V.   DISCUSSION

Improved control strategies against tuberculosis and other infections require the development of diagnostic tests and vaccines (Barnes, 2004; Dalley et al., 1999). Prevalence estimation in the captive wildlife populations has been hampered by diagnostic tests that are often difficult or impossible to conduct and/or interpret (i.e. due to the requirement for multiple immobilizations). Furthermore, the almost complete lack of validation, optimization and standardization of many of the available test methods in the species of interest (zoo animals) remain a problem. Besides, current methods for diagnosis of infection rely mainly on the detection of the pathogen (e.g. *via* ELISA or PCR) or on the detection of antibodies. Moreover, the focus of rapid diagnosis of infectious disease in the last decade has shifted from the conventional laboratory techniques of antigen detection, microscopy and culture to molecular diagnosis of infectious agents. For some diseases, however, the pathogens are not easily detectable (e.g. *Mycobacteria* or *Herpesviruses*) or antibodies are not detectable at all times of infection. It is therefore important to assess the nature of the immune responses that occur after infection or vaccination and develop diagnostic techniques, which include T cell responses.

Mediating an effective immune response against intracellular pathogens such as *Mycobacteria* or viruses requires a concert of action performed by macrophages and T lymphocytes with the help of molecular mediators such as cytokines that offer alternatives to conventional diagnostics (Barnes et al., 1990; Abbas et al., 1994). Cytokines are critical modulators of physiological and pathological immune responses, produced by a variety of cells and mediate diverse regulatory immune functions including activation, proliferation, differentiation, survival and apoptosis of immune cells. Work in murine models has made it clear that the induction of protective resistance or the exacerbation of disease is often dependent upon the pattern of cytokine genes expressed during an immune response (Urban et al., 1996; Lohoff et al., 1998). Polarized Th1 cells produce IFNγ to promote macrophage activation and in addition, development of cytotoxic T cells which support DTH reactions serving as effector mechanisms against intracellular pathogens. A hallmark of Th2 cells is the secretion of IL-4, resulting in the up-regulation of antibody production. Thus, these two cytokines mediate the most typical features of the respective helper cell subsets. In addition, they are of importance for antagonistic regulation of Th cell differentiation itself as IL-4 enhances a Th2 response and suppresses a Th1 response; conversely, IFNγ enhances a Th1 response and suppresses a Th2 response. In light of the Th1/Th2 paradigm, much work has concentrated upon these two key cytokines and I also started my work on zoo animal

immunology to clone these two cytokines. The aim of this thesis was to establish the principle of an immunologic assay suitable for the diagnosis of intracellular pathogens in zoo and wildlife animals.

Increasing availability of cytokine sequences has opened the way to a transcript-based evaluation of cytokine patterns. Unfortunately, measuring the quintessential Th2 cytokine IL-4 has proven particularly difficult. IL-4 is a labile, highly bioactive protein and generally present in relatively low quantities, making its measurement problematic (Ekerfelt et al., 2002). However, detection of cytokine message allows to-overcome this limitation, particularly where antibodies are not easily available. The general hypothesis was that there is a change in the Th1/Th2 balance during the development of a protective cellular immunity.

My choice was to set up a test system based on real-time PCR as an extremely sensitive, rapid technology capable of high throughput and relatively easy to perform in wildlife and zoo animals without the need for cross-reactive antibodies. Such assay would hopefully provide a valuable tool for the assessment of T-cell functions *in vitro* and could be standardised as quantitative assay system for the detection of intracellular pathogens. This reliable system could be applicable in the future for monitoring the animal immune status against tuberculosis, *herpesviruses* or other intracellular infections.

For enabling species-specific real-time PCR immunoassays, I cloned IFNγ as well as IL-4 cDNA in a suitable plasmid vector from mitogen-activated PBMC of different zoo species. Horse was selected to be the animal model whose, PBMC and RNA would be used to establish the assay.

Cytokines are generally well conserved and so the GenBank reported sequences of human, bovine, horse and mouse were successfully used to delineate primer pairs for a range of zoo and wildlife animal species. However, it turned out soon that a single round PCR was not sufficient to generate discrete bands of the predicted size. Accordingly, two pairs of primers for each gene were designed. The first outer primer pair was designed to flank an area outside the ORFs while the inner primer pair was designed to span precisely the size of the ORFs. Although this approach was successful for all species analysed, it was slightly disappointing that a nested-PCR was necessary. Due to this approach the 5´ and 3´ ends of all IFNγ ORFs were identical between the species. Thus, minor differences due to evolution in this area would not be detected. Such differences should not have an effect on the N-terminus of the putative protein, since the primers end well before the cleavage site of the signal peptide. However, at the putative C-terminus a point deletion could result in a frame-shift such as that

recently shown for horse GM-CSF (Mauel et al., 2006). To detect such differences further techniques need to be applied, such as 3´ end RACE cloning.

IFNγ ORF sizes obtained from all species were equal in length (501 bp) encoding for 166 aa. IFNγ sequences of *Perissodactyla* (horse, wild ass, zebras and rhinoceros) agreed very well with previous equine sequences (Mauel, 2002; Steinbach et al., 2002). Alike, both African buffalo and European bison IFNγ sequences agreed to a high extend with previous data of cattle, elk, sheep, goats and llama (Cerretti et al., 1986; Radford et al., 1991; Beyer et al., 1998; Odbileg et al., 2004). Although most basic immune mechanisms have been conserved, there are considerable diversities between the species due to their various histories of life and challenges by pathogens requiring adaptation (Steinbach et al., 2002). The same primer pairs succeeded to amplify IFNγ ORFs form all tested species and the analogous approach used for IL-4 succeeded with all species except Grevy's zebra. This IL-4 amplification failure is likely due to a point mutation at one of the IL-4 primer annealing sites. Particularly at their 3´end primers are more prone to be affected by point mutations.

Since the animal's immune systems are essential pillars of life, the genetic conservation and diversity of species are reflected therein. Within such context homology is a qualitative description of the species relation. Homology analysis by sequence alignment was considered the basis of relation analysis. Alignment of all putative amino acid sequences in comparison to reported human, bovine and mouse genes supported the notion that sequences grouped well along their zoological classification (Fig. 4 and 8). Thus, the exons of the IFNγ and IL-4 genes are to a more limited extent homologous over all species and to a higher extent identical between the members of the same family, such as within the families *Equidae* or *Bovidae* (tables 1 and 3). Moreover, animal sequences were more closely related between each other than to humans and mice represent the out-group. Accordingly, bovid proteins are more likely to react with equids proteins than with human proteins.

For IL-4 the situation was slightly more complex than for IFNγ and IL-4 ORFs differed significantly between species. Again, there was a high degree of conservation among species of the same family, where in the case of *Equidae* all ORF sequences encoded for 137 aa. There was more diversity among the families, suborders and orders than observed in the case of IFNγ. This heterogeneity was most evident as the size of cDNAs (table 2 and Fig. 6) and putative aa consequently differed (table 2 and Fig. 7). The Indian rhinoceros IL-4 ORF cDNA encoded for 139 aa (two aa more than equids) but also belongs to order *Perissodactyla*.

African buffalo and European bison IL-4 ORF cDNAs were found to be 408 bp in length, the same size as red deer (*Cervus elaphus*) IL-4 (Hook et al., 1994) and homologous to the reported bovine, ovine and caprine sequences, encoding for 135 aa (Heussler et al., 1992; Cheevers et al., 1997; Chaplin et al., 2000).

Although phylogeny was not a major objective in my thesis and despite the question whether a phylogenetic tree based on the comparison of a single gene and so few species is sufficient to represent evolution, additional information has been gained from pairwise analysis and subsequent phylogeny of protein sequences where the genetic diversity was reflected. Pairwise protein analysis of both of IFNγ (table 1) and IL-4 (table 3) divided the cloned cytokines into five main groups. The first consisted of bovids (African buffalo and European bison), the second represented by the giraffe only, the third contained the elephant while the fourth included horse, wild ass and zebras *(Equidae)* and the fifth was composed of rhinoceros only. Thus, the concise phylogenetic pattern of these five groups supported the known mammalian orders, established on zoological grounds according to generally accepted kinship. For example, IFNγ protein analysis provided higher identity between African buffalo or European bison (97 and 98%) to reported sequences of domesticated cattle as sister species in the family *Bovidae tribe bovini*. Such data were in accordance with previous studies, which suggested the genus *Bos* to be paraphyletic with respect to the genus *Bison* (Wall et al., 1992). Moreover, bovine sequences had lesser identity compared to the Nubian giraffe, which also belongs to the *tribe bovini*. This supports a recent study suggesting that *Giraffidae* and *Antilocapridae* constitute the superfamil*y Giraffoidea* as a sister group of a clade clustering *Bovoidea and Cervoidea,* as determined from mitochondrial DNA sequences (Miyamoto et al., 1989; Fernandez and Vrba, 2005). Alike was the situation among perissodactyls where the identity ranged from 98-100% among all *equus* as siblings of the family *Equidae*. This identity decreased to 91-92% comparing equids to the Indian one-horned rhinoceros, which belongs to another family (*Rhinotidae*) in the same order *Perissodactyla*.

Pairwise analysis of the IL-4 deduced proteins (table 3) revealed strong interspecies identities between *Perissodactyla species* (horse, Hartmann's mountain zebra, Somali wild ass and Indian rhinoceros). Interestingly, IL-4 identity among different bovids was as high as 100% when African buffalo, European bison and reported sequence of domesticated cattle were compared (table 3). Accordingly, the differences in the cDNA sequences all resembled silent mutations. The closer relationship between perissodactyls or artiodactyls was in accordance with the results of Odbileg et al. (2005) studying the homology and phylogeny between

artiodactyls, finding a close relationship. Likewise, they found a closer relation of tylopods (llama and camels), as ruminant-like animals, with cattle than when compared to humans or mice. It is noteworthy that true ruminants are a most diverse group, represented by 61 genera and approximately 163 species (or even 197 species including extant and recently extinct ruminants). There are four families in modern faunas; *Giraffidae* (giraffes), *Cervidae* (deer), *Antilocapridae* (pronghorn antelope) and *Bovidae* (sheep, goats, cattle, buffalo, bison and antelopes). The fourth (i.e. *Bovidae*) is a large and diverse family, currently represented by 45 genera and 137 species. The tylopods (camels and llamas) are not considered ruminants despite their ruminant-like digestive function (Grubb, 1993; Fernandez and Vrba, 2005). Also, Nubian giraffe provided a higher identity with *Perissodactya* in both genes (IFNγ and IL-4) than other *Artiodactyla*. In addition, giraffe IL-4 ORF size was 402 bp, encoding for 133 aa, which is exactly the same as the Bactrian camel (*Camelus bactrianus)* and the bottle-nosed dolphin (*Tursiops truncatus*) IL-4 (Inoue et al., 1999; Odbileg et al., 2006), but shorter than known cloned representatives of *Bovidae* or *Cervidae.*

The analysis of IFNγ and IL-4 dendrograms (Fig. 4 and 8) also reflected the kinship between the animals belonging to the same zoological suborder shown as clustering of all the perissodactyls in one group which branched into 2 distinct subgroups, each belonging to a different suborder (Fig. 4 and 8). The first included equids and other animal species of the same genus (*equus*), all part of the suborder *Hippomorpha* family *Equidae,* while the second consisted of a sole animal species (*Rhinoceros*) belonging to the suborder *Ceratomorpha,* family *Rhinocerotidae*. Analogous was the determined relation among cytokines of artiodactyls, where sequences of the same suborder *Rumenatia* (ruminants) branched into two major categories, representing members of two families; *Giraffidae* and *Bovidae.* Additionally, the ultimate ungulates (i.e. members of order *Perissodactyla* as well as of order *Artiodactyla*) were closer to each other than the almost ungulates represented by the Asian elephant as one of three species of family *Elephantidae* order *Proboscidea* (Klingel and Thenius, 1990; Rohrs and Thenius, 1990; Fernandez and Vrba, 2005).

IFNγ phylogenetic tree provides a visual account of the close relationship among IFNγ proteins of perissodactyls with few differences between equids and Indian rhinoceros. According to recent taxonomic classifications (Williams, 2002; Groves and Bell, 2004), Hartmann's mountain zebras belong to *Hippotigris* and the Grevy's zebra is the sole species of *Dolichohippus*. Grevy's zebra, however, appeared to be associated to the Hartmann's mountain zebra in the IFNγ dendrogram. This is consistent with the division of genus *Equus*

into three subgenera collecting all zebras as members in one subgenus (Skinner and Smithers, 1990).

Alternative splicing of mRNA generates protein isoforms that are preferentially expressed in different tissues or during different states of cell differentiation or activation. Such protein isoforms may have different functions (Atamas et al., 1996) and play an important role within the cell, increasing the diversity of cellular function. Alternative splicing might represent a rather common feature among cytokine transcripts. Splice variants are possibly associated with disease states and they may play an important role in immune regulation (Waldvogel et al., 2004). Splice variants have been shown for several cytokines and cytokine receptors such as IL-2, IL-4, 1L-7 and IL-13R in humans (Atamas et al., 1996; Tsytsikov et al., 1996; Krote et al., 1999; Chiaramonte et al., 2003). Recombinant proteins of human IL-4 splice variant were shown to inhibit IL-4 stimulated T cell proliferation (Atamas et al., 1996). Furthermore, IL-4δ2 inhibited the effects of IL-4 on human B cells and monocytes (Arinobu et al., 1999), antagonized the synthesis of IgE and expression of CD23 on B cells and blocked the inhibitory action of IL-4 on LPS induced cyclooxygenase-2 expression and prostaglandin E2 secretion by monocytes. It has been suggested that the balance of IL-4 isoforms may modulate asthmatic inflammation (Glare et al., 1999; Seah et al., 2001) and the disproportionate levels of IL-4 relative to IL-4δ2 may contribute to the pathogenesis of certain diseases such as tuberculosis (Seah et al., 2000) and systemic sclerosis (Sakkas et al., 1999). Hence IL-4δ2 may come within the scope of the complex network of cross talking Th1/Th2 cytokines, by acting as a further control level to determine immune response phenotypes and disease outcome. Most importantly for my work, IL-4 sequence-based detection remains the only approach available to date allowing for the discrimination between alternative splice variants, since IL-4 directed monoclonal antibodies equally detect both isoforms (i.e. IL-4 and IL-4 splice variant). Validated protein assays capable of distinguishing between the two variants have yet to be developed in order to relate transcription levels and biological activity (Gautherot et al., 2002).

In addition to human IL-4δ2, splice variants of bovine IL-4 were also detected when initial RT-PCR for IL-4 was established (Waldvogel et al., 2004). Further IL-4δ2 splice variants have been described in cynomolgus macaque (*Macaca fascicularis*), chimpanzee (*Pan troglodytes*), lagomorphs (European rabbits) and *Muridae* (woodchuck and mice) (Gautherot et al., 2002; Yatsenko et al., 2004).

I can add here the first report of giraffe Interleukin-4 alternative splice variant, where again the second of four exons (48 bp) was omitted and it was therefore designated giraffe IL-4δ2 (Fig. 10 and 11). This splice variant was observed during giraffe IL-4 RT-PCR as the dominant amplicon (Fig. 9-A). Although the RT-PCR does not act quantitatively at this attempt IL-4δ2 was clearly over-expressed. Functional studies are, however, required to confirm the IL-4δ2 antagonizing activities. Independently, this finding emphasizes the importance to consider such IL-4 splice-variants when setting up QRT-PCR (Demissie et al., 2004; Fletcher et al., 2004; Rook et al., 2004). At best, one of the primers or the probe should be placed at the border between two exons.

The comparative analysis of the exon-1 to exon-2 junction region underlined for all *Perissodactyla* species IL-4 sequences the existence of a six-base insertion at the junction site. Gautherot et al. (2002) predicted in reverse that horses (zebras, wild ass, rhinoceros as well as other *Perissodactyla* species) are not expected to have an IL-4 splice variant and indeed all my attempts to detect a second transcript upon various stimuli or time points were not successful. If prediction parameters as described are correct, pigs should also have IL-4 splice variant, but this requires further investigations.

Cytokine cloning was followed by the expression of horse IFNγ and IL-4 in an attempt to analyse their activity *via* bioassays such as described for eqGM-CSF (Steinbach et al., 2005). IFNγ-treated-monocytes altered their morphology resulting in clustered, large, adherent cells resembling macrophage-like cells. The data support the view of eqIFNγ as a stimulator of monocytes and agree with previous results (Döcke et al., 1997; Steinbach et al., 2000). The attempt to establish a bioassay based on horse monocytes MHC II expression was not so successful. Although an effect was seen (Fig. 12) it became evident that standardization between the different monocyte donors was not achievable here.

IL-4 is a major modulator of monocyte differentiation into dendritic cells (Ruppert et al., 1991; Peters et al., 1996). The TCA/DOC concentrated eqIL-4 protein was electrophoresed (Fig. 14) to check its size and approximately 18 kDa was in agreement with previous data (Dohmann et al., 2000; Mauel, 2002). Equine peripheral blood monocytes strongly express the antigen CD14 and human IL-4 has been found to affect the phenotype and a variety of functions of monocytes and macrophages. Parallel to altering monocyte morphology into dendritic cells, IL-4 induces the down-regulation of CD14 expression. Again, while I was able to observe the morphologic changes expected (Fig. 13), the modulation of CD14 was less clear-cut dose dependent. The results were somewhat inconsistent but comparable to the

human monocytic cell lines MUTZ-3 and MONO-MAC-6, where human IL-4 down-regulated the expression of CD14 on MUTZ-3, but not on MONO-MAC-6 cells (Quentmeier et al., 1994; Quentmeier et al., 1996). In the settings used in this study, it may be possible that the effects of exogenous eqIL-4 were influenced by the presence of too many remaining lymphocytes. These lymphocytes may both consume IL-4 (essentially B-lymphocytes) as well as produce IL-4 (mainly Th2 lymphocytes).

Gene expression profiles reflect the cumulative interaction of many events and mRNA levels sensitively represent the state of cell responses. Since mRNA is eventually translated into protein, one may assume that there is a correlation between the level of mRNA and protein. Based on this assumption, Mena et al. (2002) demonstrated a significant correlation between mRNA and protein expression for bovine IFNγ using real time RT-PCR. In addition, previous studies proved the usefulness as well as reliability of real-time PCR assessing Th1 and Th2 cytokines expression (Demissie et al., 2004; Li et al., 2004; Ocmant et al., 2005; Rama-Iniguez et al., 2006).

It is noteworthy that there are major advantages of using real-time PCR compared to conventional semi-quantitative PCR. First of all, the progression of the PCR reaction may be monitored after each cycle rather than at the end, thereby providing a much better quantification. A drawback of real-time PCR over northern analysis (or PCR to some extent) is that it can neither detect the quality (i.e. the size) of an RNA message, nor the number of differently sized messages (e.g. splice-variants).

Quantitative competitive (QC) RT-PCR was used earlier, but required co-amplification of a standard 'competitor', together with the unknown sample in the same reaction. For analysis, this method requires post-PCR manipulations, such as agarose gel electrophoresis. Improvements in PCR have broadened its diagnostic capabilities in clinical infectious diseases to complement and even surpass traditional methods. Automation of all components of PCR is now possible, which decreases the risk of generating false-positive results due to contamination (Pusterla et al., 2006). In addition, real-time PCR techniques are the most sensitive for the detection of low abundance mRNA when the amount of tissue sample is limited (Carvalho-Gaspar et al., 2005). QPCR does not require additional manual steps, time-consuming blots and hybridizations, radioactive isotopes or other hazardous products (Gaus et al., 1993).

As fluorescence-monitoring chemistries, the intercalating SYBR-green dye and the TaqMan chemistry utilizing FRET technology were used here. There are pros and cons to each of the chemistries employed for QPCR. The main advantage of SYBR-green over TaqMan was that the initial assay preparation required only a few days for primer design and initial validation besides, the low cost economic benefit (Ponchel et al., 2003). Despite the necessity of an additional probe, TaqMan is mostly preferred as it combines the advantage of detecting low copy numbers with increased specificity since both primers and probe must work together. Although theoretically 1 copy should be detected, in most cases the sensitivity of the assays seemed limited to >10 copies of template due to stochastic effects (Morrison et al., 1998). Increasing availability of validated TaqMan primer-probe sets will further reduce costs and increase the utilization of this method.

The various machines that were available differed in sample processing capacity, format and dynamic range (i.e. orders of magnitude). The Mx3005P is an instrument working with a 96-well format, can multiplex up to four dyes per reaction tube and deals with various sub-experiments per plate. I have compared this machine briefly with two other real-time PCR systems, the ABI prism 7000 and the Bio-Rad iCycler. Several experiments were run on each of the three systems using the same primers and templates, here IFNγ and IL-4 plasmid dilutions in TaqMan absolute quantification assays. Fortunately, the Mx3005P was the least expensive of its kind and provided equal and sometimes better results in my hands than other tested systems and thus was subsequently used.

In addition, I have evaluated primer and probe sets from different sources, based on different programmes. The ABI primer and probe sets provided good results with the horse plasmids and were used for initial evaluation of the technique and the machines, but could not be used in the SYBR-green experiments as the probes were pre-mixed with the primers. I finally selected the TIB MOLBIOL IFNγ primer and probe set and Metabion IL-4 primer and probe set for the relative quantification assays as they provided best results analysed through the standard curve and the correlation coefficient value (r^2). The accuracy (precision and efficiency) of the IFNγ and IL-4 assays was determined by standard curves from serially diluted plasmid samples (Fig. 18). The sensitivity of the IFNγ and IL-4 assay was determined by diluting plasmids of known concentrations and the limits of detection were found to be <10 molecules for each test.

Primer optimization experiments were performed using SYBR-green where all PCR conditions apart from the probe were identical to the later TaqMan approach. Dilutions of each of the primer pairs were used with a fixed concentration of the plasmids to determine optimal primer concentrations. Since accurate quantification is necessary to verify that PCR efficiency was independent of the initial amount of target DNA, each pair of primers was tested across several logs dilution series of a positive control DNA (horse IFNγ and IL-4 cDNA plasmids) and standard curves were established to determine efficiency, sensitivity and precision (Fig. 15 and 16). PCR sensitivity is directly related to the specificity of the assay, as amplification of non-specific products can compete with amplification of the specific product. Thus hot start PCR is an established necessity and was applied here to suppress generation of non-specific products. It had to be taken into consideration that the quality of detection of SYBR-green may be compromised through the formation of primer-dimer, lack of specificity of the primers and the formation of secondary structures in the PCR product, leading to the creation of unexpected double-stranded DNA product, which would incorporate SYBR-green and generate a fluorescent signal. Therefore, it was essential to analyse specificity of each set of primers through SYBR-green dissociation curves using cDNA from horse PBMC as test templates (Fig. 17). Moreover, agarose gel electrophoresis confirmed that only one PCR product was detected.

Relative quantification determines the changes in mRNA levels of a gene across multiple samples relative to the level of an internal control RNA. Therefore, relative quantification does not require standards with known concentrations. Since its basic idea is to compare the expression level of genes, or to compare the expression level in different tissues, or parts of the same organism, relative gene expression QPCR is known also as comparative real-time PCR. To accomplish such comparisons between cases of different conditions or treatments, it is a must to normalize each test sample to the total amount of nucleic acid tested, so that the differences between samples are the result of real biological difference and not of inconsistent loading. So-called "housekeeping" genes are expected to be present in the same quantity throughout tissues. Some studies have shown, however, that their individual expression may vary as a result of neoplastic growth, hypoxia or even experimental treatment and may seriously influence interpretation of results (Kunth et al., 1994; Thellin et al., 1999; Schmittgen and Zakrajsek, 2000; Wu and Rees, 2000).

Of these housekeeping genes, 18S rRNA was the preferred standard in my work. However, because of its high abundance (Fig. 19-B), the gene of interest may not be detected at lower template quantity in multiplex assays as dNTPs and enzyme may have been used up already.

The 18S rRNA detection assay, commercially sold by ABI and supplied for multiplex assays, comes with a limited primer concentration, which was confirmed by some initial dilution experiments. Since 18S rRNA is highly conserved through all species, such kit was considered suitable for both its immediate purpose and long-term aim (to be used in wildlife species). To calculate the expression of a target gene in relation to a reference gene, mathematical models were applied as integral parts of the QPCR machine software. At relative gene expression real-time PCR, $\Delta\Delta C_t$ method is a quantification method comparing the C_t value of the samples of interest with a calibrator (control) such as a non-treated sample. To calculate the increase or decrease of an expressed target gene, the C_t of the housekeeping gene (18S rRNA) was subtracted from the C_t of the target gene yielding the ΔC_t.

In this thesis a two-step RT-PCR method was used, as it had been reported to be more sensitive and less prone to the production of primer-dimer artifacts and contamination with genomic DNA (Vandesompele et al., 2002). Random hexamers bind anywhere along the RNA and accordingly result in the highest RT efficiency (Bookout and Mangelsdorf, 2003). Reverse transcription with random hexamer primers also allowed working on the same RT reaction for all of my target genes (IFNγ, IL-4 and 18S rRNA). Since DNA contamination is another important factor that affects the accuracy of gene expression analysis, RNase-free DNase I treatment of all RNA samples was performed prior to reverse transcription.

Upon activation of T cells, cytokine genes are rapidly transcribed. It is known that T cell activation induces two major signal transduction pathways, the protein kinase C (PKC)-mitogen-activated protein kinase (MAPK) pathway and the calcium-dependent calcineurin-mediated pathway. In order to obtain maximal and suboptimal T lymphocyte activation, I have compared different stimulation protocols. First, I used a monocausal chemical stimulation *via* PMA or Ca-ionophore alone and thereafter together. Some anti-CD3 mAbs are able to cross-link CD3 and resemble the physiologic stimulation *via* the TCR, since this signal is transmitted through the associated CD3 molecule. Accordingly, the use of anti-equine CD3 monoclonal antibodies (mAbs) accomplished T-cell activation *in vitro* without the need for prior sensitization (Van Wauwe et al., 1980) and was used here as well.

According to the paradigm of co-stimulation (Lafferty and Cunningham, 1975), a T cell needs two signals to become fully activated. The "first signal" arises from the interaction of T cell receptor (TCR) molecules with peptide/major histocompatibility complex (MHC) complexes on antigen presenting cells (APCs). The "second signal" is provided by the engagement of the so-called co-stimulatory receptors. The first to be discovered and still the most prominent of

these co-stimulatory receptors is CD28, whose ligation is required to rescue T-cells from anergy or apoptosis and promote T cell clonal expansion (June et al., 1994; Rudd, 1996; Frauwirth and Thompson, 2002; Sharpe and Freeman, 2002; Frauwirth and Thompson, 2004). I tested a polyclonal anti-human CD28 antiserum, whose biological activity was determined by its ability to enhance IL-2 production by human Jurkat cells stimulated with PMA and Ca-ionophore. The anti-CD28 antiserum synergized lymphocyte activation with both anti-equine CD3 and with PMA/Ca-ionophore as evident by a significant increase of IFNγ and IL-4 mRNA (Fig. 26 and Fig. 24, respectively). The anti-human CD28 polyclonal was used at different concentrations (0.5, 1.00 and 2.00 μg/ml) and the increase of cytokine expression levels peaked at 1 μg/ml (Fig. 25). This concentration was well in accordance with previous studies in the human system (Monteleone et al., 1998; Gunnlangsdottir et al., 2005). It is note worthy that it is not a simple binding but rather cross-linking of CD28 by antibody which induces signalling. Consequently, with a high surplus of antibodies, the cross-linking of CD28 no longer increased, but started to decrease and the signal diminished. Such effect was described also as the "antigen surplus phenomenon", recognized by Heidelberger and Kendall as long ago as 1935. This non-monotonous calibration effect is therefore often referred to as the "Heidelberger curve".

In addition to my data, further analysis of this polyclonal antiserum against equine leukocytes argued for its reactivity with equine CD28 (Ibrahim, 2007, unpublished data).

IFNγ was generally detected during all of the *in vitro* stimulation periods whereas IL-4 was detected in the first hours and thereafter declined. The pattern of cytokine mRNA expression was found to be dependent on the stimulus used. Mitogen stimulations proved to be more potent and lasting than biological stimulation, and therefore induction of both genes was more rapid (2, 8 hours for Ca-ionophore and PMA compared to 48 hours in case of anti-equine CD3) and accompanied by higher levels of mRNA gene expression (Fig. 25 *versus* Fig. 26). This is supported by previous data stating that time kinetics of the different cytokines are variable and also dependent on the stimuli (Jung et al., 1996; Fan et al., 1998; Abdalla et al., 2003; Harrington et al., 2006). Production of multiple cytokines in stimulated T-cell populations is not regulated by a common mechanism, but rather by independent pathways. In fact, IFNγ production is regulated *via* signal transducers and activators of transcription (STAT-1) activation (Bromberg et al., 1996; Qing and Stark, 2004) while IL-4 production is regulated by STAT-6 (Takeda et al., 1996; Aoudjehane et al., 2007), which explains such differences in time onset and accumulation time of both genes.

One objective of this thesis was to establish IFNγ/IL-4 QPCR assays to detect the cytokine balance as a prerequisite step for assessing intracellular and extracellular infections. In the last part of my work, I attempted to apply the relative gene quantification of IFNγ and IL-4 to an antigen-stimulation assay using tetanus toxoid and *EHV-1* as prototypic Th2 and Th1 antigens, respectively. Anti-CD28 was applied to mimic the co-stimulation and optimize the readout as described previously (June et al., 1989; Linsley et al., 1991; Azuma et al., 1993; Boise et al., 1995; Noel et al., 1996; Li et al., 2001; Parry et al., 2003).

Horses are commonly vaccinated against a certain set of pathogens or their products (toxins) responsible for diseases endemic within the horse population, such as *equine herpesvirus-1 (EHV-1)* or posing a common threat such as tetanus toxins. *EHV-1* resembles an intracellular pathogen while *Clostridium tetani* and its product tetanus toxoid (TT) are extracellular. Thus *EHV-1* and TT were selected as recall antigens to test cytokine mRNA expression in quantitative real-time PCR for IFNγ and IL-4. T-cell priming was performed with *EHV-1* or TT plus anti-CD28 for different times and concentrations of antigen. And it must be emphasized that CD28 alone did not show an effect, thus underlining the antigen-specificity of the results.

Upon *EHV-1* stimulation gene-expression profile revealed an increase in IFNγ mRNA expression levels possibly associated with a CMI response (Fig. 28). This was in accordance with data indicating that immunity to *EHV-1* infection is characterized by a polarized IFNγ dependent response (Coombs et al., 2006). IL-4 expression also increased, which may be explained by the vaccine inducing Th2 reaction like. This also is in accordance with previous data (Horohov et al., 2005; Kydd et al., 2006; Wagner et al., 2006; Paillot et al., 2007). Moreover, in a recent challenge-study non-immunized ponies demonstrated increase in both of IFNγ and IL-4 responses post-*EHV-1*-challenge (Coombs et al., 2006).

It was assumed that the Th2 cytokine IL-4 would play a central role in response to TT and results of real-time QPCR proved this assumption to be correct, with a slight increase in IFNγ gene expression (Fig. 29). These results were in accordance with previous results of Fernandez et al. (1994) including the production of a certain level of IFNγ as shown by an Elispot assay developed to assess the number of TT-specific CD4^{+} IFNγ producing cells (Mayer et al., 2002).

Overall, gene expression in response to different recall antigens and co-stimulation with anti-CD28 correlated well with described IFNγ and IL-4 cytokine expression. The results underline that Th1/Th2 reactions may be biased, but under field conditions in outbreed

animals both types occur to some extent in parallel. QPCR seems to be of value for the further development of a test with high specificity and sensitivity, which can be standardised under laboratory conditions for a species of interest such as zoo and wildlife animals. Gene quantitation *via* real-time PCR may also allow the detection of clinically apparent infections characterized by pathogen replication and their discrimination from chronic latent infections (Pusterla et al., 2006). Furthermore, in the case of intracellular infections Th1/Th2 cytokine balance might even be an appropriate prognostic aid, discriminating good Th1 biased responses from bad Th2 biased responses. Such assays could be established for each species after design of sequence specific primers and probes.