 |

This table shows the fTNFα concentrations measured in the serum of 20 healthy pet cats. Sixteen cats had undetectable serum fTNFa concentrations (<38.4 ng/l). In 4 cats, concentrations between 40.4 and 268.8 ng/l were measured. All samples were run in a 1 in 2 dilution and the values in this table were back-calculated by multiplying the measured values by the factor 2 since all original samples were loaded onto the assay in a 1:2 dilution with sample buffer. All samples were run in duplicates and the mean values are given. The median serum fTNFα concentration was <38.4 ng/l.

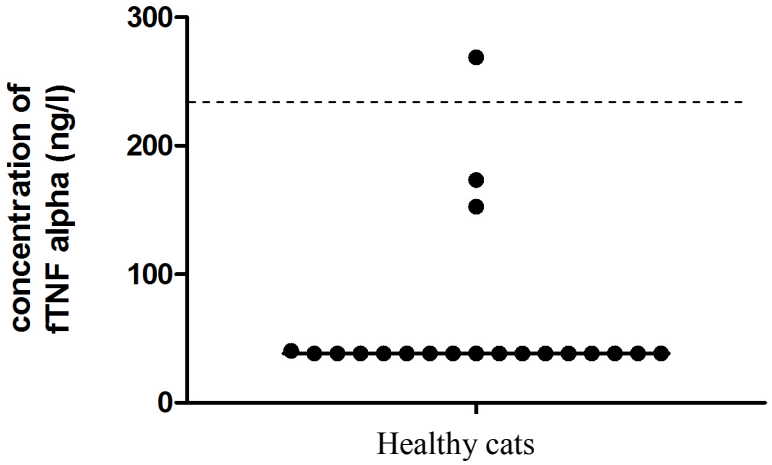


**Figure 12:** Serum fTNFα concentration in 20 healthy pet cats.

The dot-plot illustrates serum fTNFα concentrations in 20 healthy cats. Sixteen cats had a serum concentration below the detection limit of the assay (38.4 ng/l). Four cats had serum concentrations between 40.4 and 268.8 ng/l. The upper limit of the reference interval corresponds to the dotted line. The median serum fTNFα concentration (<38.4 ng/l) is displayed by the solid black line.

## 9. Serum fTNFα Concentration in Cats with Gastrointestinal Disease

Serum fTNFα concentrations were measured by ELISA in 39 cats with clinical signs of a chronic enteropathy (n = 39). All animals included in this study displayed one or more clinical signs of chronic enteropathy, predominantly diarrhea, vomiting, weight loss, but also anorexia, abdominal pain, fecal incontinence, and depression. The majority of samples from these cats showed undetectable serum fTNFα concentrations, thus the median serum fTNFα concentration in this group was <38.4 ng/l. No statistically significant difference between the group of 39 diseased cats and the group of 20 healthy cats (described in section "8.1 Serum fTNFα Concentration from Healthy Cats") could be identified ( p = 0,2181).

The 39 cats were further grouped into two subgroups based on whether they had diarrhea or not: group 1 consisted of 23 cats that showed no diarrhea, but had other signs of chronic gastrointestinal disease, while group 2 included 16 cats with diarrhea. All cats had a decreased serum cobalamin concentration (median: 159 ng/l; range: <149-290 ng/l; reference interval: 290-1499 ng/l), which is consistent with severe and longstanding distal small intestinal disease. All cats had normal serum concentrations of feline trypsin-like immunoreactivity concentrations (fTLI; median: 43.4 µg/L; range: 13.6-72.4 µg/l; reference interval: 12-82 µg/l) and feline pancreatic lipase immunoreactivity concentrations (fPLI; median: 1.9 µg/l; range: 0.7-3.2 µg/l; reference interval 0.1-3.5 µg/l). Serum concentrations of fTNFα, cobalamin, fPLI, and fTLI for each individual cat are shown in Table 12. Table 13 gives an overview of the fTNFα concentrations in the 2 subgroups.

The median serum fTNFα concentration in group 1 was <38.4 ng/l (range: <38.4 to 254.0 ng/l; i.e., most cats had undetectable serum TNFα concentrations). The median serum fTNFa concentration in group 2 was 134.0 ng/l (range: <38.4 to 2,448.5 ng/l; Figure 13).

A significant difference was identified when serum fTNFα concentrations were compared among the two subgroups and the group of healthy cats (p-value: 0.0007). A significant difference between group 2 and the group of healthy cats was shown (p-value: <0.05), but there was no significant difference between group 1 and healthy control cats. Also, there was a significant difference of serum fTNFα concentrations between groups 1 and 2 (p-value: <0.05).

Furthermore, a statistically significant difference was demonstrated between the two subgroups of p = 0.0008.

In order to compare the proportion of animals that had a serum fTNFα concentration above the upper limit of the reference interval (<223.5 ng/l) between the 2 groups a contingency

table was constructed. Using the Fisher's exact test a statistically significant difference between the proportions of increased serum fTNFα concentrations between the two subgroups was found (p-value: 0.0127; Table 14).

**Table 12:** Serum fTNFa, cobalamin, fPLI, and fTLI concentrations in 39 cats with gastrointestinal disease.

| Cat ID | fTNFα (ng/l) | Cobalamin (ng/l) | fPLI (µg/l) | fTLI (µg/l) |
|---|---|---|---|---|
| 1 | <38.4 | 186 | 1.3 | 64.6 |
| 2 | <38.4 | 214 | 2.5 | 66.4 |
| 3 | <38.4 | 168 | 1.9 | 38.2 |
| 4 | 183.4 | 149 | 2.1 | 72.4 |
| 5 | 93.9 | 149 | 1.4 | 44.3 |
| 6 | **1686.3** | 149 | 1.1 | 69.9 |
| 7 | <38.4 | 214 | 2.6 | 70.7 |
| 8 | <38.4 | 205 | 1.6 | 22.0 |
| 9 | <38.4 | 149 | 1.2 | 32.3 |
| 10 | **2000.0** | 173 | 2.0 | 49.1 |
| 11 | **246.8** | 149 | 0.6 | 26.5 |
| 12 | 174.0 | 210 | 1.8 | 33.1 |
| 13 | **1765.3** | 277 | 0.7 | 31.3 |
| 14 | **1286.7** | 227 | 1.9 | 65.9 |
| 15 | **2448.5** | 170 | 2.8 | 64.2 |
| 16 | 70.1 | 149 | 2.7 | 25.7 |
| 17 | <38.4 | 149 | 1.9 | 47.7 |
| 18 | <38.4 | 149 | 2.7 | 39.3 |
| 19 | <38.4 | 149 | 2.1 | 22.5 |
| 20 | <38.4 | 229 | 1.4 | 13.6 |
| 21 | <38.4 | 149 | 1.0 | 61.4 |
| 22 | <38.4 | 149 | 2.8 | 13.2 |
| 23 | <38.4 | 278 | 1.5 | 24.7 |
| 24 | <38.4 | 149 | 1.7 | 66.6 |
| 25 | <38.4 | 276 | 2.8 | 31.8 |
| 26 | <38.4 | 153 | 2.7 | 69.2 |
| 27 | <38.4 | 208 | 3.2 | 60.4 |
| 28 | <38.4 | 152 | 0.9 | 63.5 |
| 29 | <38.4 | 159 | 2.4 | 43.4 |
| 30 | **254.6** | 149 | 2.1 | 20.5 |
| 31 | <38.4 | 224 | 2.4 | 45.7 |
| 32 | <38.4 | 177 | 1.1 | 22.9 |
| 33 | 99.7 | 290 | 1.8 | 64.8 |
| 34 | <38.4 | 256 | 0.7 | 17.0 |
| 35 | 42.54 | 149 | 2.9 | 25.4 |
| 36 | <38.4 | 149 | 2.7 | 47.6 |
| 37 | <38.4 | 149 | 1.6 | 36.6 |
| 38 | <38.4 | 184 | 2.1 | 43.8 |
| 39 | <38.4 | 149 | 2.2 | 26.6 |

This table shows the serum concentrations for fTNFα, cobalamin, fTLI, and fPLI for 39 cats with chronic enteropathies. Twenty-six cats had undetectable serum fTNFa concentrations (<38.4 ng/l). Concentrations of fTNFα above the reference interval (<223.5 ng/l) are marked in bold.

Reference intervals: Cobalamin: 290-1499 ng/l (values established by the Gastrointestinal Laboratory; Laboratory specific range), fPLI: 0.1-3.5 µg/l (Steiner et al., 2004), fTLI: 12-82 µg/l (Steiner et al., 2000), respectively.

**Table 13:** Serum fTNFα concentration in 39 cats with gastrointestinal disease.

| Group 1; sample no. | Group 1; fTNFα (ng/l) | Group 2; sample no. | Group 2; fTNFα (ng/l) |
|---|---|---|---|
| 1 | <38.4 | 1 | <38.4 |
| 2 | <38.4 | 2 | <38.4 |
| 3 | <38.4 | 3 | <38.4 |
| 4 | <38.4 | 4 | 183.4 |
| 5 | <38.4 | 5 | 93.9 |
| 6 | <38.4 | 6 | **1686.3** |
| 7 | <38.4 | 7 | <38.4 |
| 8 | <38.4 | 8 | <38.4 |
| 9 | <38.4 | 9 | <38.4 |
| 10 | <38.4 | 10 | **2000.0** |
| 11 | <38.4 | 11 | **246.8** |
| 12 | <38.4 | 12 | 174.02 |
| 13 | <38.4 | 13 | **1765.3** |
| 14 | **254.6** | 14 | **1286.7** |
| 15 | <38.4 | 15 | **2448.5** |
| 16 | <38.4 | 16 | 70.1 |
| 17 | 99.7 | | |
| 18 | <38.4 | | |
| 19 | 42.54 | | |
| 20 | <38.4 | | |
| 21 | <38.4 | | |
| 22 | <38.4 | | |
| 23 | <38.4 | | |

This table shows serum fTNFα concentrations measured in the serum of 39 cats with clinical signs of chronic enteropathies. Group 1 consists of 23 cats without diarrhea, but other clinical signs of chronic gastrointestinal disease. The median fTNFα concentration in this group was <38.4 ng/l. Group 2 includes 16 cats with diarrhea and the median fTNFα concentration in this group was 134.0 ng/l. All serum fTNFα concentrations above the upper limit of the reference interval (<223.5 ng/l) are marked in bold.

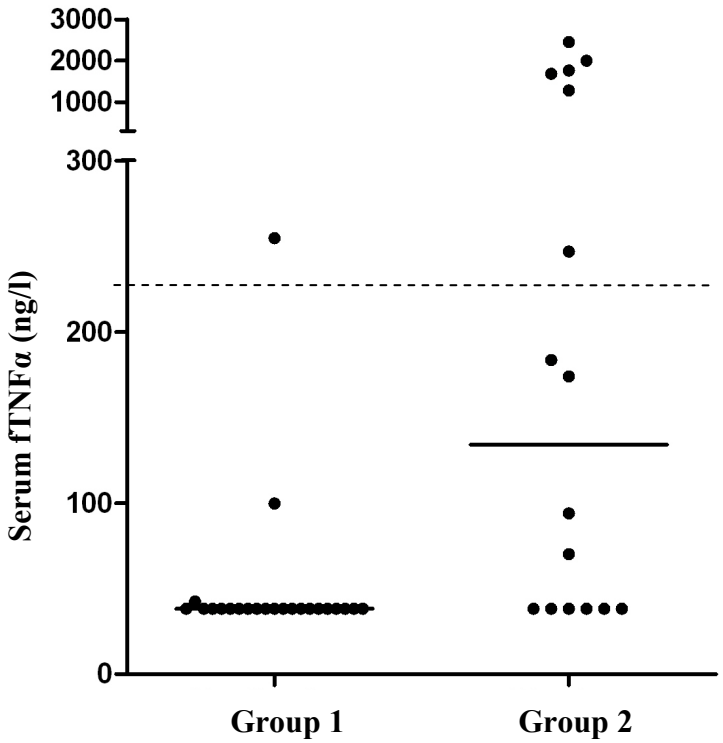


**Figure 13:** Serum fTNFα concentration in 39 cats with gastrointestinal disease.

The solid black line represents the median fTNFα concentration for each group. The dotted line represents the upper limit of the reference interval (<223.5 ng/l).

**Table 14:** Contingency table for comparison of proportions of increased fTNFα concentrations between the two subgroups.

| Data analyzed | Increased fTNFα | fTNFα within reference interval | Total |
|---|---|---|---|
| No diarrhea (group 1) | 1 | 22 | 23 |
| Diarrhea (group 2) | 6 | 10 | 16 |
| Total number of cats | 7 | 32 | 39 |

This contingency table was constructed to compare proportions of cats whose sera showed a concentration of fTNFα above the upper limit of the reference interval (<223.5 ng/l) between the 2 subgroups. Using the Fisher's exact test a statistically significant difference was identified between the two groups ($p = 0.0127$).

# V. DISCUSSION

## 1. Expression and Purification of rfTNFα

The expression and purification of rfTNFα from modified *Escherichia coli* has previously been described (RIMSTAD et al., 1995). This research group generously provided the Gastrointestinal Laboratory at Texas A&M University with cultures of their recombinant strain of *E.coli* (XL1-blue). Using the published protocol the production and purification of rfTNFα could not be reproduced in our laboratory. Thus, we developed a modified version of the published protocol in order to achieve a high yield of high purity protein.

Glutathione-S-transferase-recombinant feline TNFα (GST-rfTNFα) fusion protein was produced in modified *Escherichia coli* of the strain XL1-blue and subsequently successfully purified and separated from GST. The bacterial over-expression of the GST-rfTNFα fusion protein was induced by adding 75 µl of 1 M Isopropyl β-D-1-thiogalactopyranoside (IPTG) to each bacterial culture (final IPTG concentration: 0.3 mM). Subsequently, the cultures were incubated at 37°C for 12 hours while continuously being agitated at 180 RPM. After the induction of fusion protein expression, a lower incubation temperature of 30°C was used to decrease the rate of bacterial cell division. Often recombinant over-expressed proteins accumulate in inclusion bodies within bacterial cells when the rate of protein expression is high. By lowering the temperature, the rate of the protein synthesis is also lowered. This improves the solubility of the expressed protein. Additionally, the intracellular accumulation of the protein generally inhibits/prevents intracellular processes essential for cell function/ survival (OTTO et al., 1995). This phenomenon is known as “choking”. By lowering the temperature, a higher yield of total fusion protein was reached, probably because a greater number of bacteria survived for a longer period of time.

Recombinant fTNFα was purified, using the described purification protocol. The purity of the resultant protein was greater than 95.5%, as measured by SDS-PAGE analysis (Figure 4) and a quantification tool of the Quantity One software package. This protein was considered sufficiently pure to be used for the inoculation of live animals to produce antibodies against the protein.

Chromatography, using self-packed glutathione-agarose containing columns (8 × 40 mm), was found to yield better results than the use of an automated fast pressure liquid chromatography (FPLC) system. One explanation for this was that the self packed columns allowed for a very long incubation time (24 hours) during certain steps of the purification. A longer incubation time between the rfTNFα-GST fusion protein and the glutathione-agarose

was found to be necessary in order to ensure binding of the GST portion of the fusion protein and the glutathione binding sites of the resin. The incubation of the glutathione-agarose-fusion protein mixture was therefore performed on a rocking plate at 4°C. Using an automated FPLC system the sample would have had to been re-circulated multiple times through the column to ensure binding. Since the samples were high in protein concentration at this point, this could have led to protein accumulation in the buffer filters of the machine. Another disadvantage of the automated system is that since the machine does not offer the option to control the temperature, the incubation would have had to take place at room temperature. Over the long period of incubation this could have potentially led to the protein being damaged.

The biological activity of the rfTNFα has previously been demonstrated by the intravenous administration of the fusion protein into cats (RIMSTAD et al. 1995). The animals subsequently showed clinical signs compatible with TNFα exposure, such as depression, fever, protrusion of the nictitating membranes, and piloerection (RIMSTAD et al., 1995). Since there was no necessity for the purified protein to have biological activity to produce anti-rfTNFα antibodies, we did not repeat these experiments. In our opinion, to do so would have been ethically unjustifiable.

## 2. Partial Characterization of rfTNFα

Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) was used to determine the relative molecular mass of rfTNFα. With this technology a laser beam is directed onto a sample spot of a proprietary ProteinChip®, causing desorption and ionization of the loaded proteins. A defined laser beam pattern is used to selectively cover the entire spot surface. This allows repeated readings of a single spot, without using the same position twice. At each position, the spectrum of ionized proteins is measured, then the spectra from multiple positions are averaged in a final spectrum in which the mass-to-charge ratios (m/z) of the ionized proteins are detected. With this method, a good correlation between signal intensity and analyte concentration can be achieved for each peptide or protein in the samples. All calculations are performed automatically by a proprietary software package. Finally, a graph is generated from which the information regarding the relative molecular mass of the protein of interest can be deduced by analyzing the m/z of the generated peaks. The m/z value is not equal to, but is comparable with the unit of the relative molecular mass of the proteins in the sample.

Two distinct protein variants of rfTNFα were identified using SEDLI-TOF-MS. One had a relative molecular mass of 16.93 kDa, while the other one had a relative molecular mass of 17.57 kDa (Figure 5). These results are similar to the relative molecular masses previously reported for TNFα from other mammalian species (Table 15). By knowing the structure of the 157 amino acids that make up the native feline protein, Rimstad et al. (1995) estimated that the relative molecular mass of rfTNFα should be 17.9 kDa, which agrees very well with our findings.

N-terminal amino acid sequencing was performed on a sample containing both protein variants of rfTNFα using the Edman degradation method (EDMAN, 1950). Performing this method, the first amino acid at the N-terminus of the protein chemically reacts with phenylisothiocyanate to form a phenylthiocarbamyl derivative. The anilinothialinone derivative of trifluoracetic acid is then used to cleave this amino acid from the polypeptide strand. During this process, the derivative converts into a phenylhyantoin derivative of the amino acid, which forms a complex with the free amino acid, and thus is more stable than the anilinothialinone derivative (EDMAN, 1950). The amino acid-phenylhyantoin derivative complexes are subsequently separated by a high-performance liquid chromatography (HPLC) and detected by fluorescent detection in a fluorescence detector for high-sensitivity analysis.

Analysis revealed a slight variation at the N-terminal amino acid sequence of the two protein variants (Figure 6). The size difference of approximately 0.7 kDa can therefore in parts be explained by 3 missing serine residues (molecular weight of around 0.315 kDa) in the variant of rfTNFα with the smaller relative molecular mass. Still, there have to be other differences such as additional amino acid residues in an area which was not sequenced. A complete analysis of the molecules would be nessecary to exactly characterize the differences.

The analysis also showed a homology of 100% of both protein variants with the amino acid sequence of mature feline TNFα as predicted by the nucleotide sequence and published through the NCBI database (Figure 6; MCGRAW et al., 1990). However, it should be noted that only a small portion of the amino acid sequence of rfTNFα (11 of 157 amino acids) was analyzed and compared to the predicted sequence of fTNFα. Therefore, the homology between the rfTNFα amino acid sequence and the predicted sequence of native fTNFα could not be fully evaluated.

**Table 15:** Relative molecular mass of TNFα from different mammalian species.

| Species | Molecular weight of TNFα (kDa) | Reference |
|---|---|---|
| human | 17 | (AGGARWAL et al., 1985) |
| mouse | 17-25 | (SHERRY et al., 1990) |
| rabbit | 18 | (ABE et al., 1985) |
| guinea pig | 17 | (SHERRY et al., 1990) |
| dog | 17 | (ZUCKER et al., 1994) |

In this table the relative molecular mass of TNFα from different mammalian species are shown. Note that all the publications report masses of approximately 17 kDa. This is similar to our findings of relative molecular masses of 16.93 kDa and 17.57 kDa for the two proteins.

## 3. Production of anti-rfTNFα Antibodies

Anti-rfTNFα antibodies were successfully produced by injecting the purified protein into two New Zealand White rabbits (*Oryctolagus cuniculus*). Inoculation of the protein caused an immune reaction that led to the production of monospecific polyclonal antibodies.

Polyclonal, as well as monoclonal, antibodies can be used in immunoassays. The use of polyclonal antibodies was chosen for the fTNFα ELISA for a number of reasons. Firstly, the production of polyclonal antibodies was anticipated to be more cost effective than the production of monoclonal antibodies. Also, the production of polyclonal antibodies was anticipated to be more time efficient than the production of monoclonal antibodies. Additionally, polyclonal antibodies have been used successfully in our laboratory in previously developed immunoassays (RIA and ELISA) for the measurement of a variety of protein markers.

The production of monospecific polyclonal antibodies by inoculation of a purified antigenic protein has been well described in various species of animals. In this study the rabbit was chosen as a host species because it is smaller, easier to handle, and more cost effective than the use of larger mammalian species, such as sheep or horses. Also, rabbits can be bled repeatedly relatively safely. Furthermore, rabbit antibodies are well characterized and easy to purify (COPLEY et al., 1996). Lastly, our laboratory has a lot of experience in the use of rabbits as host species for antibody production for use in immunoassays (HEILMANN et al., 2008; STEINER et al., 2000; STEINER and WILLIAMS, 2003). Some investigators recommend using multiple animals for the production of antibodies against the same antigen in order to ensure that at least one animal produces antibodies of sufficient sensitivity (COPLEY et al., 1996). Consequently, protein was injected into 2 rabbits.

The antibody titer was considered sufficiently high to collect the serum from the animals by terminal bleeding, after 3 and 4 vaccinations, in rabbit number 1 and 2, respectively. Using affinity chromatography a total yield of 12.3 mg of anti-rfTNFα antibodies was purified from the serum of rabbit number 1 (Figure 9). This quantity was more than sufficient for the development and analytical validation of the fTNFα ELISA.

The anti-rfTNFα antibodies that were produced were shown to be species-specific for rfTNFα. Trials to detect canine TNFα in serum samples from dogs showed poor results (data not shown). Therefore, we conclude that cross reactivity with canine TNFα is very weak. These observations agree with those from a previous investigation (JONES et al., 1992). The authors of this study postulated that the production of species-specific antibodies might be

necessary because of a high interspecies variation of hydrophobic regions of the TNFα molecule (JONES et al., 1992). These highly variable hydrophobic regions are thought to be the main binding site for anti-TNFα antibodies (OTTO et al., 1997).

## 4. Development and Analytical Validation of an ELISA for the Measurement of fTNFα in Serum

A direct sandwich ELISA for the measurement of fTNFα in serum samples from cats was successfully developed and analytically validated. In order to develop this ELISA, different standard protocols and buffers were studied, in combination with varying incubation times. The protocol described here enabled us to successfully measure concentrations of fTNFα as low as 38.4 ng/l in cat serum.

For the validation of the assay, detection limit, linearity, accuracy, precision, and reproducibility were determined. Target values for the observed to expected ratios (O/E), for dilutional parallelism (linearity), and spiking recovery (accuracy) were set to be between 80 and 120%. The maximum accepted coefficients of variation (%CV) for intra-assay variability (precision) and inter-assay variability (reproducibility) were set to be 10 and 15% respectively. While there is no consensus about the validity of these criteria for assay validation in the scientific community, other immunoassays of proven clinical relevance have met these targets (FETZ et al., 2004; STEINER et al., 2003; STOLL et al., 2007).

The detection limit of the assay was determined to be 38.4 ng/l. The results of the dilutional parallelism showed that the assay had sufficient linearity to be of clinical use. The 4 samples had O/E between 75.1% and 111.9% (Table 6). These ratios were considered to be acceptable for clinical use despite the value of 75.1% being outside the target range. A likely explanation for this result was the low concentration of fTNFα at that particular dilution of 53.1 ng/l. This value is close to the detection limit of the ELISA and may lie within a range of the assay that shows a lower level of accuracy than in the middle range of the assay. Similar findings have also been reported for other assays for marker molecules (FETZ et al., 2004; STEINER et al., 2003). Measurements for spiking recovery demonstrated that the ELISA had an acceptable accuracy (Table 7). The 4 samples showed O/E between 91.3 and 113.8%. Analysis of the 4 samples that were used to evaluate the intra-assay variability demonstrated acceptable %CVs. These ranged from 4.6 to 7.6% with a mean (±SD) of 5.0% (±1.76%; Table 8). The inter-assay variability for 4 serum samples ranged from 7.6 to 12.5% with a mean (±SD) of 9.5% (±2.2%; Table 9). The results for both intra- and inter-assay variability were considered acceptable for clinical use of the assay.

## 5. Establishment of a Reference Interval for fTNFα ELISA

A reference interval of l was established using serum samples from 20 healthy pet cats. It should however be noted that serum fTNFα was only detectable in the serum of 4 cats (Table 10). The upper limit of the reference interval (<223.5 ng/l) was greater than that reported for other species (Table 4). However, it is important to note that reference intervals established for different assays cannot be directly compared to each other. The reason for this is that immunoassays, such as ELISAs do not measure the actual mass-concentration of the analyte.

## 6. Serum fTNFα Concentration from Cats with Gastrointestinal Disease

In order to determine the potential clinical usefulness of the measurement of fTNFα in cats with chronic enteropathies, serum samples from 39 cats with clinical evidence of chronic enteropathies were evaluated, using the fTNFα ELISA.

First we compared the serum concentrations of fTNFα between the group of 20 healthy cats that we had used to establish the reference interval and the group of the 39 cats with clinical evidence of chronic enteropathies. No statistically significant difference of serum fTNFα concentration was found between those two groups.

Based on their clinical presentation the 39 diseased cats were further grouped into two subgroups. Group 1 (n = 23) included cats that showed chronic signs of enteropathies, but no diarrhea, while group 2 (n = 16) included cats with diarrhea. A grouping based on other clinical signs than diarrhea like vomiting, abdominal pain, fecal incontinence, anorexia, depression, or weight loss was not undertaken, as the group sizes would have been too small for statistical analysis.

Serum fTNFα concentrations between the three groups of cats (two subgroups of cats with clinical signs of chronic enteropathies, group of 20 healthy cats) varied significantly. No statistically significant difference of serum fTNFα concentrations of cats in group 1 and the group of healthy cats could be found. However, serum fTNFα concentrations were significantly different between the two subgroups of cats with clinical signs of chronic enteropathies and also between group 2 and the group of 20 healthy cats.

Also, a statistically significant difference between the medians of the two subgroups of $p = 0.0008$ was found.

A statistically significant difference between the proportions of cats whose sera showed concentrations of fTNFα above the upper limit of the reference interval (<223.5 ng/l) and those within the reference interval between the two subgroups was found (p-value = 0.0127; Table 13). Only 3 of 23 cats of group 1 had detectable serum fTNFα concentrations and only 1 cat had a serum fTNFα concentration above the upper limit of the reference interval in this group. In contrast, 10 cats in group 2 had detectable serum fTNFα concentrations, 7 of which were above the upper limit of the reference interval.

As inflammatory bowel disease is one of the most common causes of chronic diarrhea in cats (NGUYEN et al., 2006; HALL and GERMAN, 2008) one possible interpretation of these results is that the animals in group 2 were the ones with IBD or alternatively a more severe form of IBD than the ones that did not show diarrhea at the point when serum samples were

collected. However, this distinction was beyond the scope of this clinical pilot study as results of endoscopy and histopathology were not available for any of the cats enrolled.

However, this pilot study would suggest that prospective clinical studies to further evaluate the clinical usefulness of the measurement of fTNFα concentrations in cats with clinical signs of chronic enteropathies are warranted.

## 7. Conclusion

In conclusion, recombinant feline TNFα was successfully purified from previously modified *Escherichia coli*. A direct sandwich enzyme-linked immunosorbant assay for the measurement of feline TNFα in serum of cats was successfully developed and analytically validated. Serum concentration of the fTNFα was measured in samples from 20 healthy pet cats and a reference interval was determined based on these results.

Serum concentrations of feline TNFα were also measured in serum samples from 39 cats with chronic enteropathies and the results were statistically analyzed. The cats were grouped into 2 groups. The median serum concentration of fTNFα was significantly higher in group 2 (cats with diarrhea) compared to group 1 (cats with chronic enteropathies, but without diarrhea).

Further studies are needed to determine whether the measurement of serum fTNFα concentrations in cats with chronic enteropathies is clinically useful for facilitating definitive diagnosis or assessment of progression.

## VI. SUMMARY

**Development and Analytical Validation of an Enzyme-linked Immunosorbent Assay for the Measurement of Feline Tumor Necrosis Factor Alpha in Serum**

Tumor necrosis factor α (TNFα) is a cytokine that is mainly produced by macrophages and monocytes. It is a member of a large family of proteins, the TNF family. A lot of research in different species has been undertaken regarding the usefulness of this protein as a marker for a variety of diseases. The aim of this study was to develop a simple, reproducible protocol for the purification of recombinant feline TNFα from modified *Escherichia coli*, the partial characterization of the rfTNFα protein, the production of anti-rfTNFα antibodies, as well as the development and analytical validation of an ELISA for the measurement of feline TNFα in serum from cats. We also aimed to measure serum fTNFα concentrations in cats with chronic enteropathies.

An efficient and reproducible protocol for the purification of rfTNFα from *Escherichia coli*, modified to express fTNFα was established. The specific absorption and the molecular weight of the protein were evaluated. An ELISA for the measurement of fTNFα concentrations in serum from cats was developed and analytically validated. A reference interval was established by determination of the central 95$^{th}$ percentile in a group of 20 healthy cats. Lastly, serum concentrations of fTNFα in cats with chronic enteropathies were evaluated.

The specific absorbance of rfTNFα was found to be 1.75. The relative molecular mass was estimated at 16.93 and 17.57 kDa for 2 isoforms of rfTNFα. The N-terminal amino acid sequences for both isoforms of the protein were found to be R-T-P-S-D-K-P-V-A-H-V and S-S-S-R-T-P-S-D-K-P-V, respectively. Both sequences showed 100% homology with the amino acid sequence of mature feline TNFα, as predicted by the nucleotide sequence of the protein. The isoelectric point of rfTNFα was estimated at approximately 5.3.

The ELISA for the measurement of serum concentrations of fTNFα in cats was determined to be sufficiently sensitive, linear, accurate, precise, and reproducible for clinical use. The reference interval for fTNFα in serum from healty cats was established at <223.5 ng/l. Feline TNFα was only detectable in 33.3% of cats with chronic enteropathies.

In conclusion, a sensitive, linear, accurate, precise, and reproducible assay for the measurement of TNFα in cat serum was developed. Further studies are needed to explore if serum fTNFα concentrations could serve as a useful marker for the diagnosis, staging, and monitoring of feline patients with chronic enteropathies.

# VII. ZUSAMMENFASSUNG

## Entwicklung und Validierung eines Enzyme-linked Immunosorbent Assays zum Messen von felinem Tumornekrosefaktor alpha in Serum

Tumor Nekrose Faktor α (TNFα) ist ein Zytokin, welches hauptsächlich von Makrophagen und Monozyten produziert wird. Das Protein gehört zu einer größeren Proteinfamilie, der TNF-Familie. Es wurden zuvor viele Untersuchungen an verschieden Spezies durchgeführt, um den Nutzen des Proteins als Marker für unterschiedliche Erkrankungen zu evaluieren. Ziel dieser Studie war es, ein einfaches, reproduzierbares Protokoll für die Reinigung von rekombinantem felinem TNFα (rfTNFα) aus modifizierten *Escherichia coli* zu etablieren, das rfTNFα-Protein teilweise zu charakterisieren, anti-rfTNFα-Antikörper zu produzieren und einen ELISA für das Messen von Serumkonzentrationen von felinem TNFα in Proben von Katzen zu entwickeln und zu validieren. Des Weiteren sollten Serum-fTNFα-Konzentrationen von Katzen mit chronischen Enteropathien bestimmt werden.

Ein effizientes, reproduzierbares Protokoll für die Reinigung von rfTNFα aus modifizierten *Escherichia coli* wurde angefertigt. Der Extinktionskoeffizient, sowie die relative molekulare Masse des Proteins wurden bestimmt. Ein ELISA für das Messen von fTNFα-Konzentrationen in Katzenserum wurde entwickelt und validiert und ein Referenzbereich wurde durch die Bestimmung von Serumkonzentrationen von TNFα bei einer Gruppe von 20 gesunden Katzen bestimmt. Zudem wurde die Serumkonzentration von fTNFα bei Katzen mit chronischen Enteropathien gemessen.

Ein Extinktionskoeffizient von 1,75 wurde für rfTNFα ermittelt. Die relative molekulare Masse zweier Isoformen von rfTNFα war 16,93 bzw. 17,57 kDa. Die N-terminale Aminosäurensequenz der beiden Isoformen des Proteins war R-T-P-S-D-K-P-V-A-H-V und S-S-S-R-T-P-S-D-K-P-V. Beide Sequenzen zeigten eine 100%ige Homologie zu der Aminosäurensequenz von felinem TNFα, welche mit Hilfe der bekannten Nukleotidsequenz des Proteins ermittelt worden war. Der isoelektrische Punkt von rfTNFα liegt bei etwa 5,3.

Der ELISA für das Messen von fTNFα-Serumkonzentrationen bei Katzen war ausreichend sensitiv, linear, akkurat, präzise und reproduzierbar, um klinisch anwendbar zu sein. Das Referenzintervall von fTNFα bei gesunden Katzen betrug <223,5 n/l. Messbare Konzentrationen von fTNFα fanden sich nur bei 33,3% der Katzen, welche an chronischer Enteropathie litten.

Ein sensitiver, linearer, akkurater, präziser und reproduzierbarer Assay für die Messung von TNFα in Katzenserum wurde entwickelt. Weitere Studien sind notwendig, um zu erforschen, ob Serum-fTNFα als Marker für die Diagnose, Charakterisierung oder Verlaufskontrolle von chronischen Enteropathien bei der Katze Anwendung finden kann.

## VIII. REFERENCES

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# VIII. APPENDIX

## A.1. List of figures

## A.2. List of Tables

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**SELBSTSTÄNDIGKEITSERKLÄRUNG**

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

Viktoria M. Schwierk