I. INTRODUCTION

Immunity is the protection against foreign organisms. Vertebrates have two types of immunity, innate and adaptive, which do not operate independently of each other but rather in cooperative and interdependent ways make them both more effective. Adaptive immunity as one part exhibits four attributes: memory, specificity, diversity and self/nonself recognition. Functionally, an adaptive immune response consists of two interrelated events, which are recognition of antigen and response to that antigen (Goldsby et al., 2000). Memory T-cells are qualitatively distinct from naïve T cells in that they have less stringent requirements for activation and respond more rapidly than naïve T cells (Ahmed and Gray, 1996). Memory (of both T and B cells) is therefore manifested by stronger and quicker responses upon secondary exposure to antigen. Although mechanisms that maintain memory are not completely understood, effective immune memory relies on the maintenance of the T helper phenotype (Asnagli and Murphy, 2001; Messi et al., 2003). In part, T-cell memory reflects an increase in the frequency of precursor cells. Accordingly, naïve CD4 and CD8 T cells initiate a program for their clonal expansion and development into functional effector and memory cells after interaction with stimulatory antigen-presenting cells (APCs). The strength and duration of antigenic plus co-stimulatory stimulation can affect the development and differentiation process and regulate the functional quantities of the effector and memory cells (Swain et al., 1990a; Salgame et al., 1991; Lanzavecchia and Sallusto, 2002; Gett et al., 2003).

After differentiation and emigration from the thymus to the peripheral immune organs, $CD4^+$ T helper cells are termed naïve T helper precursor (Thp) cells. Thp cells are functionally immature and only capable of secreting interleukin 2 (IL-2). The exact *in vivo* mechanism by which naïve $CD4^+$ T cells differentiate into one of two broad (perhaps overlapping) populations (Th1 and Th2) effector cells is still a matter of some controversy. Naïve T cells differentiate first into an intermediate cell type, termed Th0, with phenotypic characteristics of both Th1 and Th2 cells. Functional diversity of T lymphocytes may be relevant, since Mosmann and colleagues (1986) described that murine Th lymphocytes could be divided into two subsets Th1 and Th2. Th1 cells mainly produce IFNγ and IL-2 and have a key role in the cellular immune responses. Conversely, Th2 cells produce IL-4, IL-5, IL-6 and IL-10, and promote the humoral immune responses (Mosmann and Coffman, 1989b; Bullens et al., 1999).

Early observations suggested that naïve $CD4^+$ T cells could be directed toward Th1 or Th2 lineage *in vitro* by the addition of exogenous cytokines (Swain et al., 1990b; Seder and Paul, 1994; Constant and Bottomly, 1997) and it is widely accepted that IL-12 and IL-4 are "master

signal" cytokines for Th1/Th2 polarization. While IL-12 induces T cells to secrete IFNγ and differentiate *via* the Th1 lineage, IL-4 stimulates its own synthesis and polarizes T cells to commit to the Th2 lineage. If IL-4 is also present during the priming period, the resulting $CD4^+$ T cells produce IL-4 and the development of IFNγ-producing cells is subsequently inhibited by IL-4. In the absence of IL-4, priming for IFNγ-production occurs, but this is markedly enhanced by IL-12. The role of IFNγ in enhancing priming for IFNγ-production is not fully resolved. In some *in vitro* systems, it appears to act together with IL-12 to enhance such production. Anti-IFNγ diminishes priming for IFNγ production *in vivo* (Mosmann and Coffman, 1989a; Seder and Paul, 1994).

The recognition of antigens (Ag) presented by dendritic cells (DCs), is capable of inducing naïve T lymphocyte sensitization (Croft et al., 1994). To acquire this capacity, DCs must undergo a maturation process, characterized by the loss of their Ag-capturing capacity and the increase of their expression of co-stimulatory and adhesion molecules including integrin (Puig-Kroger et al., 2000). Effector T cells are triggered when their antigen specific receptors and either CD4 or the CD8 co-receptors bind to peptide, major histocompatibility (MHC) complexes. Nevertheless, ligation of the T-cell receptor does not, on its own, stimulate naïve T cells to proliferate and differentiate into effector T cells. Clonal expansion of effector T cells requires a co-stimulatory signal. CD8 T cells appear to require a stronger co-stimulatory signal than CD4 cells and their clonal expansion may be aided by CD4 cells interacting with the same antigen-presenting cell (Dutton et al., 1998; Croft, 2003).

The T cell receptor (TCR) is a multi-component signalling complex (Weiss et al., 1986; Janeway, 1992). After activation, a cascade of events is set in motion, including activation of protein kinase C (PKC) and an increase of the intracellular calcium content. The strength of antigenic stimulation, the duration of T cell receptor engagement, the presence of different cytokines, and the participation of co-stimulatory molecules are critical in determining the phenotype of differentiated T cells (Linsley et al., 1991). CD28 activation results in one of the best and most characterized co-stimulatory signals (Linsley and ledbetter, 1993; Shahinian et al., 1993; Verwilghen et al., 1993). An optimal T cell activation requires engagement of the co-stimulatory molecule CD28 by its ligand B7-1 (CD80) on DC in addition to TCR cross-linking (as a first signal) (Freeman et al., 1993b; Lenschow et al., 1996; Parra, 2002).

CD28 is a homo-dimeric glycoprotein expressed on the surface of a major subset of T cells and has been identified as a member of the immunoglobulin supergene family. The physiological ligands for CD28, B7.1 and B7.2 are only expressed at high levels by DC upon

prior activation, thereby controlling the initiation of the T cell response (Beyersdorf et al., 2005). The "MYPPPY" motif in the extra cellular domain is critical for CD28 ligand, B7.1 and B7.2 binding (Peach et al., 1994; Wang et al., 2002; Lühder et al., 2003).

Human CD28 is expressed on 95% of $CD4^+$ lymphocytes, approximately 50% of $CD8^+$ lymphocytes, and on plasma cells (Aruffo and Seed, 1987; June et al., 1989; June et al., 1990). In horses, a unique T cell surface molecule was suggested before to be the ortholouge of human CD28. Although it had similar biochemical characteristics to CD28, the number of equine peripheral blood lymphocytes (PBL) expressing this molecule differed considerably from human lymphocytes and lacking of *in vitro* expressed equine CD28 made it difficult to designate this antigen (Kydd et al., 1994; Byrne et al., 1997; Lunn et al., 1998).

Early studies had shown that the combination of CD28 stimulation and PKC activation by PMA results in T cell proliferation that is independent from both accessory cells and activation of the TCR/CD3 complex. After 3-hours activation of T cells by PMA alone, a slight (8%) increase in cell volume occurred that did not progress to DNA synthesis. CD28 in combination with PMA resulted in DNA synthesis at 12 to 14 h after stimulation (Thompson et al., 1989). In addition, stimulation by PMA alone resulted in the accumulation of IL-2 receptor transcripts but no detectable IL-2 mRNA. T-cell stimulation by the combination of CD28 and PMA resulted in the appearance of IL-2 transcripts. Furthermore, stimulation by CD28 in combination with immobilized CD3 antibodies caused a striking enhancement of IL-2 mRNA expression. Accordingly, binding of mAb to the CD28 surface antigen can augment the activation of human T cells stimulated with suboptimal doses of mitogens or anti-TCR/CD3 complex antibodies (Thompson et al., 1989; Olive et al., 1994; Bian et al., 2005). The importance of co-stimulatory molecules in Th cell skewing is clear as CD28 and B7 knockout mice have significantly reduced immune responses with especially pronounced defects in generating the Th2 compartment (Freeman et al., 1993a; Green et al., 1994; Schweitzer et al., 1997). A related co-stimulatory receptor, ICOS, interacts with its ligand B7-H1 (Dong et al., 1999; Hutloff et al., 1999; Yoshinaga et al., 1999; Ling et al., 2000) and was thought to preferentially stimulate Th2 differentiation. Th2 cells are also preferentially expanded by CD28-dependent OX40 signalling (Akiba et al., 2000; Lane, 2000; Murata et al., 2000; Linton et al., 2003). CD2 has a relatively large and highly conserved cytoplasmic domain (Rabb et al., 1995; Schneider et al., 1995) and further enhances TCR/CD3 and CD28 mediated responses, particularly in resting and naïve T cells (Bierer et al., 1989; Kabelitz, 1990).

Tuberculosis is the leading cause of death in the world from a single infectious agent, killing about 3 million individuals every year. Although several *Mycobacterium species* can cause tuberculosis, the species *M. tuberculosis* is the principle causative agent in humans (Goldsby et al., 2000; Parsons et al., 2002). *M. tuberculosis* is considered one of the most successful pathogens, judged by the infectious dose (1–10 inhaled organisms in humans), proportion of the species infected, duration of infection (lifelong) and induction of pathology that promotes dissemination (liquefaction of lung and provocation of cough) to generate infectious aerosols (Cooper et al., 1993). Inhaled bacilli are ingested by alveolar macrophages and are able to survive and multiply intracellularly by inhibiting formation of phagolysosomes (Goldsby et al., 2000). The activation state of macrophages determines whether *M. tuberculosis* proliferates or lies dormant. It initially encounters "resting" alveolar macrophages, in which it is thought to replicate with little restriction for several weeks, leading to spread through the blood and uptake into macrophages throughout the body. With the onset of cell-mediated immunity (CMI), macrophages acquire the capacity to kill most of the bacteria and restrict the replication of the remainder, usually for the lifetime of the host. IFNγ, the principal macrophage activating factor (Cooper et al., 1993), is required for macrophages to attain a tuberculostatic or tuberculocidal state, as judged by the exuberant growth and early lethality of *mycobacteria* in IFNγ-deficient mice (Flynn et al., 1993). Tuberculosis caused by *Mycobacterium bovis (M. bovis)*, known as bovine tuberculosis (BTB), with cattle as a principal reservoir, remains a major zoonotic and economic problem in many countries (Cosivi et al., 1998; Amadori et al., 2002; Michel et al., 2006) as 5% of TB deaths in the world are reported due to infection with BTB (Acha and Szyfres, 2003). A feature of BTB infections is the variation in the appearance and distribution of lesions in the different host species (de Lisle et al., 2002). Diagnosis of *M. bovis* in free-ranging wildlife often relies on post-mortem examination.

The standard test for detection of tuberculosis infection *in vivo* without biopsies is the intradermal delayed type hypersensitivity (DTH) skin test known as “tuberculin test”, but it suffers from poor sensitivity and specificity (Lepper et al., 1977; Francis et al., 1978), and possibly alters the subsequent immune status of the tested individuals (Radunz and Lepper, 1985).

Cross-reactivity of immune responses induced by other species of *Mycobacteria* complicates the interpretation of the tuberculin test and may be responsible for at least some of the false positive reactions. Where purified antigens were compared, it was clear that there was variation between animals in the antigen specificity of the responses, suggesting that by using

a combination of antigens it might be possible to increase the level of sensitivity (Vordermeier et al., 2000).

The lymphocyte transformation test has been the *in vitro* correlate of the DTH reaction and was used to detect cellular reactivity to tuberculin PPD antigen in both humans and cattles (Oppenheim, 1968; Outteridge and Lepper, 1973; Jensen et al., 1977). These assays have been adapted for use with whole blood samples (Viljanen and Eskola, 1977; Theon et al., 1980), but still the test is rather unspecific and does not discriminate naïve and memory T-cells. The assay has limited scientific value and is not used for routine diagnosis because it is time-consuming and the logistics are complicated (it requires long incubation times and the use of radio-active nucleotides). In addition, the test is relatively expensive and has not been subject to inter-laboratory comparisons (Griffin et al., 1994).

Recent advances in molecular biology and immunology have led to a promising generation of alternative tests such as the *in vitro* T cell based IFNγ assays (Andersen et al., 2000; Barnes, 2004; Lalvani, 2003; Pai et al., 2004). Cytokine measurements in naturally infected *M. bovis* reactors showed that assays based on the detection of either IFNγ or IL-4 responses were equally able to differentiate infected from uninfected animals. Rothel et al. (1990) described an *in vitro* sandwich enzyme immunoassay (EIA) for bovine tuberculosis, detecting gamma-interferon released in response to specific antigen in a whole blood culture system. The IFNγ assay is based on the concept that T cells of individuals sensitized with *M. tuberculosis* release IFNγ Th1 cytokine, when they re-encounter mycobacterial antigens. A high level of IFNγ response is likely to indicate previous sensitization with *M. tuberculosis*, but does not necessarily imply active disease, thus, the IFNγ assay cannot easily distinguish latent from active disease and acute from chronic infections (Wood et al., 1990; Pai, 2005). This test uses the same antigen (*M. bovis* PPD) employed in skin testing and also requires parallel use of avian PPD to distinguish specific from non-specific reactions. In field trial comparison, the IFNγ test was more sensitive than the intradermal test (Wood et al., 1991; Wood et al., 1992). The positive IL-4 responses in low-dose *M. bovis* challenge animals that remained SCITT (single comparative intradermal tuberculin test) negative suggests that the measurement of IL-4 could provide additional information (Rhodes et al., 2000).

M. tuberculosis causes progressive pulmonary disease in Asian elephants (Montali et al., 2001). The deaths of two Asian elephants (*Elephas maximus*) in August 1996 led the United States department of Agriculture to require the testing and treatment of elephants for tuberculosis. Guidelines for the control of tuberculosis in elephants were issued in 1997 for

screening all elephants for TB. From August 1996 to September 1999, *M. tuberculosis* infection was confirmed by culture from 12 of 118 elephants in six herds. Eight diagnoses were made ante-mortem on the basis of isolation of *M. tuberculosis* by culture of trunk wash samples; the remaining cases were diagnosed post-mortem (Larsen et al., 2000; Mikota et al., 2001).

The problem of tuberculosis in animal species is a worldwide problem. In the monitoring period between 1990 and 1999, *M. tuberculosis* was isolated in two European countries (Poland and Slovak Republic) from 16 animals with tuberculous lesions. Among them were an African elephant (*Loxodonta africana*), an agouti (*Dasyprocta aguti*) and a terrestrial tapir (*Tapirus terrestris*) (Pavlik et al., 2003). In addition, a new *Mycobacterium* strain was isolated from a lung abscess in an elephant that died from chronic respiratory disease (Shojaei et al., 2000).

Sternberg et al. (2002) tested 214 animals in three Swedish zoos between the years 1993 and 2000. A total of five skin test reactors were found: three cotton-top tamarins (*Saguinus Oedipus*) and two tapirs (*Tapirus terrestris*). Furthermore, between 2001 and 2003, there were several incidents of tuberculosis in Stockholm zoo where five elephants and one giraffe had been infected by four different strains of *M. tuberculosis* (Lewerin et al., 2005).

While *Mycobacteria* resemble a most prominent example of intra-cellular bacteria and their threat directly initiated this study, viruses are generally replicating inside cells only and the concept of measuring cytokine levels to determine cellular immunity is transferable.

Herpesviruses are a large group of ubiquitous viruses within the family *Herpesviridae* that occur in most if not all species of animals (Roizman and Pellett, 2001). *Equine herpesvirus-1 (EHV-1)*, a member of the subfamily *Alphaherpesvirinae*, is one of the most important pathogens of equine populations worldwide. *EHV-1* typically enters the body *via* respiratory mucosal surfaces, establishes latency in peripheral blood mononuclear cells and trigeminal ganglia (Slater et al., 1994; Baxi et al., 1996; van der Meulen et al., 2002).

Cell mediated immunity (CMI) is thought to play a major role in protection against infection and *EHV-1* challenge infection of ponies induced an increase of IFNγ production of virus-specific T lymphocytes (Breathnach et al., 2005; Paillot et al., 2005). The protection against *EHV-1* infection and disease, however, depends on both humoral and cellular immune responses and non-immunized ponies demonstrated an increase in both of IFNγ and IL-4 responses post-*EHV-1*-challenge (Coombs et al., 2006; Kydd et al., 2006).

Viral latency and reactivation are important features of *EHV-1* epidemiology, allowing virus to persist in previously infected horses (Allen et al., 2004; Allen, 2006). The respiratory

symptoms of *EHV-1* cannot easily be differentiated from those of influenza or other viruses on the basis of clinical signs. Accordingly, there is a clear need for specific, sensitive techniques that allow the rapid diagnosis of clinical disease, as well as surveillance of susceptible populations (Wagner et al., 1992; Borchers and Slater, 1993; Henninger et al., 2007). In addition, in the case of experimental studies of vaccine efficacy, a critical parameter for measuring immunological control of *EHV-1* infection is the detection of cell-mediated immunity (Allen et al., 2004).
It is well known that *EHV-1* or closely related *herpesviruses* can also infect different captive species of zoo equids (Przewalski's wild horse, Damara zebra, Grant's zebra, Burchell's zebra, Grevy's zebra, onager and domestic ass) (Chowdhury et al., 1988; Crandell et al., 1988; Rebhun et al., 1988; Blunden et al., 1998; Borchers et al., 2006). The infection of Thomson's gazelles was initially attributed to *gazelle herpesvirus type 1* (*GHV-1*), which was later classified as *EHV-9,* based on nucleic acid identity in the gene encoding glycoproteins B and G of 97.5% and 92.4% to *EHV-1*, respectively (Fukushi et al., 1997). In addition, serosurvey for selected infectious disease agents in free-ranging black and white rhinoceros in Africa revealed a prevalence of *EHV*-1 Ab of 8.8% (Fischer-Tenhagen et al., 2000).
In mid 1988, a 3-years-old Asian elephant (*Elephas maximus*) from a circus in Switzerland died following generalized manifestation of a *herpesvirus* infection. *Endotheliotropic Elephant Herpes Virus (EEHV)* causes a rapidly progressing and severe disease that finally results in the death of the animal, especially in young Asian elephants. Worldwide, 26 elephants confirmed *EEHV* cases are documented. Since 1995, 7-cases have occurred in North America, 10 in Europe and 2 in Asia. Carried by otherwise healthy African elephants they can be fatal mainly for young Asian elephants (Fickel et al., 2001; Reid et al., 2006), as the virus reaches endothelial cells and causes capillary leakage and haemorrhage, subsequently resulting in death of shock (Montali et al., 2001). Diagnosis is based mainly on pre-mortem/post-mortem findings as the virus has not yet been isolated, but samples taken during viremia may be subtracted to a PCR. Up-to-date there are no further standardized techniques to screen for *EEHV* infections (Montali et al., 2001; Reid et al., 2006).

Cytokines are low molecular weight secreted proteins mediating cell growth, inflammation, immunity, differentiation and repair. They are highly potent and often acting in *femtomolar* (10^{-15} M) concentrations. The majority of cytokines normally act locally in a paracrine or even autocrine fashion. They act in a highly complex coordinated network in which they induce or repress their own synthesis as well as that of other cytokines and cytokine receptors. Many

cytokines appear to be pleiotropic since there is considerable overlap and redundancy between the functions of individual cytokines (Bidwell et al., 1999). The importance of various cytokines has been aptly proven using transgenic knockout (KO) mice in which one or more cytokine or cytokine receptor genes have been disrupted (Appelberg et al., 1994). Recombinant cytokines are valuable tools for functional studies and candidates for vaccine additives for various diseases. Cloning and expression of cytokine genes permit the analysis of their immune function and role in the control of the immune response to disease and vaccination (Radford et al., 1991). They can also be used to generate specific antibodies to analyze the roles of different cytokines during immune responses (Wagner et al., 2005).
Interferons (IFNs) represent an evolutionary conserved family of cytokines originally discovered 50 years ago on the basis of their antiviral activity therefore named interferons (Isaacs and Lindenmann, 1957). While type I IFNs such as IFN-*alpha* and *-beta* (IFNα and IFNβ) are secreted by virtually all cells in response to viral infection, type II (IFNγ) is mainly produced as a result of stimulation of T lymphocytes and NK cells (Goodbourn et al., 2000).

Interferon *gamma* (IFNγ) is a pleiotropic cytokine with immunomodulatory activities that are crucial for the regulation of immune responses (Farrar and Schreiber, 1993; Huang et al., 1993). It plays an unanticipated but substantial role in Th2 priming, although it is an important Th1 cytokine, and under certain circumstances a Th1 inducer (Bocek et al., 2004). IFNγ induces the production of cytokines, up-regulates the expression of class I and II MHC antigens, Fc receptor and leukocyte adhesion molecules. It modulates macrophage effector functions, influences isotype switching and potentiates the secretion of immunoglobulins by B cells. IFNγ also augments Th1 cell expansion and may be required for Th1 cell differentiation (Farrar and Schreiber, 1993; Kamijo et al., 1993; Bach et al., 1997; Wu et al., 2002). IFNγ increases the cytotoxic activity of macrophages *via* the induction of reactive oxygen intermediates (ROI), reactive nitrogen intermediates (RNI), inducible nitric oxidase and cytokines such as TNF (Nathan et al., 1983; Chan et al., 1992; Farrar and Schreiber, 1993; Jungi et al., 1997; Ehrt et al., 2001). As a result, activated macrophages show greatly enhanced microbicidal activity for many intracellular pathogens including *Mycobacteria, Leishmania, Toxoplasma, and Trypanosomes* (Murray, 1988; Boehm et al., 1997; Moreira et al., 2000; Pearl et al., 2001; Sharma et al., 2007), in addition to its cell-specific antiviral activity (Devos et al., 1992). The functional significance of IFNγ in antimicrobial defence is demonstrated by the increased susceptibilities of IFNγ and IFNγ receptor (IFNγR) KO mice to a variety of infections, particularly to intracellular organisms such as *Listeria* and

Mycobacteria (Shtrichman and Samuel, 2001). Accordingly, IFNγ plays pivotal role in controlling intracellular infections. In addition, IFNγ induces the B7 antigens that function as co-stimulatory molecules with the CD28 ligand on T lymphocytes (Billiau, 1996).

IFNγ exerts its biological activities by binding to specific high affinity cell surface receptors consisting of α and β subunits. The α subunit of IFNγ receptor is constitutively present on almost all cell types except mature erythrocytes. The expression of the β subunit is regulated by various external stimuli. IFNγ signals through a multimeric receptor complex consisting of a ligand binding chain and a transmembrane accessory factor. IFNγ signalling is initiated when IFNγ binds to its receptor, thereby inducing its dimerization (Farrar and Schreiber, 1993; Kontenko et al., 1995; Marsters et al., 1995; Bach et al., 1997; Wu et al., 2002).

The production of IFNγ by NK cells during the innate phase of the immune response influences Th-cell differentiation by promoting development of a Th1 phenotype (Scott and Trinchieri, 1995). IFNγ is secreted by NK cells primarily in response to IL-12, a *heterodimeric* cytokine that is secreted by cells such as Mø and DCs early during infection. However, recent evidence has demonstrated a role for other cytokines and co-stimulatory molecules in enhancing the activation and function of NK cells. In particular, cytokines such as IL-1 and TNF-α have been shown to augment both NK cell proliferation and IFNγ production. Similarly, type I IFNs have been shown to enhance IL-12-driven IFNγ production *in vitro*. Another cytokine with particular relevance to innate immunity is IL-18 (IFNγ-inducing factor), which has been characterized as a potent enhancer of IL-12-induced IFNγ production by Th1 cells and has been shown to enhance NK cell proliferation and IFNγ synthesis (Tomura et al., 1998).

Unlike, IFNα and IFNβ, IFNγ does contain introns. Human and murine IFNγ molecules consist of two identical noncovalently linked polypeptide chains of 17 kilo-Daltons (kDa) and 16 kDa, respectively. Mature forms of natural human and murine IFNγ generally display molecular weights of 50 kDa when analyzed under non-reducing conditions. Both human and murine proteins had been expressed in *Escherichia coli (E. coli*) and highly purified, recombinant forms are available. Cerretti et al. (1986) isolated bovine IFNγ cDNA *via* screening a complementary DNA (cDNA) library with a human IFNγ cDNA probe. Mature bovine IFNγ is composed of 143 amino acids (aa) with a predicted molecular weight of 16.858 kDa and an amino acid homology of 63% with human IFNγ and 47% with murine IFNγ (Cerretti et al., 1986). Cloning of IFNγ in various species as well as availability of IFNγ

specific mAb had permitted the development of a variety of assays to quantify this protein. Most developed IFNγ immunoassays are based on the use of ELISAs, which are species-specific.

Interleukin 4 (IL-4) is a T-cell-derived glycoprotein initially described in 1982 as a cofactor in the proliferation of resting B-cells stimulated through the cross-linkage of their membrane immunoglobulin M (IgM) by anti-IgM antibodies. IL-4 is also a factor that induced B-cell differentiation into plasma cells secreting IgG (Howard et al., 1982). The expression of IL-4 is restricted to a subset of CD4 (Th2) and CD8 (Tc2) T cells, NK, mast cells, basophils and eosinophils (Abbas et al., 1996; Bullens et al., 1999; Rautajoki et al., 2007).

Th2-cell secretion of IL-4 and IL-10 leads to the suppression of Th1 responses by down-regulating the production of macrophage-derived IL-12 (Brown and Hural, 1997), inhibiting the differentiation of Th1-type cells (Kelso, 1995). Furthermore, IL-4 is able to block or suppress the monocyte-derived cytokines, including IL-1, TNF-α, IL-6, IL-8, and macrophage inflammatory protein (MIP) (Paul, 1991; Brown and Hural, 1997). Functions of IL-4 explain the strong pleotropic effects on different cell types and sometimes overlapping biological functions.

The receptor complex consists of the high affinity receptor IL-4-binding protein (IL-4R) and the γ chain of the IL-2 receptor complex (Russell et al., 1993). Experiments by He and Malek (1995) provided evidence for the presence of two distinct IL-4R-mediated signalling events, γ-chain-dependent and γ-chain-independent.

IL-4 also promotes antigen presentation by B cells and DCs (Stack et al., 1994). Moreover, IL-4 acts as a chemoattractant for neutrophils and eosinophils (Le-Gros et al., 1990). IL-4 is required for protection against some ectoparasites (Matsuda et al., 1990) and many gastrointestinal worm infections where Th2 and IgE responses are helpful (Else et al., 1994; Waldvogel et al., 2004). But IL-4 inhibits hydrogen peroxide (H_2O_2) production and the anti-leishmanial capacity of human cultured monocytes mediated by IFNγ (Lehn et al., 1989). Additionally, IL-4 alters inflammatory cell activity, inhibits cytotoxic activity, suppresses the inflammatory responses associated with type I cytokine production (IL-12, TNF-α and IFNγ) and inhibits killing of intracellular organisms (Vannier et al., 1992; deWaal Malefyt et al., 1993). In human B cells, IL-4 is not only a stimulator of Ig class switch from IgM to IgE, but also up-regulates CD23 and MHC II (Lundgren et al., 1989). Comparison of the ratio of IL-4/IFNγ in supernatants of T cell cultures reveals a skewing towards IL-4 production by PBMC from patients with atopic dermatitis, while T cells from (non-atopic) healthy adults

predominantly produce IFNγ (Nakazawa et al., 1997). In addition, IL-4 detection can contribute to the evaluation of cellular immune responses during infectious diseases, immunological disorders or vaccination (Wagner et al., 2006; Roberts et al., 2007).

A strong Th2 biased immune response may have a negative impact on an animal's immune system. It is unclear whether the increased production of IL-4 causes, or merely reflects, severe tuberculosis. IL-4 may reduce killing of *mycobacteria* by phagocytes through inhibition of IFNγ production (Heinzel et al., 1989; Lucey et al., 1996). However, severely ill patients with miliary TB did not show increased expression of IL-4, suggesting that IL-4 is not just a marker of disease severity (Hernandez-Pando et al., 1998). Likewise, over-expression of IL-4 in PPD-sensitized mice increased the size and cellularity of PPD-induced granulomas (Lukacs et al., 1997) and thus pointed to involvement of IL-4 in tissue damage in mycobacterial infections of mice. However, IL-4-/- mice displayed normal instead of increased susceptibility to *mycobacteria* in two studies, suggesting that IL-4 may be a consequence rather than the cause of tuberculosis development (van Crevel et al., 2000; van Crevel et al., 2002).

Hook et al. (1994) isolated mRNA from mononuclear leucocytes of the red deer (*Cervus elaphus*), cloned and sequenced IL-4. IL-4 cDNA was 408 bp in length and its deduced aa sequence was 92% homologous with the published bovine IL-4 aa sequence. Inoue et al. (1999) cloned and sequenced a bottle-nosed dolphin (*Tursiops truncatus*) IL-4 cDNA. Dolphin IL-4 cDNA was 528 bp in length and contains an ORF of 402 nucleotides coding an IL-4 precursor of 133 aa with the putative signal peptide of 24 amino acids. On the basis of the conserved transcription start point (tsp) region of human and bovine IL-4 gene transcripts, Khatlani et al. (1999) designed gene specific primers with an adaptor primer pair and subsequently generated the full-length cDNA sequence encoding for canine IL-4 (cIL-4) from mitogen stimulated canine peripheral blood mononuclear cells (PBMC). The cIL-4 ORF was composed of 399 nucleotides. Caprine IL-4 cDNA was amplified from RNA of mitogen-stimulated goat PBMC. Sequence of caprine IL-4 cDNA corresponds to a 535 nucleotide mRNA including 5' and 3' untranslated regions and a 405 nucleotide ORF, the first 66 nucleotides encoding a putative signal peptide. Mature IL-4 was a 12.8 kDa protein containing six cysteine residues and two potential N-linked glycosylation sites and was highly homologous with other ruminant IL-4 (Snekvik et al., 2001). Overall, IL-4 sequences were more heterologous than IFNγ between species.

Alternative splicing of pre-mRNAs is a powerful and versatile regulatory mechanism that can exert quantitative control of gene expression and functional diversification of proteins (Lopez, 1998). Human IL-4 splice variant (IL-4δ2) was the first reported to generate protein isoforms derived from alternative splicing of cytokines. Thereafter, human IL-2 was next with two additional isoforms (IL-2δ2 and IL-2δ3) and as in the case of IL-4 assumed to be naturally occurring antagonists (Sorg et al., 1993; Tsytsikov et al., 1996). Human IL-7 is probably the most variable spliced cytokine to date, with no less than six reported isoforms (Korte et al., 1999). Occurrence of alternative splicing in three major cytokines, playing pivotal roles either in polarization/activation of immunity (IL-4, IL-2), or in earlier stages of B- and T-cell development (IL-7), may indicate a significant involvement of this transcriptional control mechanism in determining immune response phenotype and outcome of certain immune-related diseases. Increased expression of IL-4δ2 in stable asthmatic subjects suggests that the balance of IL-4 and IL-4δ2 may modulate asthmatic inflammation (Glare et al., 1999).

In human IL-4 splice variant, the second of four exons is omitted, therefore designated as IL-4 delta2 (hIL-4δ2). Skipping exon 2 occurs without disruption of the IL-4 reading frame (Sorg et al., 1993; Zav'yalov et al., 1997). Although deletion of the 16-amino acid exon 2 product was thought to affect the two postulated binding sites of the cytokine to its receptors (Powers et al., 1992; Kruse et al., 1993) the binding ability of IL-4δ2 splice variant to IL-4 receptors appears to be retained, albeit somewhat decreased compared with IL-4, thus making it a potential competitive inhibitor of IL-4/IL-4R interaction. Further research on human IL-4δ2 (hIL-4δ2) has proven that it is a potent naturally occurring antagonist of human IL-4 as its recombinant proteins inhibit IL-4 stimulated T cell proliferation (Atmas et al., 1996). It antagonized the IL-4 induced 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 subsequent prostaglandin E2 secretion by monocytes (Arinobu et al., 1999). In addition, IL-4δ2 was reported to compete for the IL-4 receptor and to bind to the IL-4Rα chain (Arinobu et al., 1999; Vasiliev et al., 2003).

Extending the study to various mammalian species led to the assumption that the generation of IL-4 splice variants may be common to primates, lagomorphs (rabbit) and rodents of the family *Sciuridae* (*woodchuck*), but was unlikely to occur in mice and rats, family *Muridae* (Gautherot et al., 2002). However, the unexpected IL-4δ2 mRNA was described from mouse cells in a more recent study (Yatsenko et al., 2004). Several alternatively spliced variants of IL-4 were also observed during its cloning from lymphocytes of the European rabbit

(*Oryctolagus cuniculus*) (Perkins et al., 2000). The splice variants of bovine IL-4 were detected when an RT-PCR for IL-4 was established. Studying the role of IL-4 along the course of experimental infection of *Fasciola hepatica* in cattle, Waldvogel et al. (2004) detected two splice variants of bovine IL-4 mRNA designated as (boIL-4δ2 and boIL-4δ3). In order to distinguish IL-4 and IL-4δ2 mRNA in respiratory tract tissue, Glare et al. (1999) used competitive PCR assay, established with primers designed on either side of the alternative splice junction of the IL-4 gene. This allowed the simultaneous quantitation of both IL-4 and IL-4δ2 mRNA from one reaction. Although boIL-4δ2 splice was detected in a number of samples, quantitative analysis did not yield any clue to its function, further research is therefore needed to study the significance of bovine IL-4 splice variants (Waldvogel et al., 2004).

Although research into the role of cytokines in equine immunity and disease is still in its infancy, considerable advances have been made. The emphasis of the earliest work was on the characterisation of the cytokine response through the use of bioassays (Stott and Osburn, 1988; May et al., 1990; Morris et al., 1990; Morris et al., 1992) and later, with the growing availability of recombinant cytokines and mAbs, through immunoassays (MacKay and Socher, 1992; Franchini et al., 1998) and molecular analyses (Giguere and Prescott, 1999; Leutenegger et al., 1999; Swiderski et al., 1999). Cloning, sequencing and expression of a number of equine cytokines have been accomplished including equine IL-1, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-18, IFNα, IFNβ and TFNα. The availability of these reagents will help lead to a better understanding of the equine immune system and, perhaps, of the immunological basis of a number of important equine diseases (Steinbach et al., 2002). Wagner et al. (2005) developed a mammalian expression system for recombinant cytokines using the equine IgG1 heavy chain constant region as a tag for detection and purification.

Equine IFNγ cDNA was successfully cloned and thereafter expressed as biologically active recombinant equine IFNγ by two different gene expression systems (Steinbach et al., 2002; Wu et al., 2002). While rec. eqIFNγ did not act antivirally, it was effective as an immune modulator of monocytes *in vitro*. To measure IFNγ production of equine lymphocytes (eqIFNγ), Gutmann et al. (2005) developed a quantitative ELISA, with mAbs produced against bacterially derived eqIFNγ. The mAbs recognised recombinant and lymphocyte-derived eqIFNγ in ELISA, Western blotting, as well as flow cytometric and microscopic analysis. In contrast to bacterially derived material, mammalian and insect cell-derived eqIFNγ was biologically active but could be neutralised by one of the monoclonal antibodies.

Unexpectedly, glycosylation seemed to be required for antiviral activity of eqIFNγ. Equine IFNγ (eqIFNγ) shares approximately 61%, 71% and 78% amino acid sequence homology with human, porcine and feline IFNγ, respectively. The protein also exerts anti-proliferative, immuno-regulatory and proinflammatory activities and is thus important in host defence mechanisms (Farrar and Schreiber, 1993; Bach et al., 1997; Wu et al., 2002).

Equine interleukin-4 (eqIL-4) is a presumably glycosylated, 13-18 kDa secreted monomeric polypeptide. The molecule is synthesized as a 137 amino acid (aa) precursor that contains a 23 aa signal sequence and a 114 aa mature segment. There are two potential N-linked glycosylation sites and seven cysteines, six of which likely form three intrachain disulfide bridges, creating a series of α helices. The mature form is 69%, 61%, 61%, 59%, 46%, 44% and 42% aa identical to porcine, feline, human, canine, rat, cotton rat and mature mouse IL-4, respectively (Steinbach et al., 2005). Equine IL-4 was expressed in three different sizes of 17.1, 19.6 and 22.1 kDa, probably due to different glycosylation modifications. Furthermore, eqIL-4 biological activities were tested by proliferation assays using prestimulated equine PBMC, where IL-4 induced dose-dependent lymphocyte proliferation (Dohmann et al., 2000).

Real-time PCR

Polymerase chain reaction (PCR) is a sensitive and rapid method that can be performed starting with a variety of biological samples, including peripheral blood cells, but requires electrophoretic analysis of the amplification products and is usually not suitable for quantification of the template DNA. In contrast quantitative (real-time) PCR allows the continuous monitoring of the accumulation of PCR products during the amplification reaction. Since the amplification products are monitored in "real-time", no post-amplification handling is required. Advantages compared to standard PCR are a reduction of both the assay's time and contamination risks with improved sensitivity and specificity (Bruijnesteijn-Van-Coppenraet et al., 2004; Alvarez-Martinez et al., 2006; Dussault and Pouliot, 2006). Moreover, real-time PCR has a broad dynamic range of 6 to 10 log orders of magnitude (Heid et al., 1996; Morrison et al., 1999; Wong and Medrano, 2005; Abdul-Careem et al., 2006). Despite reverse-transcription PCR analysis of mRNA is referred to as RT-PCR unfortunately "real-time PCR" sometimes is also abbreviated as RT-PCR resulting in some confusion.

Theoretically, there is a quantitative relationship between amount of starting target sequence and amount of PCR product at any given cycle. However, by nature, an exponential amplification is not ideally performing at all times. Therefore, quantification is achieved by measuring the increase in fluorescence during the linear phase of real-time PCR. While

different chemistries and technologies have been set up for the amplification monitoring, based on the use of fluorescence, TaqMan probes and SYBR-green are the most commonly used types.

The simplest of several available fluorescence-monitoring chemistries to be used are the DNA-intercalating dyes like ethidium bromide. SYBR-green is a non-specific detection system based on a fluorescent double-stranded DNA (dsDNA) intercalation and it is applicable to all potential targets. It is a fluorogenic dye that exhibits little fluorescence when in solution, but emits a strong fluorescent signal upon binding to double-stranded DNA (Ponchel et al., 2003; Ma et al., 2006). Since only double-stranded DNA increases exponentially, so will the fluorescence signal of SYBR-green (Lekanne Deprez et al., 2002). SYBR-green detection is prone to lack of specificity but its comparative low price and ability to detect any given PCR product in a sequence-independent manner often outweigh its potential disadvantages, provided optimal conditions are assured (Ferreira et al., 2006). SYBR-green requires less specialist knowledge than the design of fluorogenic oligoprobes and does not suffer when the template sequence varies, which may abrogate hybridisation of an oligoprobe. SYBR-green is excited at 497-nanometer (nm) and emits at 520 nm. If the PCR reaction is fully optimised it is possible to produce a melting peak profile that contains only a single peak that represents the specific product expected from the primer pair. In this situation, SYBR-green can be useful for quantification and also for mutation detection. Even amplicons that differ by a single nucleotide will melt at slightly different temperatures and can be distinguished by their melting peaks. In this way it is possible to distinguish homozygote (single peak) from heterozygote (2 peaks). SYBR-green can also be useful for optimising a PCR reaction, checking that the primers are working well before moving on to use a probe-based method. Hot-start enzymes have been used effectively to prevent primer-dimer formation in SYBR-green assays, therefore increasing sensitivity (Hein et al., 2001).

TaqMan technology is more specific than SYBR-green, since it uses a fluorescent probe to hybridize to the target sequence between the primer pair. Accordingly, TaqMan probes have been widely used in detection and quantification of gene expression and quantification of pathogens (Hermansson and Lindgren, 2001; Hristova et al., 2001; Kikuchi et al., 2002; Bach et al., 2003; Mocellin et al., 2003). The TaqMan probes rely upon fluorescence resonance energy transfer (FRET) usually between one fluorophore and a dark or ‘black-hole’ non-fluorescent quencher (NFQ) that disperses energy as heat rather than fluorescence (Diagram-1).

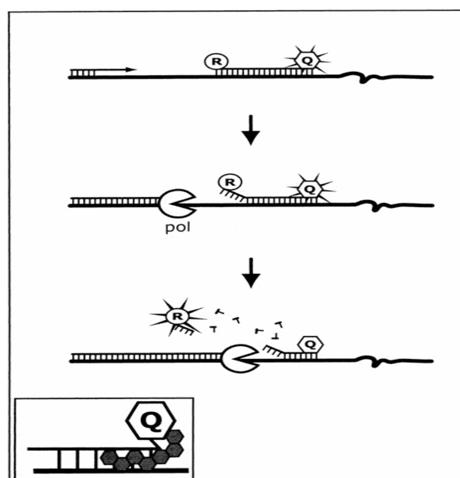

Diagram (1) The 5′ nuclease oligoprobe chemistry. When the DNA polymerase (pol) progresses along the relevant strand, it displaces and then hydrolyses the oligoprobe *via* its 5′ 3′ nuclease activity. Once the reporter (R) is removed from the extinguishing influence of the quencher (Q, open), it is able to release excitation energy at a wavelength that is monitored by the instrument and different from the emissions of the quencher. Inset shows the nonfluorescence quencher (NFQ) and minor groove binder (MGB) molecule that make up the improved MGB nuclease-oligoprobes.

TaqMan probes release their fluorescence signal upon the hydrolysis of the probe by Taq polymerase 5' to 3' exonuclease activity. Thus most but not all Taq polymerases are suitable for quantitative real-time PCR (Giulietti et al., 2001). The desirable criteria for an oligoprobe label are (i) easy attachment of the label to DNA, (ii) detectability at low concentrations, (iii) biological safety, (iv) stability at elevated temperatures and (v) an absence of interference with the activity of the polymerase (Holland et al., 1991). General design requirements include (i) a length of 20–40 nucleotides (ii) a guanine cytosine (GC) content of 40–60%, (iii) no repeated sequence motifs, (iv) absence of hybridisation or overlap with the forward or reverse primers and (vi) a TaqMan probe melting temperature (T_m) at least 5°C higher than that of the primers to ensure the oligoprobe has bound to the template before extension of the primers can occur (Landt, 2001).

Reporter fluorophores that are covalently attached at the 5' end include FAM (6-carboxyfluoroscein), TET (tetrachloro-6-carboxyfluorescein), JOE (2,7-dimethoxy-4, 5-dichloro-6-carboxyfluorescein) and HEX (hexacholoro-6-carboxyfluorescein). The reporter is usually quenched by TAMRA (6-carboxy-tetramethyl-rhodamine) or 4-(4'-dimethylamino-phenylazo)-benzene (DABCYL) at the 3' end. DABCYL in contrast to TAMRA is a nonfluorescence quencher (NFQ).

TaqMan probes are normally modified at the 3' end with a blocking phosphate to prevent probe extension during amplification.

Fluorescence emission is measured on a cycle-to-cycle basis. Hydrolysis probes eliminate the need for subsequent PCR product verification steps, thus reducing the time scale of analysis.

The non-PCR related fluorescence fluctuations are usually normalised using a non-participating or 'passive' internal reference fluorophore such as 6-carboxy-*N*,*N*,*N*,*N*-tetramethyl-rhodamine (ROX). The corrected values, obtained from a ratio of the emission intensity of the reporter signal and ROX, are called RQ^+. To further control amplification fluctuations, the fluorescence from a 'no-template' control reaction (RQ^-) is subtracted from RQ^+ resulting in the RQ value that indicates the magnitude of the unspecific fluorescence generated for the given PCR (Gelmini et al., 1997).

Relative quantification real-time PCR is already the most frequently applied method to compare gene expression levels to an internal control gene, usually cellular maintenance (housekeeping) genes, whose expression levels remain roughly constant in all samples. These housekeeping genes are selected to normalize for the variability between clinical samples. These genes regulate basic and ubiquitous cellular functions and code, for example, for components of the cytoskeleton such as *Beta* actin (β-actin), β-2-microglobulin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or 18S rRNA. If the analytical goal is absolute quantification of a nucleic acid sequence of interest, then a dilution of known standards must be used (Killeen, 1997; Suzuki et al., 2000). It is generally accepted that DNA standards are not an optimal choice because they do not compensate for the variations in the reverse transcription reaction, which has been shown to be the source of most variability in RT-PCR experiments. This primary cause of error is the efficiency with which individual RNA molecules are converted to amplification-competent cDNA molecules. The "gold-standard" normalization or standardization genes are still the subjects of debate.

The most important parameter for real-time RT-PCR quantification is the threshold cycle (C_t) value. This value indicates the cycle at which a statistically significant increase in normalized reporter is first detected. This occurs when the signal increases significantly above the level of threshold set by negative controls. The more the template is present at the beginning of the reaction, the fewer cycles it takes to reach a point in which the fluorescent signal is first recorded as statistically significant above background (Gibson et al., 1996). The C_t value is always calculated during the exponential phase of the amplification reaction and may be transformed into absolutely or relatively quantitative result taking the different (positive) controls into account.